

CANADENSOLIDE AND OTHER METABOLITES

OF

PENICILLIUM CANADENSE

A Thesis presented by

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to

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Degree

of

Doctor of Philosophy

The Chemistry Department

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This thesis is mainly concerned with the elucidation of the structure and of the relative and absolute stereochemistry of the antifungal bis lactone antibiotic canadensolide which is found in the culture filtrates of the fungus Penicillium canadense. This work is interlinked with structural studies on five metabolites isolated from the same source. Of these, one, the bis lactone dihydrocanadensolide, had been previously isolated. The remaining four, canadensic acid, isocanadensic acid, dihydroisocanadensic acid and hydroxyisocanadensic acid were all shown to be related monolactones. The absolute stereochemistry of the natural monolactones and of various monolactones derived from canadensolide was established using ORD and CD, and in one case (canadensic acid) by multistage degradation to (-) γ -caprylolactone.

The absolute and relative stereochemistry of canadensolide was derived from the following : chemical and spectroscopic evidence of cis fusion of the two lactone rings ; determination of the absolute configuration at C-4 by conversion to a monolactone with one asymmetric centre; elucidation of the stereochemical relationship of canadensolide and dihydrocanadensolide, including the first reproducible conversion of the former to the latter; determination of the absolute configuration at C-3 of

hydroxyisocanadensic acid; assignment of the absolute configuration at C-3 in dihydrocanadensolide following chemical conversion of hydroxyisocanadensic acid to dihydrocanadensolide. The latter reaction also established the configuration at the carbinol carbon atom (C-4) in hydroxyisocanadensic acid, the result being at variance with that predicted from application of the Horeau method.

A number of degradative schemes suitable for determining the distribution of label in canadensic acid biosynthesised from radioactive precursors were studied and application of one of these gave results in accord with biosynthesis via an alkylcitric acid type of intermediate.

Synthetic studies and ultraviolet spectra proved to be important in establishing the structure of a new phenolic minor metabolite isolated from the culture filtrates as 3-chlorogentisyl alcohol.

Synthetic work carried out on the unusual benzoylated metabolite, candipolin, previously found in the mycelium of P. canadense, has disproved a peptide like structure and shown it to be N-benzoyl-L-phenylalanine N-benzoyl-L-phenylalaninyl ester.

I should like to express my sincere thanks and appreciation to my supervisor, Dr. N.J. McCorkindale, for his constant guidance and advice during the course of this work and also in the presentation of this thesis.

I should also like to thank the Carnegie Trust for the Universities of Scotland for maintenance during the last three years and to Professor R.A. Raphael, F.R.S., for providing the opportunity to carry out this research.

I am also indebted to Miss M. McKenzie and staff of the Mycology Department for technical assistance in the preparation of fungal extracts. Thanks are also due to Mr. J. Cameron, B.Sc., and his staff (micro-analyses), to Miss F. Lawrie (infra red spectra), Dr. J. Roberts and staff (mass spectra), to Mr. J. Gall (n.m.r. spectra) and to my laboratory colleagues for their assistance and advice.

Finally, I should like to acknowledge the efforts of my wife in the typing of the manuscript, and my brother Robert G. Roy, B.Sc., for assistance with diagrams, etc.

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INTRODUCTION.

THE FUNGI.

No account of the fungal metabolites can be commenced without a brief account of the character and role of fungi in nature. This is no easy task since the very definition of the term "fungi" is imprecise. Thus Christensen¹ was led to remark, "The rules of procedure of the United States Senate are as the play of prattling children compared with the Rules of Nomenclature of the fungi".

The fungi² are classified as a member of the vegetable kingdom, and range in size from species with large fruiting bodies to those appearing as minute dots or cobweb-like coverings. They differ fundamentally from other plants in that they do not possess chlorophyll, the green colouring matter found in land plants, and, hence, cannot utilise light energy to synthesise carbon compounds from carbon dioxide and water. Fungi obtain these basic organic compounds (sugar, starch, etc.) ready made from dead plant or animal tissue, when they are called saprophytic or by attacking living plants as parasites. Often an originally parasitic fungus can obtain a state of equilibrium with the host plant, whereby, both derive benefit. This association, symbiosis, is typified by the Lichens

(algae and fungus) and also the "fungus roots" of many forest trees.

The adsorption of nutrients takes place through minute tubes (hyphae) which form the mycelium. In most species this is invisible as it spreads in the soil or within the host organism. Decaying leaves, however, can occasionally be seen with a covering of white mycelium. From the mycelium arises the fruit bodies which consist of densely interwoven hyphae. The fruit bodies differ according to the species but all produce a microscopically small spore which on germination gives rise to a new mycelium.

Fungi are of course responsible for most of the known crop damage ³. Rusts (like black stem rust or white pine blister rust) and smuts cause extensive damage to cultivated crops. Armillaria mellea ("Honey Fungus") not only attacks coniferous trees but also herbaceous plants such as potatoes, strawberries, etc. Other parasitic species like the Polypores grow on living trees, killing the host. Saprophytic fungi attack constructional timber; similarly, the "dry rot" fungus, Serpula (Merulius) lacrymans, can cause extensive damage to house property where timber is exposed to moisture.

Fungi, however, are also associated with deterioration of foodstuffs and with the decomposition of organic debris of dead

plants and animals. Similarly the part played by yeast in the making of bread and the preparation of alcoholic drinks has long been known and utilised. Citric acid, originally obtained from the juice of lemons, is now prepared commercially on a large scale by using a common black mold, Aspergillus niger.

Not so apparent is the role played by fungi in the destruction and conservation of the organic matter of the soil. Here their effect on the humus level is vital in soil fertility and plant nutrition. Many soil organisms have also proven useful sources of antibiotics⁴. A genus of filamentous soil organism, the actinomycetes (streptomycetes) is a particularly prolific source of organisms adaptable to antibiotic production. Research with these has led to the discovery of agents (chlorotetracycline, oxytetracycline, etc.) effective against a broad spectrum of pathogens. These have supplemented the effectiveness of the mould product penicillin in treating diseases in man.

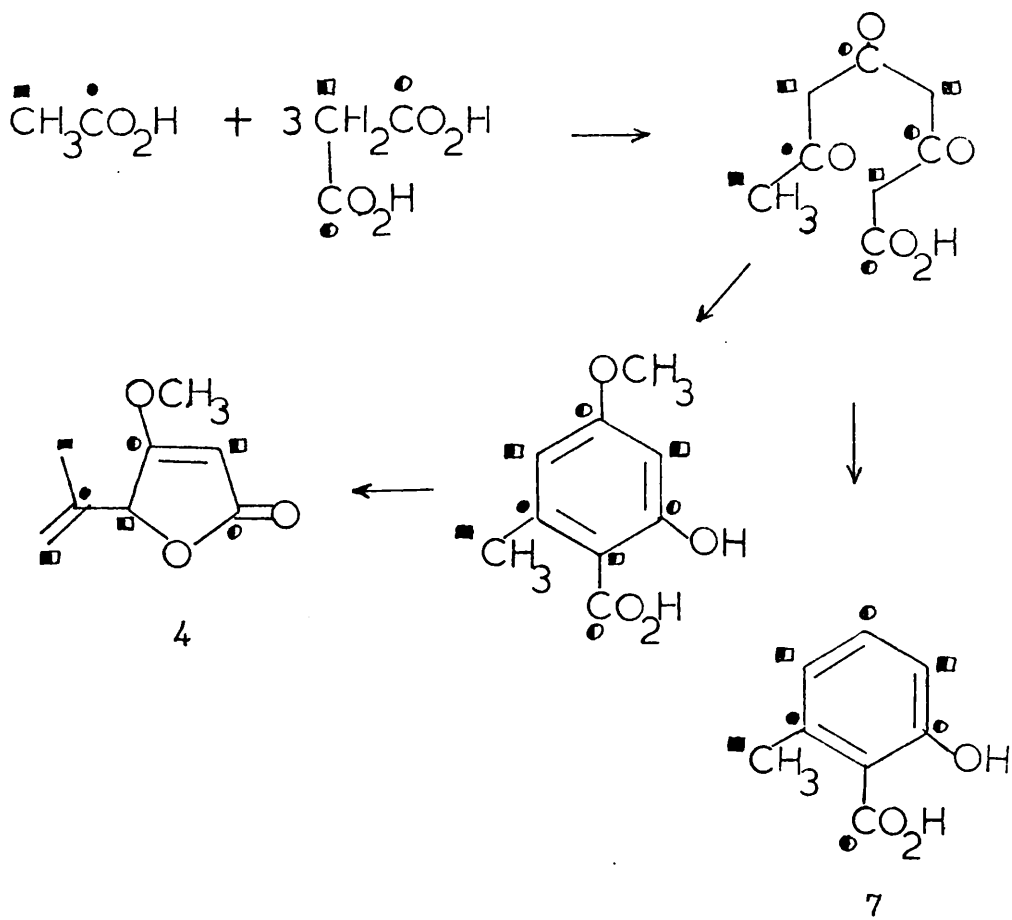
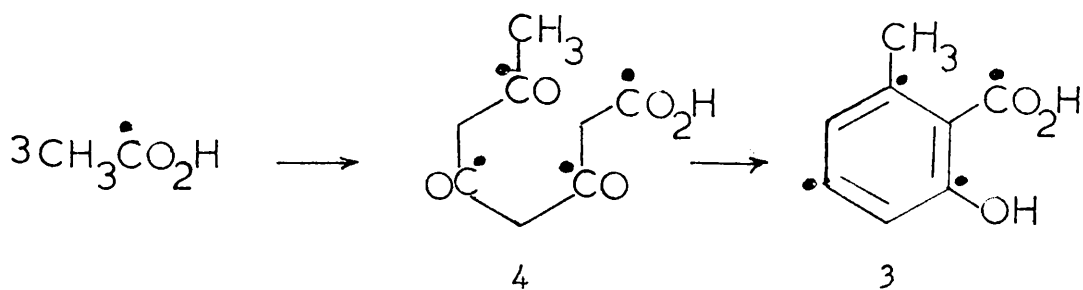
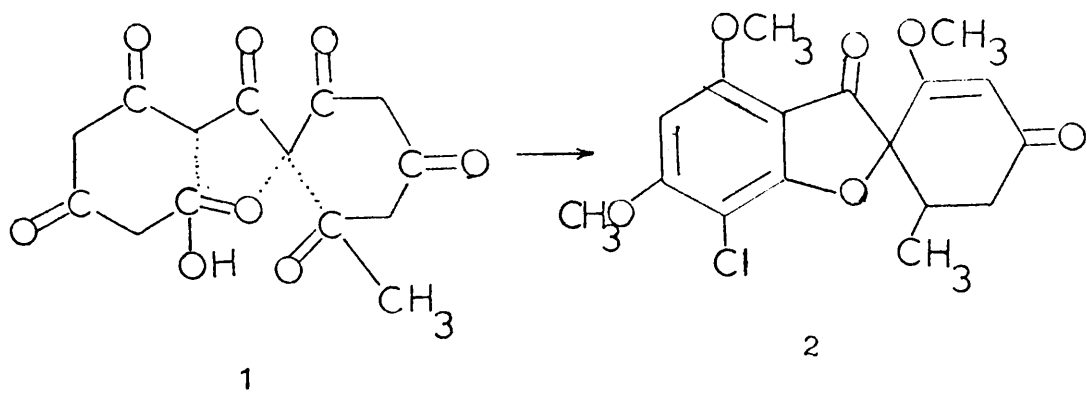
The screening of fungi for antibiotic activity is now carried out in laboratories all over the world. The present work also concerns an antifungal substance resulting from a relatively modest survey of this type carried out in Glasgow University's Mycology Unit.

FUNGAL METABOLITES.

The fungal cell not only produces the primary materials essential for life (proteins, nucleic acids, lipids, etc.) but also synthesises a wide range of additional chemical compounds - the so called "secondary" metabolites. These are often species dependent and little is known of their metabolic function. They constitute to a large extent the natural products of organic chemistry.

The range of chemical structure encountered in secondary metabolism is extensive. So impressed was Raistrick by the heterogeneity of compounds synthesized by molds that he designated this feature "polychemism". His group at the London School of Hygiene alone isolated over 200 fungal metabolites. By 1960, the known number of fungal metabolites had exceeded 1,000 and has increased rapidly since. Only a brief introduction will therefore be given outlining the main lines of classification of these metabolites according to the underlying chemical reaction processes involved in their biogenesis. Selected examples will be provided where appropriate to illustrate the various classes.

Many secondary metabolites can be shown to have acetic acid as their biosynthetic precursor. The polyketide hypothesis was first postulated by Collie⁵ (1907) who deduced that a large number of natural products could be derived from head to



tail acetate polymers. It was Birch ⁶ (1953) who related the hypothesis to a number of known structures, and who provided experimental proof of it. By examining a number of naturally-occurring aromatic compound he found that the position of the oxygen atoms was that to be expected if the compounds were formed by cyclisation of a polyketomethylene acid $\text{CH}_3 (\text{CO}.\text{CH}_2)_n \text{COOH}$. Thus griseofulvin (2) a metabolite of Penicillium griseofulvum could be derived from (1), which itself could be formed from seven molecules of acetic acid. Similarly 6- methylsalicylic acid (3) from the same fungus could be derived by condensation of the tetraacetyl chain (4). This was verified experimentally by growing the fungus upon a medium containing carboxyl labelled acetic acid when the isolated 6- methylsalicylic acid (3) was shown to have the predicted labelling pattern.

The fatty acids could be considered as a special case of a fully reduced polyketide chain, and, hence, the recognition of the involvement of malonyl-Co.A in fatty acid biosynthesis resulted in a modification of the polyketide hypothesis.

A summary of the mechanism ⁷ involved in the formation of fatty acids is given in fig. 1 . In this an acetyl thiol ester forms the starting point in the chain, and condensation occurs with a malonyl thiol ester to form a β -keto thiol ester.

Fig. 1. Mechanism of fatty acid biosynthesis.

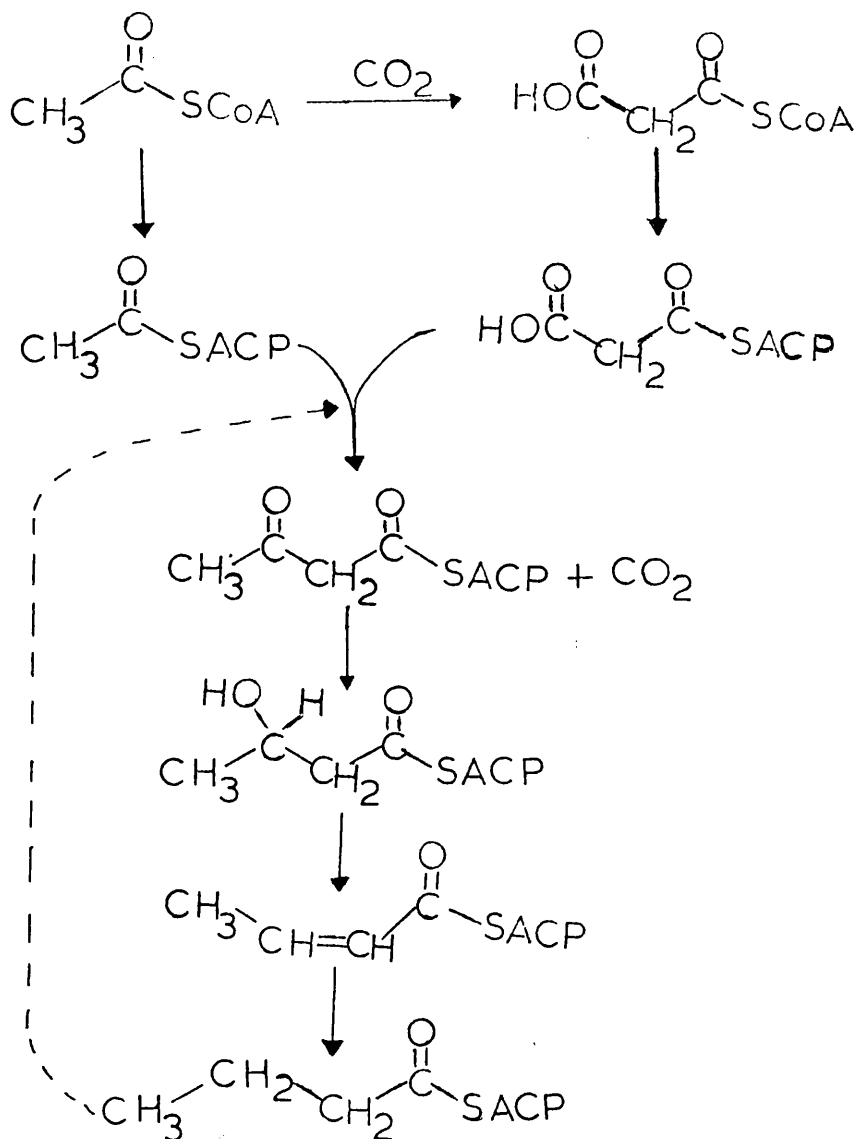
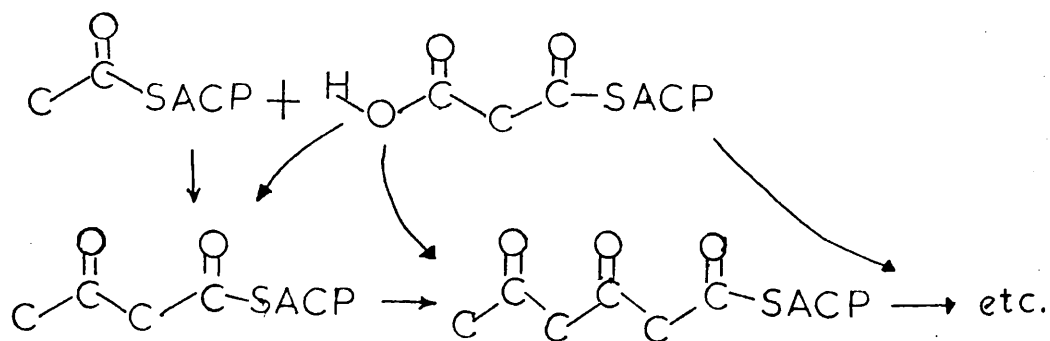


Fig. 2. Formation of polyketide chain.

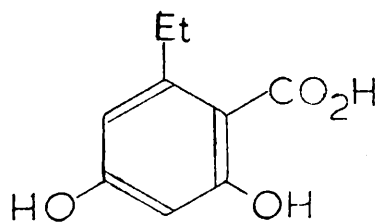
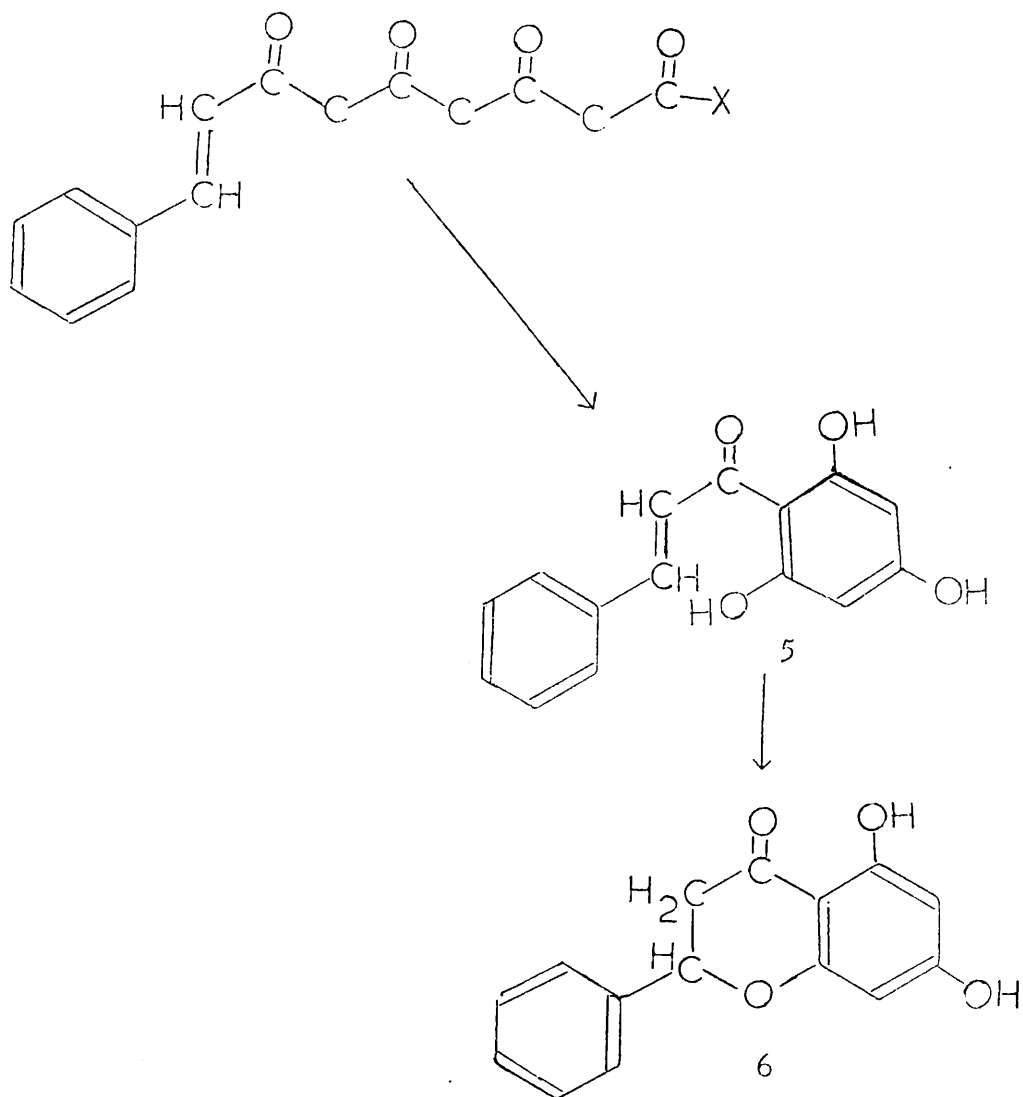


Reduction, dehydration and reduction steps occur to form a saturated (butyryl) thioester, and the process is repeated, each cycle adding two carbons to the chain. The intermediates are bound as thiol esters to a protein called acyl carrier protein ⁷ (ACP), this linkage being formed by an exchange reaction between the protein and the corresponding CoA ester. A polyketide chain could be formed in a similar manner if reduction of the intermediate β -keto thiol ester did not result but instead a second addition of malonyl CoA occurred (fig. 2).

Tracer experiments with ¹⁴C-malonate and acetate ⁸ provided evidence that penicillic acid (4) was formed from one acetyl and three malonyl groups. Since then the "acetyl-polymalonyl" labelling pattern has been established for many polyketides. The biosynthesis of polyketides is hence by an acetate-polymalonate pathway in which acetyl-CoA is the starter unit and malonyl-CoA the chain extending agent. The condensation, accompanied by loss of CO₂, leads to a polyketide chain.

Starter groups other than acetate (as in fatty acid biosynthesis) are of course possible. Thus addition of three malonyl groups to a cinnamoyl starter group (fig. 3) could produce a polyketide which would give chalcone (5) and then

Fig. 3. Polyketide formed from cinnamoyl starter unit.



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Fig. 4 Biosynthesis of methymycin

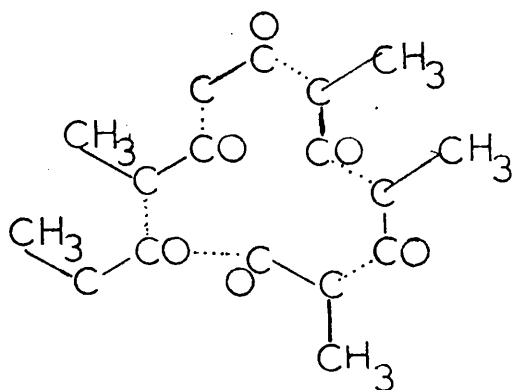
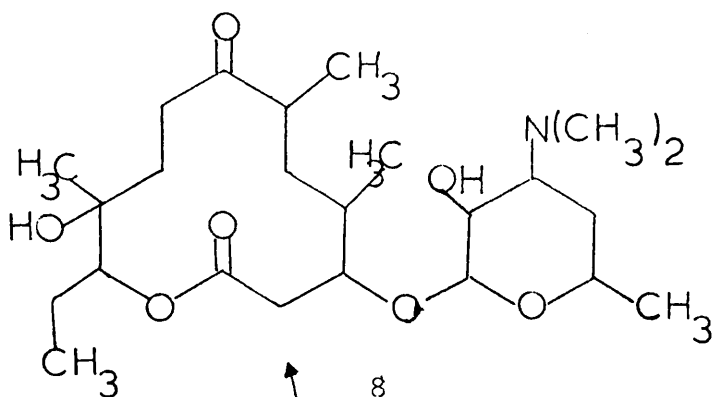
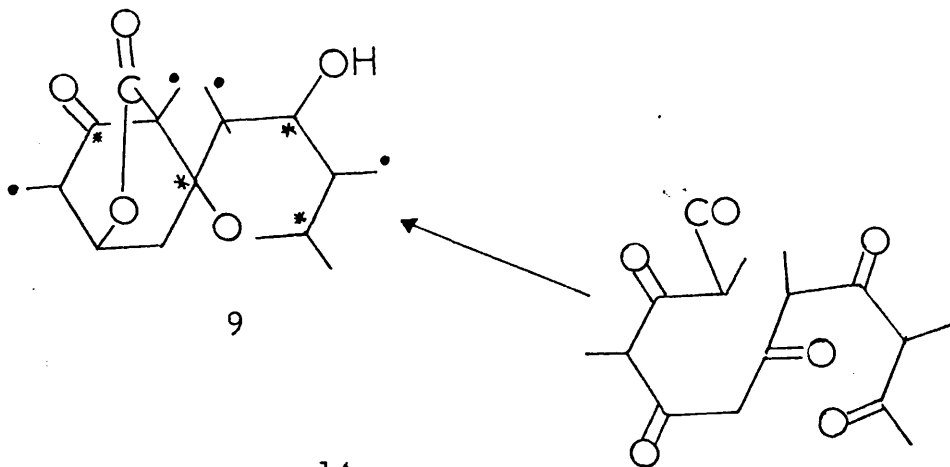


Fig. 5 Biosynthesis of portentol



* Sodium [1 - ^{14}C] acetate

• [Me - ^{14}C] methionine

compounds of the flavanoid series such as flavanone (6)⁹. Homoorsellinic acid (7)¹⁰ is biosynthesised in Penicillium baarnense from a molecule of propionate and three malonate units. Many of the macrolide antibiotics⁵³ are found not only to have propionate as the starter unit but also α -methylmalonate as the chain extender giving structures which appear to be derived partly from propionate. Thus the biosynthesis of methymycin (8) has been shown¹¹ to occur via 5 x C₃ units + 1 x C₂ unit as shown (fig. 4). Although methionine -Me-¹⁴C was even better incorporated into methymycin (8) than propionic acid -l-¹⁴C the labelling was localised exclusively in the dimethylamino group of desosamine. This is in direct contrast to the lichen metabolite portentol (9)¹², whose structure suggests that it may be formed via a linear polyketide (fig. 5) consisting of one acetate and five propionate units. In fact neither propionate nor methylmalonate were incorporated and the molecule appears to be synthesised in vivo from acetate and methionine as shown, (i.e. 6 x C₂ units plus 5 x C₁ units).

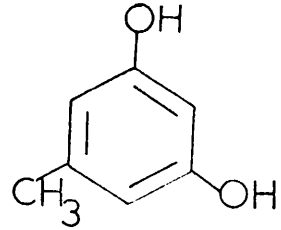
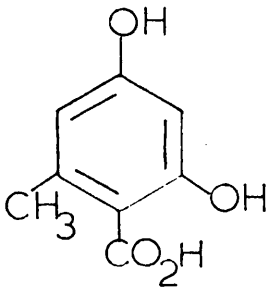
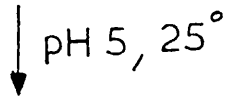
The polyketide intermediates predicted by the hypothesis are known to be highly reactive molecules and speculation has existed about the manner in which they are stabilised during chain extension. Thus in attempts to prepare 3,5,7-trioxo-octanoic acid (11) from diacetyl acetone (10), only traces

Diacetyl acetone

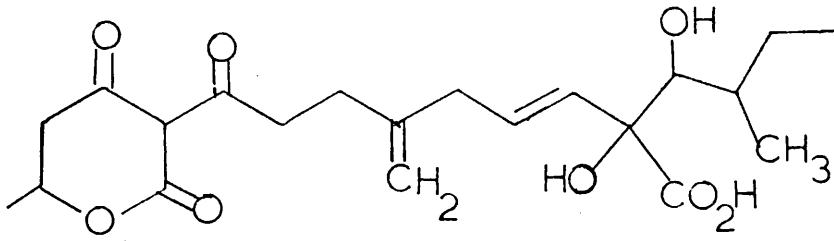
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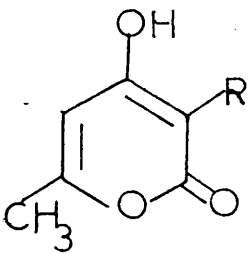
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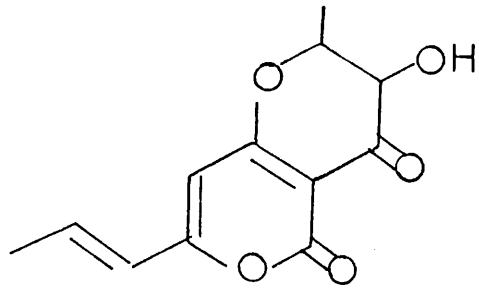
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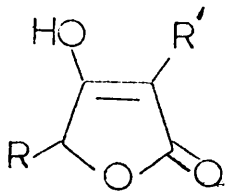
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of an impure compound were obtained ¹³. On standing in aqueous solution at pH 5 this polyketide cyclised to orsellinic acid (12) in good yield. Similarly attempts to hydrolyse tetraacetic lactone (13) led to the formation of orsellinic acid and its decarboxylated product orcinol (14). In view of their instability it has been assumed that polyketides do not occur in the free state but as enzyme bound entities.

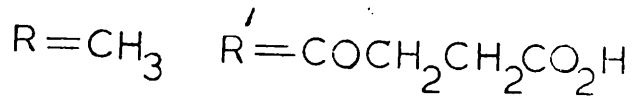
Although there is little evidence for these enzyme bound entities as yet, it is interesting to note that some polyketide lactones have been isolated from fungi. Thus both triacetic lactone (15, R=H) and its methyl derivative (15, R=Me) have been isolated from Penicillium stiptitatum by Tannenbaum and his colleagues ¹⁴. Similarly from an ethionine inhibited strain of the same fungus the previously unknown tetraacetic lactone (13) accumulated. ¹⁵

These simple lactones are important in that they are direct dehydration products of polyketides. They represent the closest approximation yet made to actual isolation of polyketides. Three other structurally related fungal products are alternaric acid (16) ¹⁶, radicinin (17) ¹⁷, and citreoviridin (18) ¹⁸. The actual significance of these is uncertain as tetraacetic lactone, which would appear to be the precursor of radicinin, is not incorporated.

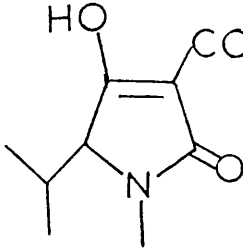
The polyketide chain can apparently be handled by the fungal



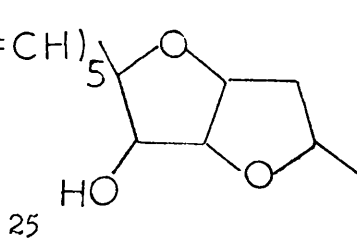
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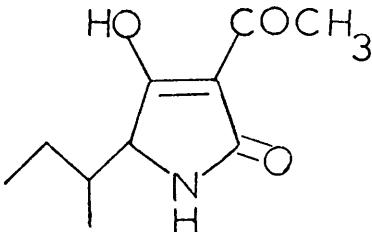
Carolinic acid



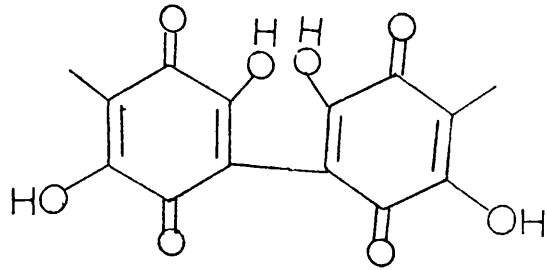
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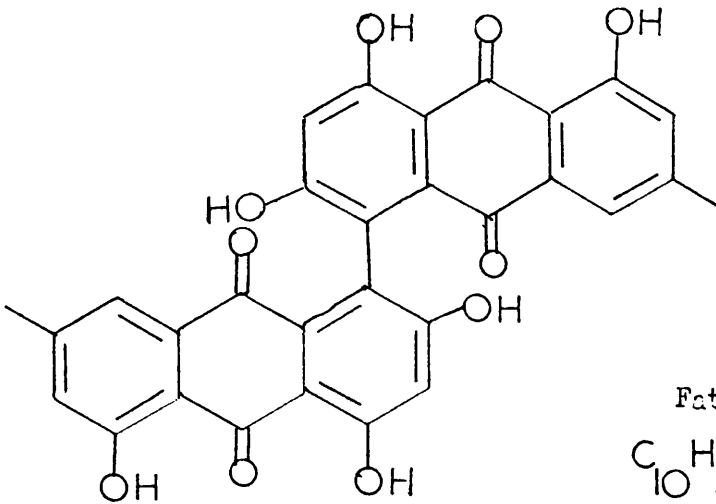
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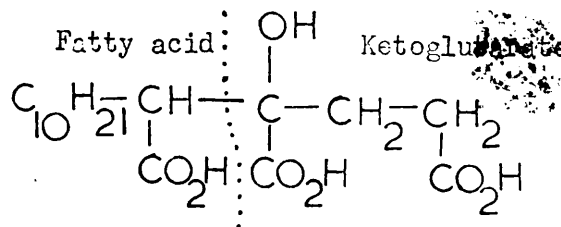
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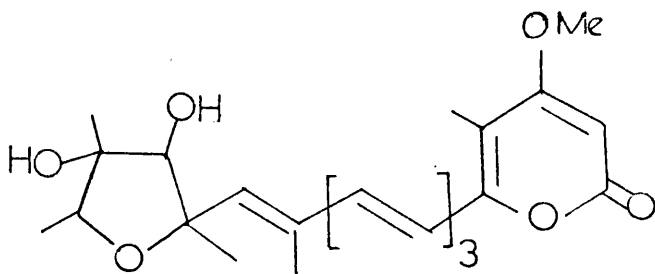
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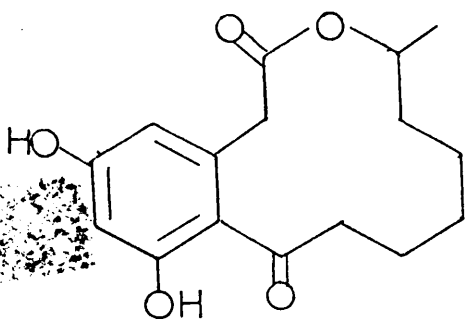
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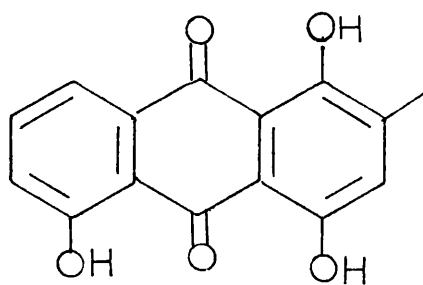
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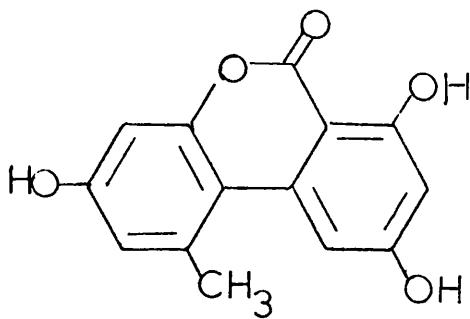
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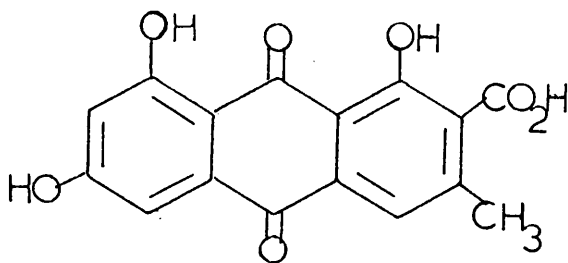
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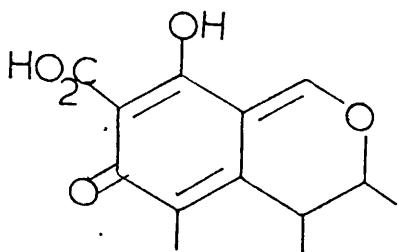
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cell in three different ways. Firstly part or all of the polyketide chain can self-condense to form aromatic species. Examples of these are endocrocin (19)¹⁹ and islandicin (20)²⁰ as well as alternariol (21).²¹

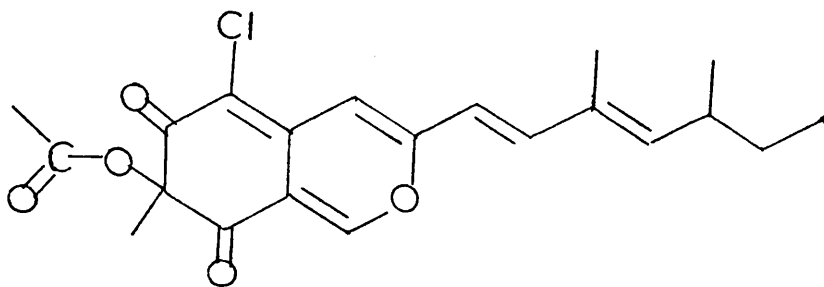
Secondly a portion of the polyketide chain may be reduced presumably by processes comparable to those involved in fatty acid biosynthesis. Curvularin (22)²² and the side chain of alternaric acid (16)¹⁶ exemplify this type of situation.

Finally the polyketide chain may undergo intermolecular nucleophilic condensation as opposed to the intramolecular process involved above in aromatic ring synthesis. The basic skeletons of the tetrone acids (23)²³ and related tenuazonic acids (24)²⁴ and erythroskyrin (25)²⁵ appear to be formed from condensation of a polyketide chain with a molecule of an aliphatic or amino acid. Spiculisporic acid (26)²⁶ represents another group of this type, and its skeleton presumably arises from the condensation of a fatty acid with a molecule of α -ketoglutarate.

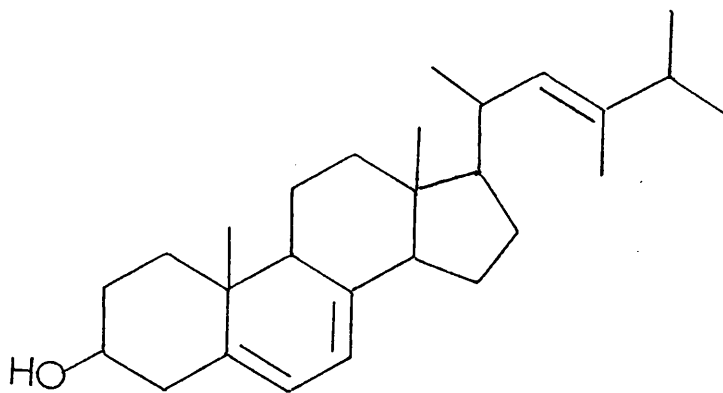
Further modification to polyketide derived skeletons can evidently be produced by phenol-oxidative coupling processes²⁷. Representatives of this class of metabolites are oosporein (27)²⁸ and skyrin (28)²⁹. Also oxidative fission of an aromatic ring can occur to give fungal products which do not at first



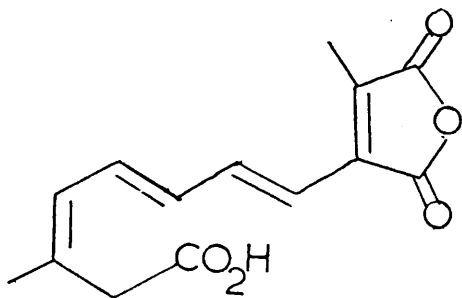
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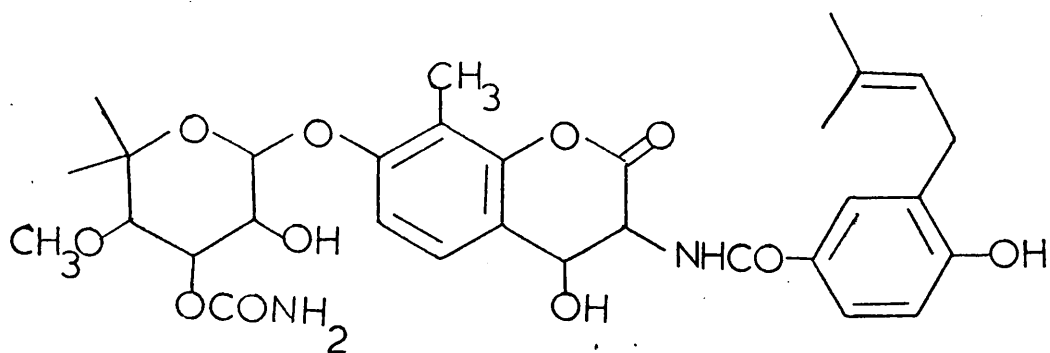
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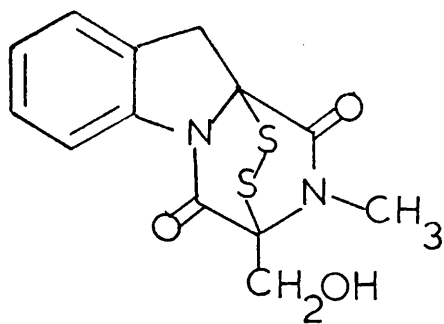
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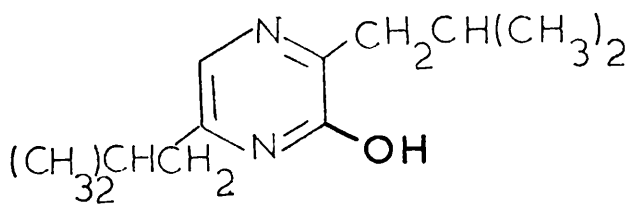


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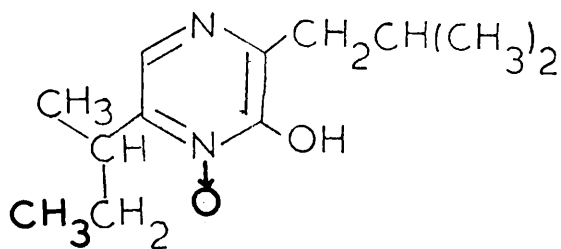
hand appear to be polyketide derived. Penicillic acid (29)²⁰ might appear to contain an 'isoprene' unit but does not in fact involve biosynthesis from mevalonate. Patulin (30)³¹ may be similarly derived from oxidation of 6-methylsalicylic acid.

As has been mentioned, many fungal metabolites are found to possess C₁ carbon residue which appear additional 'extra' to the basic carbon skeleton of the major precursor units, and whose oxygenation level can range from CH₃, through =CH₂ to COOH. Examples of these include citrinin (31)³², sclerotiorin (32)³³, ergosterol (33)³⁴ and itaconitin (34)³⁵. The compounds believed to be the precursors of the C₁ units are formate and the methyl group of methionine. By the pathways of C₁-metabolism formate is assumed to give rise to methionine and hence 5-adenosylmethionine, the immediate methyl donor.

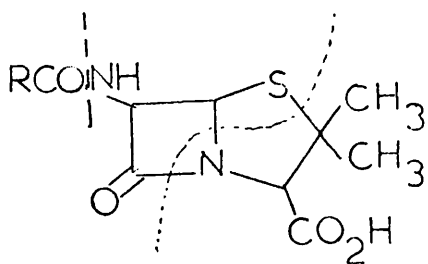
In addition to the peptides a large number of fungal products can be derived wholly or partly from amino acids, from shikimic acid or both. Aromatic amino acids such as tyrosine are, of course, biosynthesised via shikimic acid (35)³⁶. Although the aromatic rings of many fungal metabolites are derived from acetate, the unusual aminocoumarin ring system of the antibiotic novobiocin (36)³⁷ is derived from tyrosine. Similarly labelling experiments³⁸ have shown that phenylalanine is incorporated into the indole ring of gliotoxin (37) the sulphur containing metabolite of Trichoderma viride. The remaining skeletal carbon



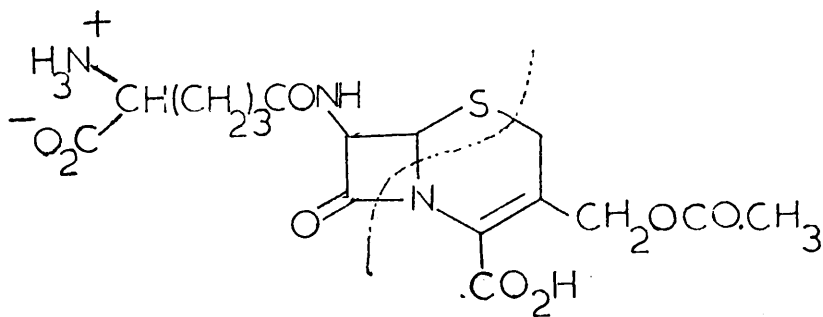
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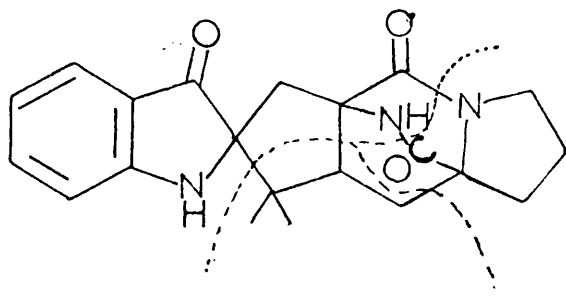
Aspergillic acid.



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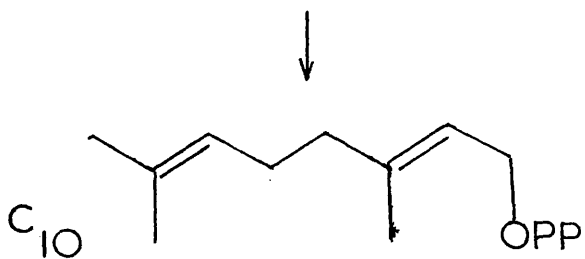
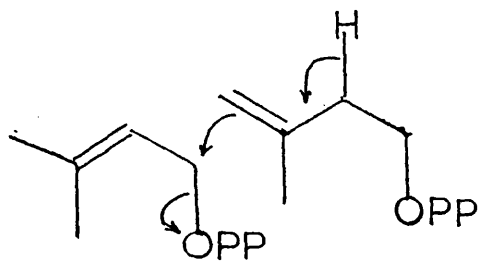


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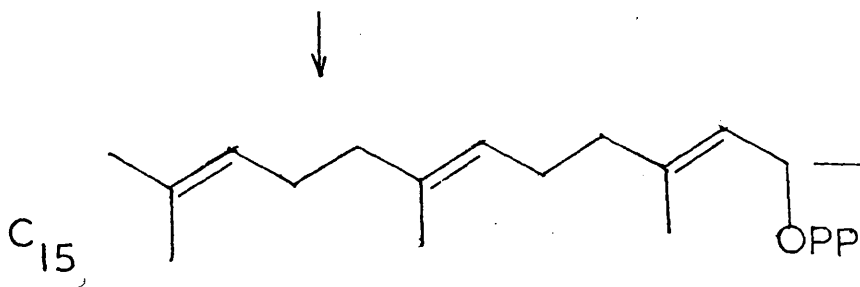
atoms are derived from serine.

Many fungal products appear to be derived directly from aliphatic amino acids. Flavacol (38)³⁹ (from two molecules of leucine) and aspergillic acid (one molecule of leucine and one of isoleucine) represent relatively simple examples. The extremely important antibiotics, the penicillins and cephalosporins must also be included in this class. The penicillin structure (39) can be formally dissected into residues of a monosubstituted acetic acid, L-cysteine and D-valine respectively, as shown. Labelling experiments⁴⁰ have shown that these two amino acids are incorporated into the 6-aminopenicillanic acid structure. Cephalosporin C (40) appears to be derived from D- α -amino adipic acid, L-cysteine, α , β -dehydrovaline and acetic acid as shown. The specific incorporation of labelled amino acids⁴¹ into this antibiotic has likewise confirmed this biogenesis.

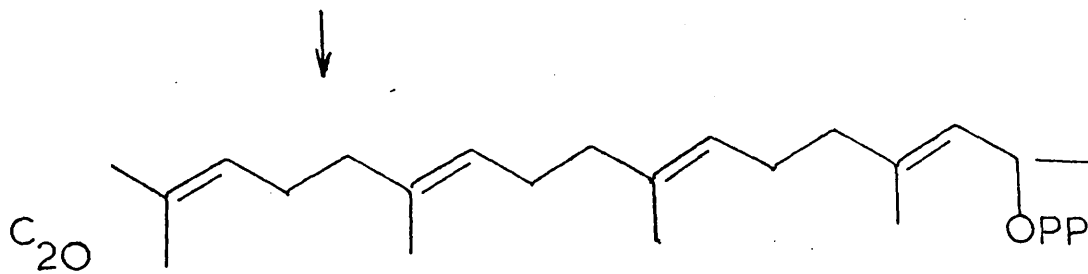
An example of amino acid derived metabolites of a more complex nature are the brevianamides⁴² from Penicillium brevicompactum. These metabolites had also been encountered during isolation work carried out in our own laboratories on a similar strain of the same fungus. Brevianamide A (41) was shown by Birch to be biogenetically derived from tryptophan, proline and mevalonic acid, as indicated. Brevianamides A - D all exhibited the characteristic u.v. chromophore of the



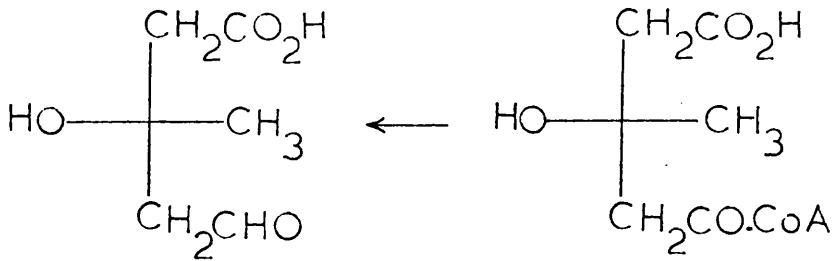
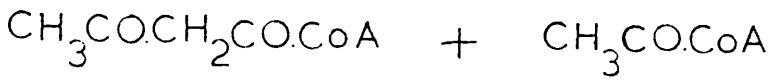
monoterpenes



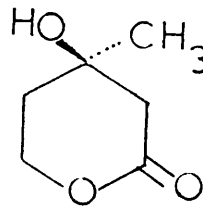
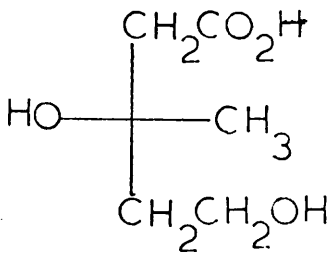
sesquiterpenes



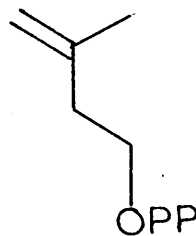
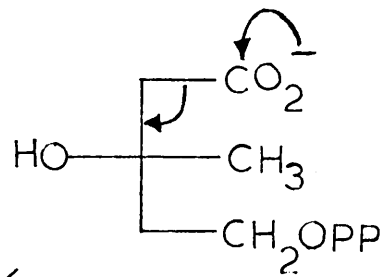
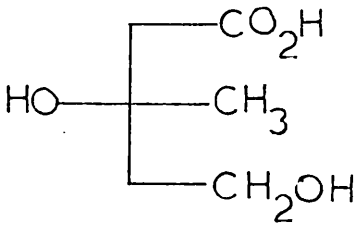
diterpenes



43



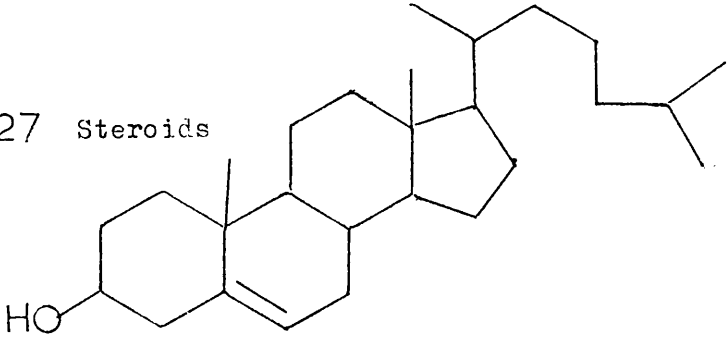
42



44

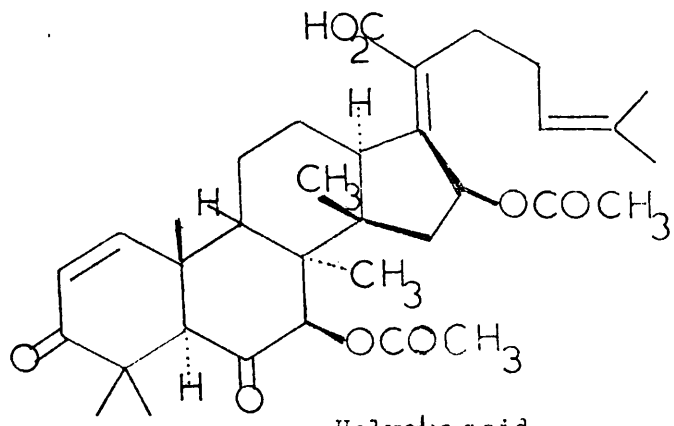
Diag. 1. Examples of terpenoid fungal metabolites.

C₂₇ Steroids



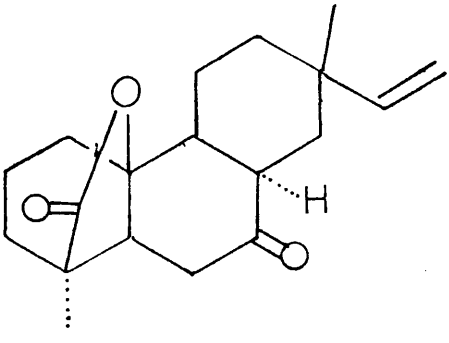
Cholesterol

C₃₀ Triterpenes

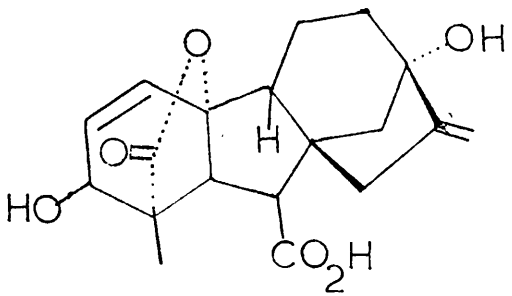


Helvolic acid

C₂₀ Diterpenes

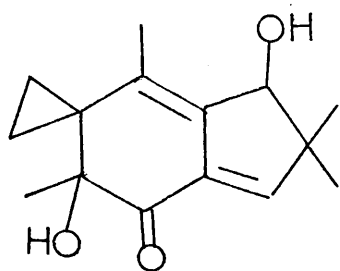


Rosenonolactone

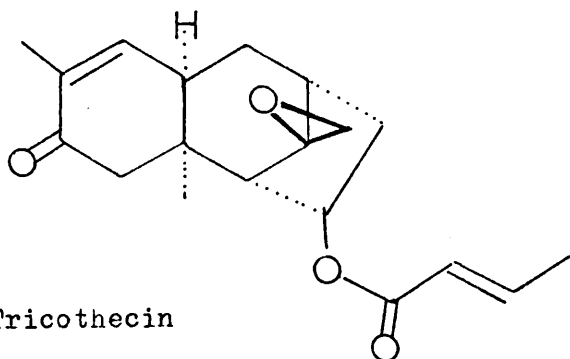


Gibberellic acid

C₁₅ Sesquiterpenes

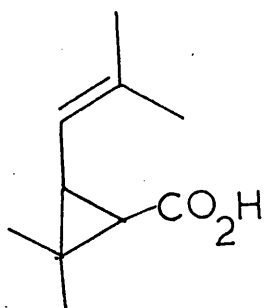


Illudin

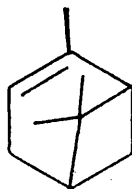


Tricothecin

C₁₀ Monoterpenes



Chrysanthemic acid



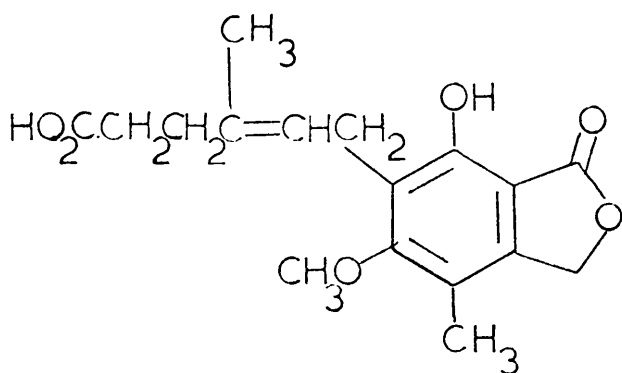
α - pinene

2,2 disubstituted Ψ -indoxyl system.

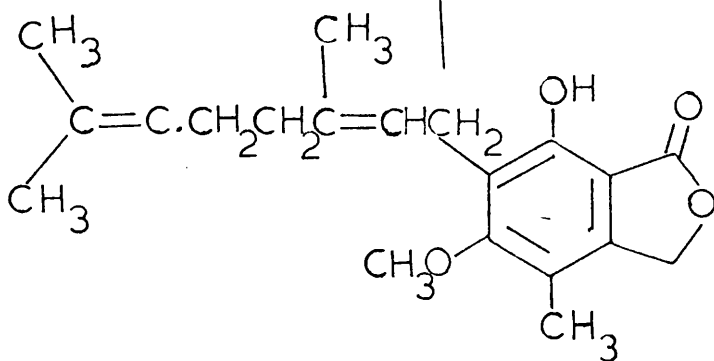
One process which competes with polyketide synthesis for the acetyl -CoA available in fungi is the formation of terpenoids via mevalonolactone (42). Claisen condensation of acetyl-CoA with acetoacetyl-CoA gives the CoA ester of hydroxymethylglutaric acid (43) (or the corresponding enzyme bound species). Subsequent reduction to mevalonic acid is reputedly irreversible and it is a maxim that mevalonate is a poor precursor of polyketides.

Condensation⁴³ of various numbers of isopentenyl units (44) derived from mevalonic acid gives rise, as shown, to geranyl pyrophosphate (C₁₀), farnesyl pyrophosphate (C₂₀). Subsequent modifications of these give various terpenes and steroids⁴⁴. (Some examples are given in diag. 1.)

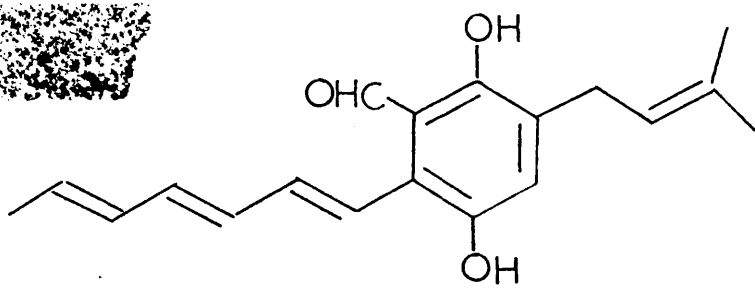
In addition to its role of precursor of the terpenoids mevalonate is frequently involved as a precursor of secondary metabolites carrying a side chain of one or more isoprene residues. The allylic ester 3,3-dimethyl pyrophosphate or its derived cation is an effective alkylating agent and is the presumed precursor of the mevalonate-derived isopentenyl groups which appear in many natural products. These are usually attached to non-terpenoid units at positions susceptible to electrophilic attack - phenolic oxygen, activated methylene,



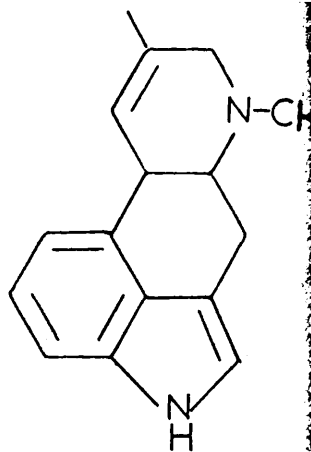
49



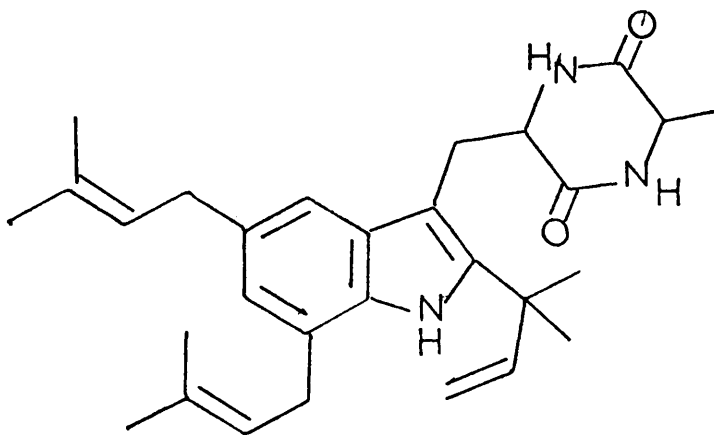
50



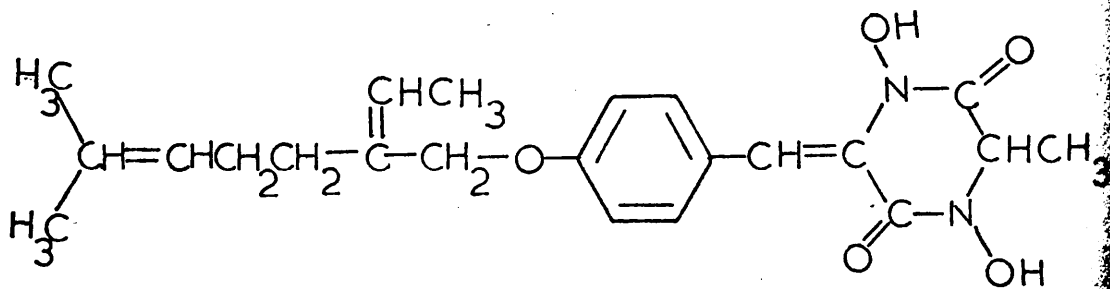
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47



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nucleophilic sites on aromatic rings etc. Examples of these are auroglaucin (45)⁴⁵ with its attached isoprene residue, agroclavine (46)⁴⁶ in which the isoprene unit forms part of a ring, and echinulin (47)⁴⁷ another metabolite derived from amino acid residues and mevalonic acid like the brevianamides discussed above. Similarly higher isoprenoids also can react as allylic pyrophosphate to attach polyisoprenene residue. The antibiotic mycelianamide (48)⁴⁸ could be derived biogenetically by alkylation of the phenolic group with a geranyl pyrophosphate type residue. Labelling experiments⁴⁹ indicate that the aromatic ring is probably shikimate derived. Mycophenolic acid (49)⁵⁰, a metabolite of Penicillium brevicompactum is another example of this type. Its probable biosynthesis is by alkylation of the polyketide aromatic framework with geranyl pyrophosphate to give a precursor of type (50) followed by oxidation of the isopropyl group to generate mycophenolic acid (49). The author is unaware of any case in which similar C-alkylation of a non-aromatic polyketide by 3,3-dimethylallyl pyrophosphate seems apparent.

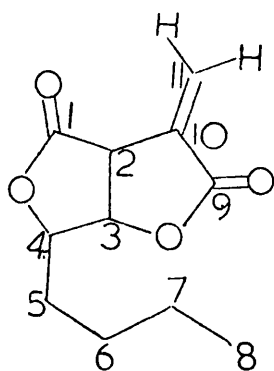
Until recently natural products containing aryl or heterocyclic rings substituted by 1,1-dimethylallyl groups, for example echinulin (47), were usually considered to arise by reaction with 3,3-dimethylallyl pyrophosphate at its tertiary

centre ⁵¹. An alternative biosynthetic route involving a Claisen rearrangement has been confirmed by labelling experiments in plants ⁵².

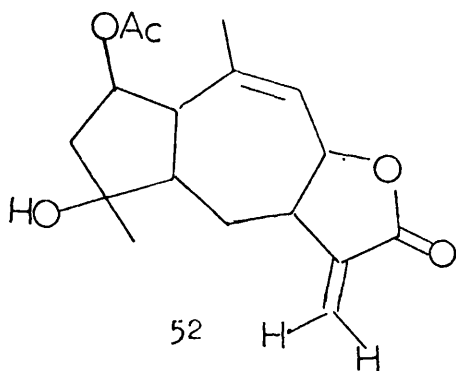
The rapid increase in the study of fungal products in recent years can be partly ascribed to the highly sophisticated physical tools now available to the organic chemist. As a consequence of this, structure elucidation, even on minor quantities of material, has almost been reduced to a routine level.

Hand in hand with this has gone the advances in mycological techniques which has enabled most organisms to be cultured on a synthetic medium in the laboratory under standard conditions. This has made available larger amounts of material for study. The feeding of radio tracers to fungi with a view to elucidating biosynthetic pathways can be readily carried out, the short life cycle of most fungi facilitating the process. This is borne out by the good incorporations achieved in part of this investigation.

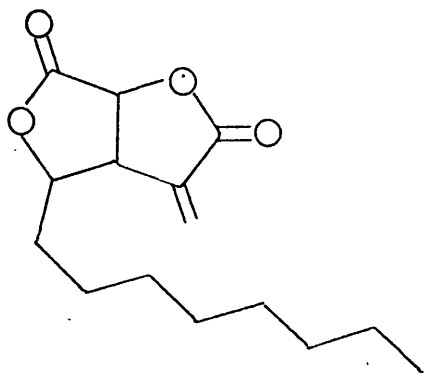
PRELIMINARY STUDIES ON CANADENSOLIDE
DIHYDROCANADENSOLIDE AND 'CANADENSIC ACID'.



51



52



53

The work to be described in this thesis concerns the antibiotic canadensolide which is present in culture filtrates of the fungus Penicillium canadense and various other metabolites produced by this organism. Canadensolide was first isolated in these laboratories by Wright⁵⁴, together with two related metabolites, dihydrocanadensolide and 'canadensic acid'. Tentative structures were assigned to these compounds almost entirely on the basis of spectral evidence and the relative and absolute stereochemistry remained to be established.

The dilactone structure (51) postulated for canadensolide was consistent with analytical and mass spectral evidence. The i.r. spectrum shows double bond absorption at 1663 cm^{-1} (ϵ 75) typical of a double bond exocyclic to a five membered ring⁵⁵ and a single carbonyl bond at 1775 cm^{-1} (ϵ 1320, $\Delta\nu_{\frac{1}{2}}$ 23 cm^{-1}). It is not evident from this that the double bond is in conjugation with one of the carbonyl groups as is shown by the high intensity end absorption in the u.v. spectrum (213nm., $\log \epsilon$ 3.93). The same features are, however, shown in the spectra of gaillardin (52)⁵⁶, which exhibits α, β unsaturated γ -lactone

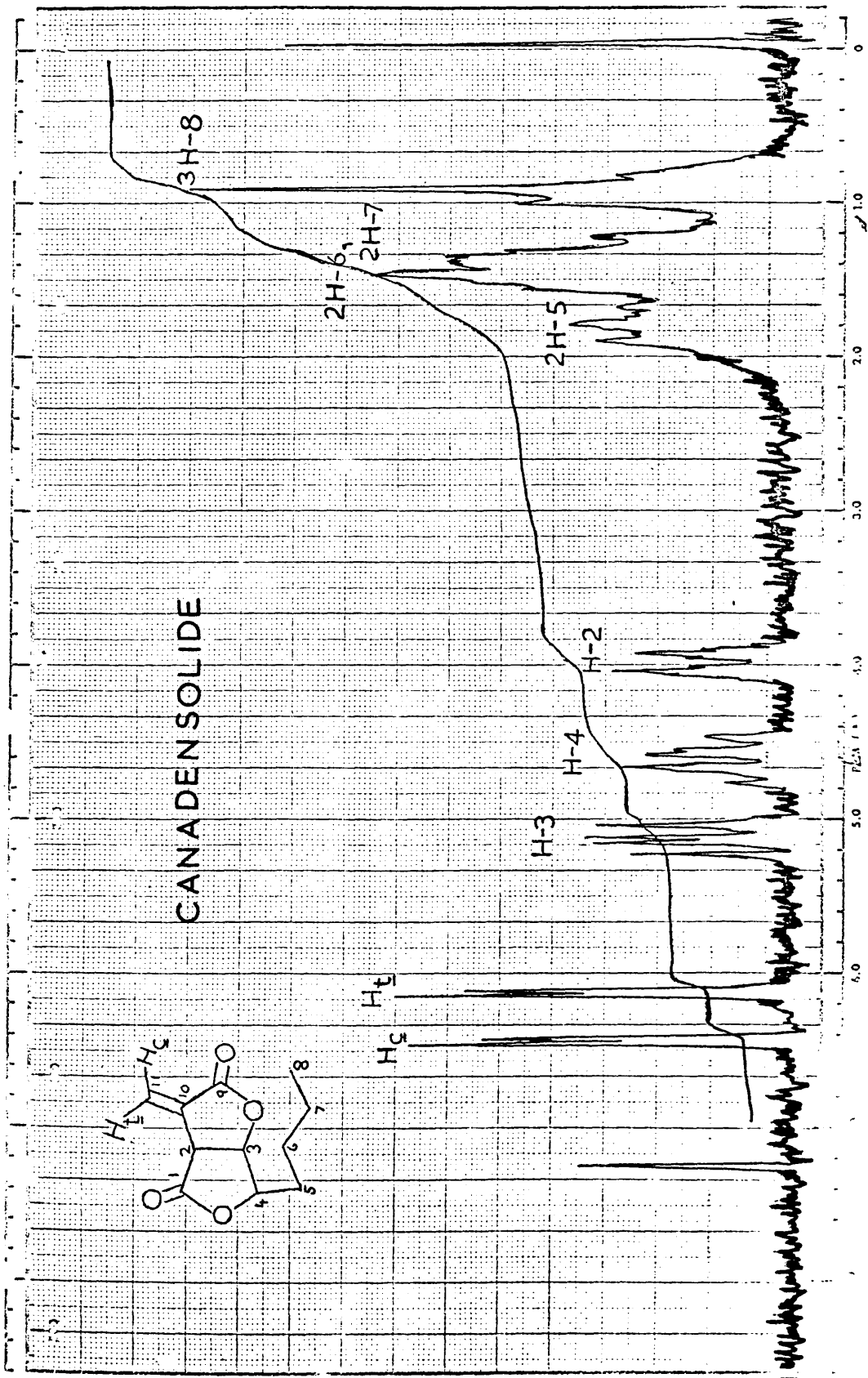


Fig. 6.

TABLE: 1

n.m.r. data for metabolites of *Penicillium canadense*.

	H-2	(2)H-3	H-4	2H-5	2H-6	2H-7	3H-8	H-10	H-11
<u>Canadensole</u>	6.0 m ^{ac}	4.88 dd ^a	5.98 dt ^b	8.2 m ^a	8.5 m	8.5 m	9.04 t ^a	-	3.58 ± 3.97 d ^c
J(Hz.)	6.5, 4.5						6.0		3.0
<u>Dihydrocanadensole</u>	6.83 dd ^a	4.85 dd ^b	5.44 m ^b	8.2 m	8.5 m	8.5 m	9.06 t ^a	7.06 dq ^a	8.53 d ^a
J(Hz.)	6.0, 1.0	6.0, 4.0					6.0	8.0, 1.0	8.0
<u>'Canadensic Acid'</u>	6.34 dd	7.3-8.2 m ^b	5.50 m	8.3 m	8.5 m	8.5 m	9.10 t	-	ca. 3.47 ± ca. 4.05 25
J(Hz.)	9.0, 11.0						6.0		25

Collapse to signals of the expected multiplicity upon irradiation at one or both vicinal centres is indicated by a or b respectively. Similar demonstration of allylic coupling is indicated by c.

absorption at 1764 cm^{-1} and 1667 cm^{-1} , the u.v. spectrum showing high intensity absorption at 209 cm^{-1} . Similarly the antifungal antibiotic avenaciolide (53)⁵⁷ has only a single band in the carbonyl region (1786 cm^{-1}) while double bond absorption occurs at 1667 cm^{-1} .

The n.m.r. spectrum (fig.6) shows well separated resonances. The numbering system used in referring to the skeletal carbon atoms of the antibiotic is as shown in (51). It is based on the postulated biogenetic origin of these metabolites. The exocyclic methylene protons appeared as fine doublets at 3.54τ ($J = 3.0\text{H}$) and 3.88τ ($J = 2.5\text{H}$). The double doublet at 4.88τ (H-3) and the double triplet at 5.4τ (H-4) were each assigned to the protons geminal to the oxygen atoms of the two γ -lactone functions. The remaining 1H resonance at 6.0τ (H-3) corresponded to the allylic proton. The protons of the n-butyl chain exhibited the expected pattern for this system with resonances at 8.2τ (2H), 8.5τ (4H) and 9.0τ (3H). These assignments were supported by decoupling experiments, summarised with the n.m.r. data in table. 1.

Further corroboration was obtained from the mass spectrum. The most favoured cleavage for 3-alkyl substituted γ -lactones

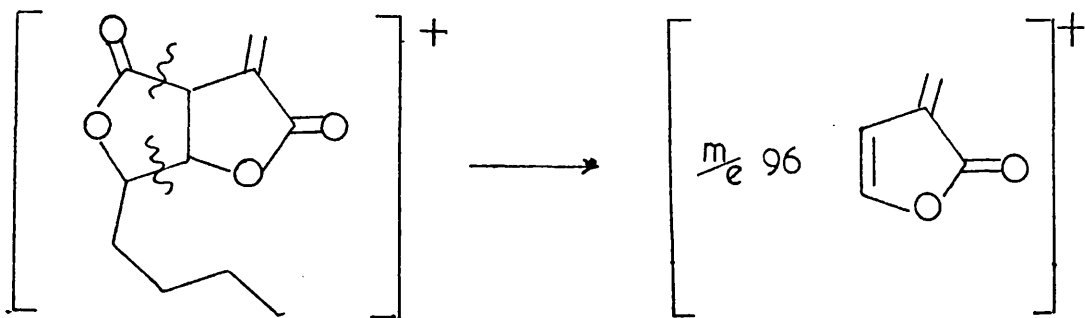
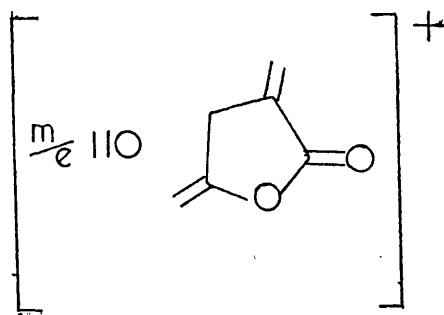
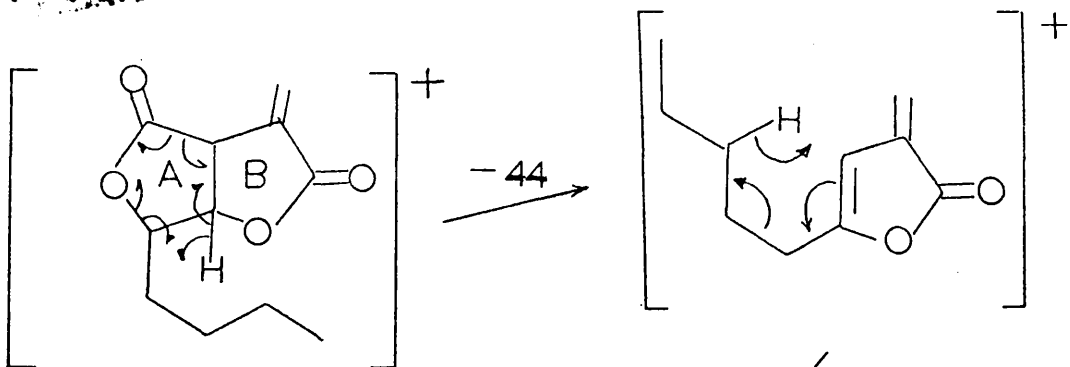
TABLE: 2

Mass Spectral Data.

Peak	Canadensolide		Dihydrocanad.		Epidihydrocanad.	
	m/e	% Abundance	m/e	% Abundance	m/e	% Abundance
M ⁺	210	0.5	212	5	212	4
M-28	182	1.5	184	0.8	184	0.3
M-44	166	3	168	0.7	168	0.5
M-57	153	2	155	3	155	1.3
M-(44+15)	151	3	153	4	153	1.2
M-(44+29)	137	7	139	4	139	1.6
M-(44+43)	123	18	125	10	125	
M-(44+57)	109	19	112	7	111	2
Ring A Cleavage	110	40	112	12	112	5
Scheme I		96	100	98	100	98

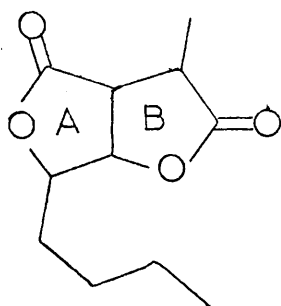
Scheme 1.

Cleavage of ring A.

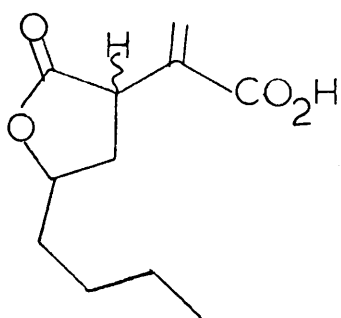


arises by loss of the side chain, while the parent ion itself is usually of low abundance.¹³¹ In keeping with this the mass spectrum of canadensolide (Table 2) showed an ion at m/e 153 (M-57) (loss of the n-butyl side chain), which was several times more abundant than the parent ion. Furthermore there were losses of 57, 43 and 29 mass units from the ion at m/e 166 (M-44).

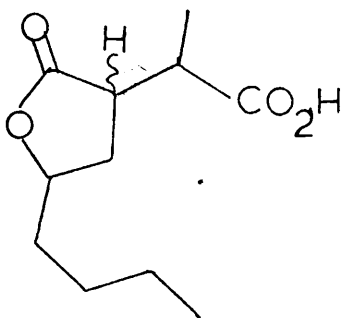
The presence of the exocyclic methylene group in canadensolide (51) was also confirmed by the formation of formaldehyde (characterised as its dimedone derivative) on ozonolysis. The low yield (10%) is reputed to be characteristic of α -methylene γ -lactones⁵⁸. Further chemical evidence in accordance with this structure was also obtained by treatment of canadensolide with two moles of sodium metaperiodate to yield n-valeraldehyde. This product was characterised as its d.n.p. by paper chromatography, and directly as n-valeraldehyde by g.l.c. comparison with an authentic sample. Borohydride reduction of the aldehyde gave n-pentanol which was also characterised by g.l.c. comparison with an authentic sample. This degradation provided chemical evidence for the presence of an n-butyl side chain. This grouping was subsequently confirmed in the present work when cleavage of the α -diol system



54



55



56

was carried out using sodium periodate under slightly alkaline conditions (pH9) with continuous removal of the liberated aldehyde into a chloroform solution containing 2,4-dinitrophenylhydrazine. The valeraldehyde dinitrophenylhydrazone obtained by p.l.c. was identified by comparison with an authentic sample.

Dihydrocanadensolide (54)⁵⁴, the second metabolite isolated by Wright, was slightly less polar than the antibiotic (by t.l.c.) and analysed as $C_{11}H_{16}O_4$. It was readily recognised as a dihydroderivative of canadensolide from similarities in i.r., n.m.r. and mass spectra. The spectral features characteristic of the terminal methylene group were absent in this compound. Its i.r. spectrum exhibited two carbonyl absorptions at 1789 cm^{-1} ($\epsilon 850$) and 1783 cm^{-1} ($\epsilon 925$) but no peak attributable to a double bond. This apparent lack of the exocyclic methylene group present in canadensolide was confirmed by the u.v. spectrum which showed no absorption maxima above 200 nm.

The n.m.r. spectrum (fig. 7) likewise exhibited no resonance indicative of a terminal methylene group but a secondary methyl group was evident as a 3H doublet at 8.50τ coupled to a 1H multiplet at 7.06τ . The remaining resonances were similar in appearance and chemical shift value to those in the n.m.r.

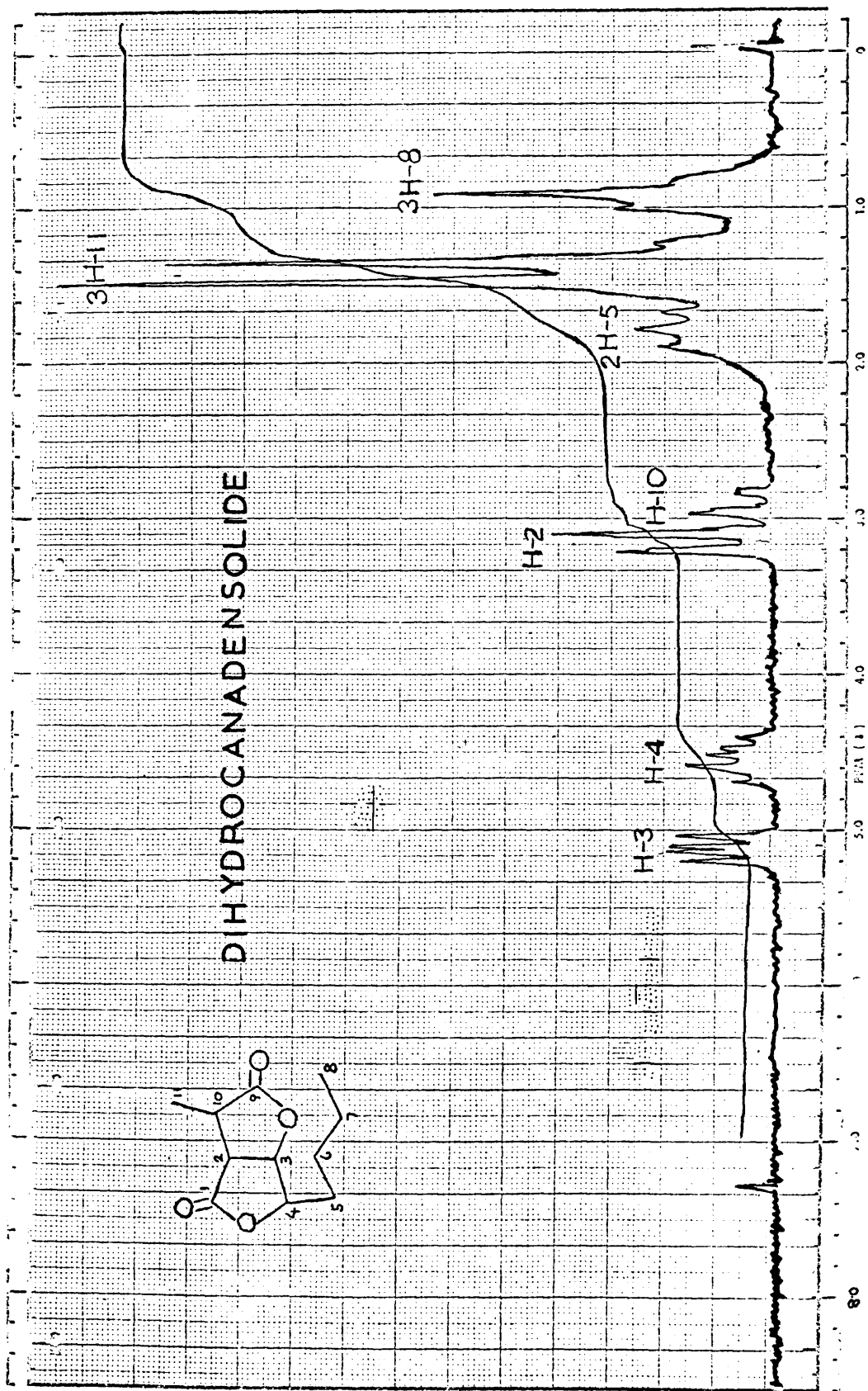


Fig. 7.

spectrum of canadensolide except for the allylic proton (H-2) of the antibiotic. This appeared at higher τ value than the corresponding proton in canadensolide as a doublet, superimposed upon the quartet of the other proton α to carbonyl (H-10). Spin decoupling studies, similar to those carried out on canadensolide, established the structure (54) for dihydrocanadensolide. (The n.m.r. data for this metabolite is given in table 1.).

The structure was further corroborated by the mass spectral data. Dihydrocanadensolide undergoes the same type of cleavage as canadensolide giving a series of mass spectral ions which differ by two mass units from the corresponding ions in the spectrum of canadensolide (Table 2). Dihydrocanadensolide (54) showed a parent ion at m/e 212 (m/e 210, for canadensolide), and the characteristic loss of the butyl side chain (M-57) resulted in an ion at m/e 155 (m/e 153, for canadensolide). Cleavage of ring A would again account for the intense ion at m/e 112 and the base peak at m/e 98 in the mass spectrum of dihydrocanadensolide. The corresponding ions in canadensolide occurred at m/e 110 and m/e 96.

Interconversion of these two metabolites was also reported. Thus catalytic reduction of canadensolide (51) gave a product which was more polar than the antibiotic. The n.m.r. spectrum

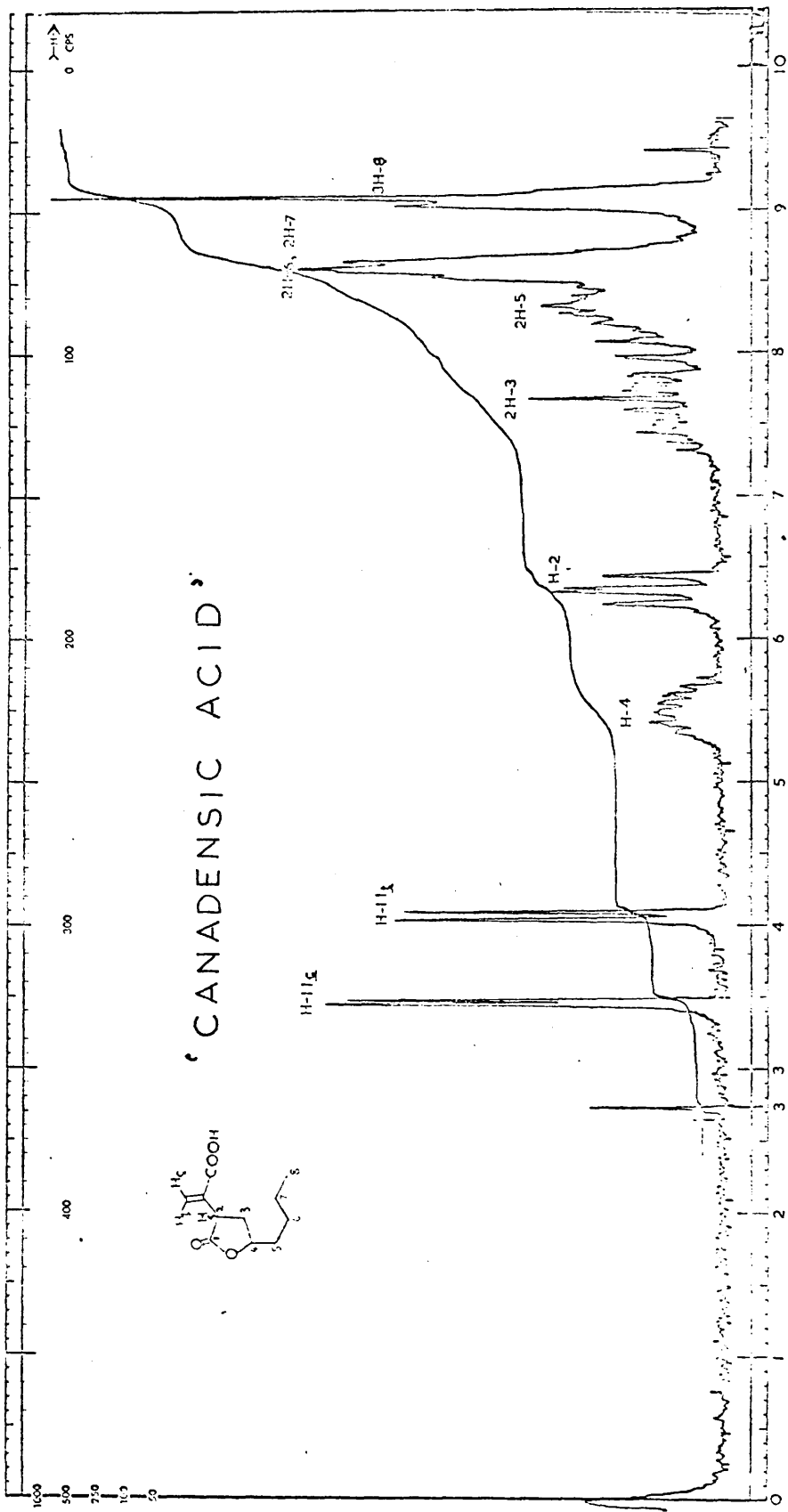


fig. 8.

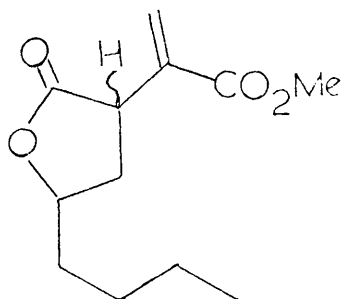
showed that reduction of the terminal methylene group had been accompanied by the reduction of one or both lactone rings to give a mixture of lactols. Following oxidation of the mixture with Jones' reagent dihydrocanadensolide (54) was isolated as the major product. (This result will be discussed in more detail in the following section).

The third substance isolated by Wright was a crystalline acid, $C_{11}H_{16}O_4$, which was named "canadensic acid". This was shown in the present work to be a 1:1 mixture of the naturally occurring canadensic acid with its epimer formed under the conditions used by Wright in the isolation procedure (cf. chapter 3).

It was possible to postulate structures (55) for the mixture of epimeric acids from the data obtained. The i.r. spectrum showed absorption at $3,200-2,600\text{ cm}^{-1}$ and carbonyl absorption at 1700 cm^{-1} ($\epsilon\ 396\ \Delta\nu_{\frac{1}{2}}\ 20\text{ cm}^{-1}$) indicative of the carboxylic acid grouping. Further, absorption at 1768 cm^{-1} ($\epsilon\ 522,\ \Delta\nu_{\frac{1}{2}}\ 28\text{ cm}^{-1}$) and 1630 cm^{-1} ($\epsilon\ 11$) indicated the α -lactone function and conjugated terminal methylene group respectively. The conjugation was confirmed by the u.v. spectrum which showed high intensity absorption at 214 nm.

Of particular importance in structure determination was the n.m.r. spectrum (fig. 8) to which assignments were made as

indicated. The resonances were more complex than in the n.m.r. spectra of canadensolide (51) and dihydrocanadensolide (54) mainly due to the presence of C-2 epimers. The most interesting feature in this spectrum was what appeared to be the "two doublets" at 3.47 and 4.07 τ (each 1H), which were assigned to the two protons of the terminal methylene group. Each doublet is in fact comprised of two singlets corresponding to the resonance of one terminal methylene proton from each epimer. Evidence that these were not those of two rotamers (caused by restricted rotation about the C-2 — C-10 bond for example) was provided by high temperature n.m.r. experiments when no collapse of the two doublets was observed. The resonance of the proton geminal to oxygen (H-4) appeared as the complex multiplet at 5.5 τ , while that of the proton α to carbonyl and allylic (H-2) occurred as the double doublet at 6.38 τ . The complex region from 7.3 — 8.2 τ (2H) was assigned to the protons β to carbonyl (H-3). The remaining resonances from 8.2 — 9.2 τ exhibited the typical resonance pattern and integral expected of a n-butyl group. These assignments were supported by decoupling evidence (Table I). Support for the presence of the n-butyl side chain was again provided by the mass spectrum which showed an intense ion at



57

155 (M-57) due to cleavage of this group.

Confirmation of the presence of the terminal methylene group in the canadensic acid epimers was provided by ozonolysis, when formaldehyde dimedone was isolated as its dimedone derivative in reasonable yield. Similarly, hydrogenation of the mixture of acid epimers resulted in the uptake of two moles of hydrogen, to give a mixture of dihydrocanadensic acid stereoisomers (56), $C_{11}H_{18}O_4$, ν_{max} . 1764 cm^{-1} (α -lactone) and 1711 cm^{-1} (CO_2H). Its n.m.r. spectrum confirmed the disappearance of the resonances of the terminal methylene protons present in the mixture of canadensic acid epimers, and the presence of a secondary methyl group β to carbonyl at 8.77 τ (doublet $J = 7.0$ Hz.). This 3H doublet was found to collapse to a singlet on irradiation of a 2H proton multiplet at 6.92 τ assigned to the protons α to carbonyl (H-2 and H-10). This supplied further proof as to the type (terminal methylene group) and position (α, β to a carbonyl) of the double bond present in the mixture of canadensic acid epimers (55).

Attempts were made to separate the two canadensic acid epimers (55) as their methyl ester derivatives. Esterification using the methyl iodide, potassium carbonate, acetone procedure⁵⁹ afforded the mixture of methyl canadensate C-2 epimers (57)

as an oil, ν_{\max} . 1767 cm.^{-1} (ϵ 540) and 1721 cm.^{-1} (ϵ 430) corresponding to the δ -lactone system and unsaturated methyl ester group respectively, and at 1633 cm.^{-1} corresponding to the terminal methylene group. This was confirmed by the n.m.r. spectrum which showed the 3H singlet at 6.15 τ corresponding to the resonance of the methoxyl group, and the characteristic pair of 'doublets' at 3.4 and 4.0 τ due to the resonances of the two terminal methylene protons. The mass spectrum showed a parent at m/e 226 and ions at m/e 195 (M-31) and m/e 169 (M-57) corresponding to the anticipated losses of methoxyl and the side chain group. The mixture of epimeric methyl esters, however, failed to separate on p.l.c. and on g.l.c. using various columns. The isolation of pure natural canadensic acid is described in a later section.

CANADINESOLIDE AND DIHYDROCANADINESOLIDE.

Table 2.

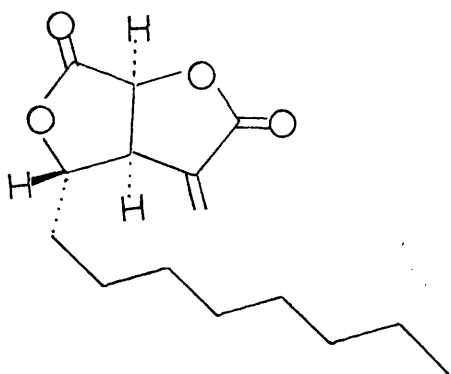
Application of the Karplus equation to the calculation of coupling constant.

<u>Canadensolide</u>						
	θ cis	J cis	θ trans	J trans	J observed	
H-2/H-3	ca. 12°	ca. 9.5 Hz.	ca. 170°	ca. 14 Hz.	6.5 Hz.	
H-3/H-4	ca. 35°	ca. 6.5 Hz.	ca. 115°	ca. 3.5 Hz.	4.5 Hz.	
<u>Dihydrocanadensolide</u>						
	θ cis	J cis	θ trans	J trans	J observed	
H-2/H-10	ca. 15°	ca. 9 Hz.	ca. 110°	ca. 1 Hz.	1.0 Hz.	
H-2/H-3	ca. 15°	ca. 9 Hz.	ca. 170°	ca. 14 Hz.	6.0 Hz.	
H-3/H-4	ca. 30°	ca. 7 Hz.	ca. 115°	ca. 3.5 Hz.	4.0 Hz.	
<u>10-epidihydrocanadensolide.</u>						
	θ cis	J cis	θ trans	J trans	J observed	
H-2/H-10	ca. 15°	ca. 9 Hz.	ca. 110°	ca. 1 Hz.	10.5 Hz.	
H-2/H-3	ca. 15°	ca. 9 Hz.	ca. 170°	ca. 14 Hz.	6.0 Hz.	
H-3/H-4	ca. 30°	ca. 7 Hz.	ca. 115°	ca. 3.5 Hz.	4.0 Hz.	

At first sight the fused dilactone system of canadensolide (51) appears fairly rigid and extremely suitable for the n.m.r. determination of its relative stereochemistry using the observed coupling constants and the Karplus equation⁶⁰. There is, however, a certain amount of flexibility in the molecule and hence dihedral angles were calculated for the conformation involving the least amount of non-bonded interactions.

The first conclusion which can be drawn from a model of the antibiotic is that the two lactone rings must be cis-fused, since it is almost impossible to construct the severely strained trans-fused system. Besides the dihedral angle between H-2 and H-3 in this latter system would approach 170° , for which a large coupling constant (ca. 14 Hz) would be expected, more than double the observed value (6.5 Hz.).

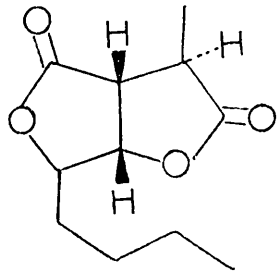
If the rings are cis-fused, the dihedral angle between protons H-2 and H-3 appears from models to be around 12° for which a predicted coupling constant of 9.5 Hz. is obtained from the Karplus equation. Since the presence of electronegative substituents is known to decrease the coupling constant⁶¹ between vicinal protons, this is in much better agreement with the observed value (cf. Table 3). If protons H-3 and H-4 are cis to each other, the dihedral angle in the most stable conformation



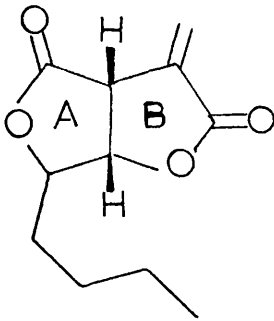
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would appear to be ca. 35° giving a postulated coupling constant of 6.5 Hz. while for the structure in which H-3 and H-4 are trans the theoretical value for the coupling constant is ca. 3.5 Hz. The observed value for this coupling constant is 4.5 Hz. From this data it is very difficult to predict whether these protons are cis or trans since the Karplus equation is only an approximation and must be treated with reserve in view of distortion of bond angles and lengths. However, since both carbon atoms in question carry electronegative substituents which, as stated previously, should decrease the size of these predicted coupling constants the cis configuration would appear more likely. On the other hand, it should be noted that the analogous trans orientated protons of avenaciolide (58) have a similar coupling constant (4.0 Hz.)⁵⁷, the calculated values for this molecule are J_{cis} (ca. 15°) 9.0 Hz. and J_{trans} (ca. 120°) 5.0 Hz.

A similar treatment of dihydrocanadensolide (54) was also carried out (Table 3), but again it was not possible to assign with certainty the relative stereochemistry of H-3 and H-4, although the trans configuration of H-2 and H-10 was established. Thus if protons H-2 and H-10 are cis to each other the dihedral angle appears to be ca. 15° giving a predicted coupling constant



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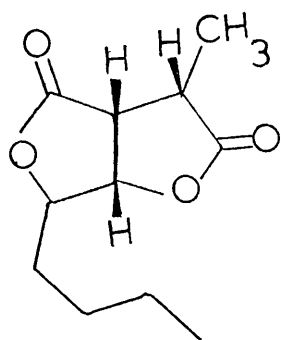


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of 9.0 Hz. The dihedral angle for the trans configuration is ca. 110° , giving a predicted value of 1 Hz. close to that observed (1.0 Hz.), proving dihydrocanadensolide has the relative stereochemistry at C-2 and C-10 shown in (59).

A chemical investigation of canadensolide (51) was therefore, initiated with a view to determining the unknown relative and absolute stereochemistry of the molecule as well as that of dihydrocanadensolide (54). Examination of a model of canadensolide (51) suggested that hydrogenation would probably be stereospecific. Adsorption of the antibiotic onto the catalyst surface must occur from the least hindered side, from above the plane of lactone ring assuming the absolute stereochemistry depicted in (60) and proved later. The dominating factor is the steric size of the atoms comprising lactone ring A as opposed to the methine protons of the bridge. The stereochemistry of the butyl group would appear to be relatively unimportant as it is the non-conjugated lactone ring itself which prevents the molecule being adsorbed flat onto the catalyst surface, if approach from the α side occurs.

This theory was fully borne out when hydrogenation of canadensolide was carried out using glacial acetic acid with 5% palladium on charcoal as catalyst. A crystalline solid of



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identical R_f to canadensolide (yellow/brown with 'ceric') but of similar staining to dihydrocanadensolide (purple/brown with 'ceric') was obtained. No dihydrocanadensolide appeared to have been formed.

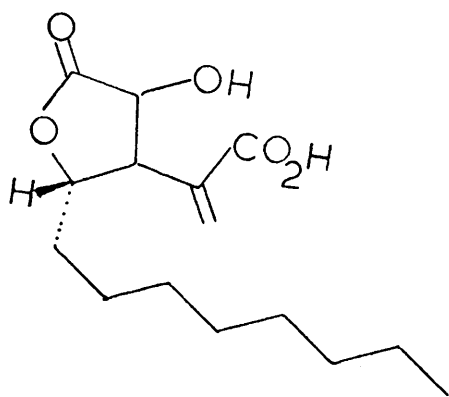
The i.r. spectrum (K.Br) of this compound, $C_{11}H_{16}O_2$, lacked the absorption of the exocyclic double bond present in the i.r. spectrum of canadensolide (51). Peaks at 1780 and 1762 cm^{-1} indicated the presence of two α -lactone groups, while the remainder of the spectrum was similar but not identical to that of dihydrocanadensolide (54). That it was epimeric at C-10 with natural dihydrocanadensolide (54) was confirmed by a comparison of their n.m.r. spectra. These were almost superimposable except that the protons on carbon atoms α to carbonyl, H-2 and H-10, which appeared essentially as a doublet and quartet respectively in dihydrocanadensolide (54), now appeared as a double doublet at 6.53 τ ($J=6.0, 10.5$ Hz.) and a double quartet ($J= 7.5, 10.5$ Hz.) at 6.93 τ respectively. The large coupling constant between these protons (10.5 Hz.) in the hydrogenation product indicates a cis relationship (calculated J value 9.0 Hz.) (cf. Table 3). This latter dihydroderivative of canadensolide (51) is hence 10-epidihydrocanadensolide (61).

As mentioned earlier Wright reported an interconversion of canadensolide (51) and dihydrocandensolide (54) by hydrogenation of the antibiotic in ethanol employing 5% palladium on charcoal

as catalyst. This was concluded to have reduced not only the double bond but also the lactone rings to give a mixture of lactols. This deduction was supported by i.r. and n.m.r. spectral evidence. Subsequent oxidation of this mixture of lactols with Jones reagent and p.l.c. gave dihydrocanadensolide in 20% overall yield. This material had identical i.r., m.s. and mixed m.p. to that of an authentic sample.

As this data appeared to be contrary to the high stereospecificity encountered above, an attempt was made to repeat this experiment described by Wright. Hydrogenation of canadensolide was carried out in ethanol using 5% palladium on charcoal. The material obtained, however, was again pure epidihydrocanadensolide. No polar material was in evidence and no trace of dihydrocanadensolide was apparent (by g.l.c., t.l.c., n.m.r.).

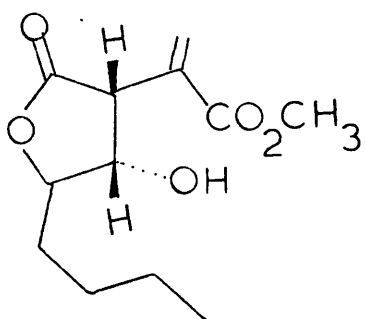
It is obvious that the particular grade of catalyst used in the earlier work did reduce the lactone rings on overnight hydrogenation to lactols. These might have reduced the stereospecificity of hydrogenation of the double bond by involvement of the open chain hydroxyaldehyde tautomer thus allowing formation of some dihydrocanadensolide (54). It does, however, seem surprising to say the least that reduction of the lactone to a lactol should have occurred prior to reduction of the double bond.



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In view of the difficulty in detecting small amounts of dihydrocanadensolide (54) present in canadensolide (51), the sample of the antibiotic used for the reaction could have been contaminated. This might account for this result if the yield of dihydrocanadensolide (54) (4 mg.) in the small scale reaction (20 mg.) carried out by Wright was overestimated. 10-epidihydrocanadensolide (61) formed in the reaction could possibly have been mistaken for unreacted canadensolide (51) due to their identical R_f values. Re-examination of the reported results of this experiment showed that a substantial quantity of material with the same R_f and staining characteristics as 10-epidihydrocanadensolide (61) was in fact present⁶². Reproducible conversions of canadensolide (51) to dihydrocanadensolide (54) were, however, achieved in the manner described below.

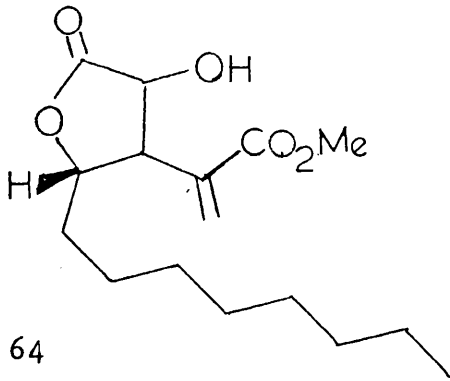
The double bond exocyclic to the lactone ring in canadensolide (51) must introduce a certain amount of strain into the molecule. This would be relieved by the ring opening. Thus the antifungal antibiotic avenaciolide (58) on mild alkaline hydrolysis formed the hydroxy acid (62), which regenerated avenaciolide (58) on refluxing in ethyl acetate⁶¹. Canadensolide (51), however, appeared to be relatively base stable. On alkaline hydrolysis (refluxing $N/10$ NaOH) two equivalents of



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alkali were consumed, but acid treatment resulted in recyclisation to the antibiotic although a trace of acidic material was detected.

Methanolysis of canadensolide (51) was slightly more successful. Treatment with methanol containing sulphuric acid as catalyst at R.T. for seven days partially converted canadensolide into a more polar compound believed to be the desired hydroxyester. (Attempts to increase the yield of this compound by prolonged heating only resulted in the formation of a product less polar than canadensolide (51) which will be discussed later). Attempts to obtain a pure sample of this polar compound were unsuccessful because of decomposition on t.l.c. In attempts to isolate the postulated hydroxyester (63) as a stable derivative various acetylation procedures were carried out, again without success. However, evidence that ring opening had taken place was shown by the i.r. spectrum (thin film) of the crude reaction mixture of canadensolide (51) and polar compound. Absorption at $3,490 \text{ cm}^{-1}$ indicated the presence of a hydroxyl group, while in addition to the normal γ -lactone frequency at 1770 cm^{-1} , a peak at 1715 cm^{-1} , characteristic of an α, β unsaturated ester was apparent. A new double bond absorption also appeared at 1638 cm^{-1} in addition



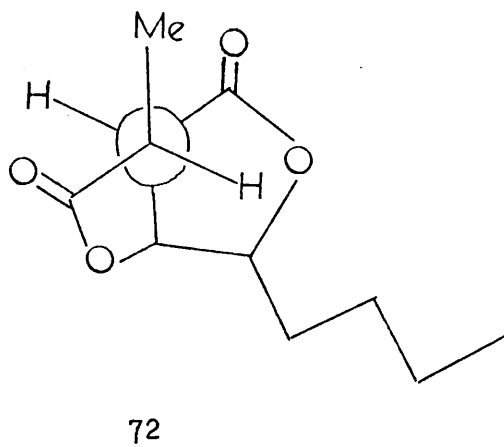
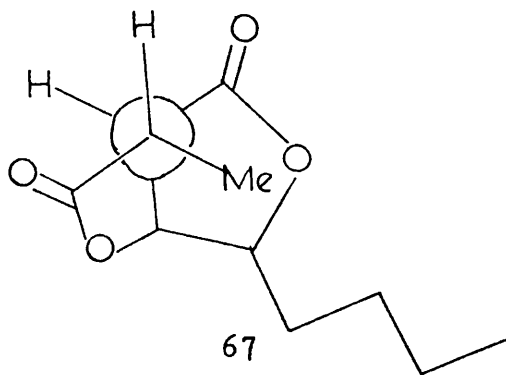
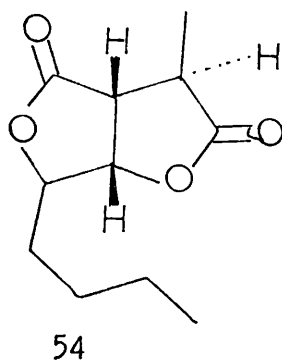
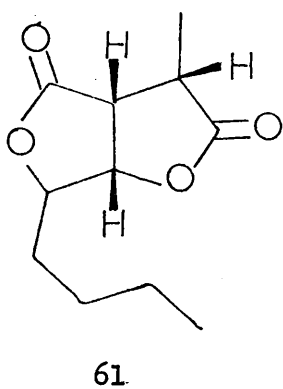
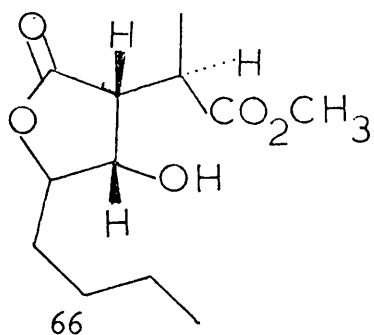
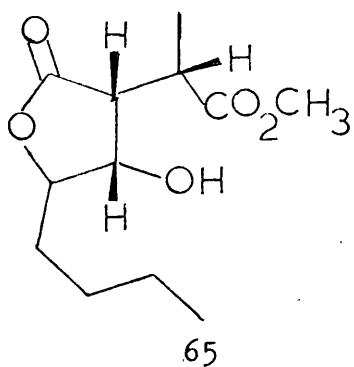
64

to that of the exocyclic methylene group of canadensolide at 1660 cm.^{-1} .

Confirmation that the hydroxyester (63) had been formed was obtained from the n.m.r. spectrum of a methanolysis mixture which contained approximately 50% of canadensolide (51), a typical yield for a short reflux in methanol. The resonances of the conjugated double bond appeared as one proton singlets at 3.33 and 3.90τ , while a 3H singlet at 6.15τ indicated the presence of the methyl ester group. The remaining resonances of the hydroxy ester (63) could not be distinguished from those of the canadensolide (51) present in the mixture. Avenaciolide (58) under similar conditions was found to form⁶¹ the hydroxy methyl ester (64) in good yield exhibiting carbonyl absorption at 1782 cm.^{-1} and weak double bond absorption at 1635 cm.^{-1} .

The poor overall conversion of canadensolide into the hydroxy ester can be explained if recyclisation readily occurs by what is essentially a trans esterification mechanism. The result would be an equilibrium mixture of canadensolide and hydroxyester in the methanol solution.

This was demonstrated by refluxing a crude sample of ester in an inert solvent (benzene) when recyclisation took place



to give canadensolide in good yield. The ready cyclisation of the hydroxyester (63) confirms the cis relationship of the C-2 and C-3 substituents deduced on the basis of the n.m.r. spectrum.

An important result of this ring opening to give the hydroxy ester (63) would be that the double bond is no longer held in a rigid configuration (as in canadensolide). Free rotation about the carbon-carbon bond joining the double bond to the ring means that hydrogenation should no longer be stereospecific. Both the 10S(65) and 10R(66) dihydrohydroxy esters should be formed. These should readily cyclise to form 10-epidihydrocanadensolide (61) and dihydrocanadensolide (54) respectively.

Direct hydrogenation of a methanolysis reaction solution with 5% palladium on charcoal as catalyst gave two compounds of similar R_f as the only major products. No hydroxy ester (63) appeared to remain. P.l.c. gave epidihydrocanadensolide (61) and a 20% yield of dihydrocanadensolide (54) which had identical i.r., m.pt. and mixed m.p. to authentic samples.

This reaction thus realised the predictions concerning the hydroxy ester (63) and provided the first reproduceable interconversion of canadensolide (51) and dihydrocanadensolide

(54). A more efficient means of interconversion was, however, achieved as follows.

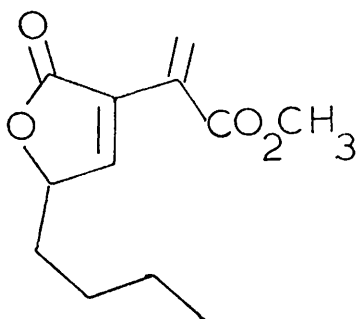
One of the effects of the cis orientation of the protons at C-2 and C-10 in epidihydrocanadensolide (61) is to increase the non-bonded interactions at these centres relative to dihydrocanadensolide. This can best be represented by the Dewar formula for 10-epidihydrocanadensolide (67) and dihydrocanadensolide (72) in which we look along the (C-10) - (C-2) bond. From this it can be seen that dihydrocanadensolide lies in a natural anti configuration as compared to the skew configuration of epidihydrocanadensolide, and is hence thermodynamically more stable.

Epimerisation at C-10 should thus produce an equilibrium mixture in which the more stable epimer, dihydrocanadensolide, should predominate. Epimerisation at the bridge head proton C-2 is, of course, prevented by the requirement of a cis fused lactone system.

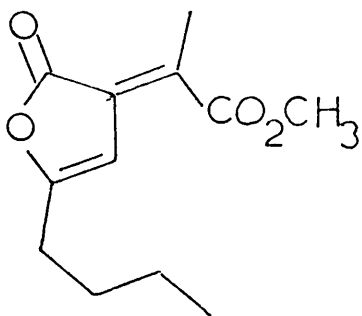
Epidihydrocanadensolide was found to be unaffected on stirring with anhydrous potassium carbonate in analar acetone (a reagent often used for epimerising α to a ketone⁶³). The more powerful base methanolic potassium hydroxide, however, carried out the equilibrium in a few hours. Comparison of the relative quantities was obtained using g.l.c. with a 1% S.E. 30 column when

the ratio of dihydrocanadensolide (R_t 11.0 mins.) to 10-epidihydrocanadensolide (R_t 13.7 mins.) was found to be 4.5/1. Pure dihydrocanadensolide was obtained on crystallization from benzene light-petroleum in 70% yield, and was identical in every respect with an authentic sample. It will be seen later that this interconversion represents a vital link in establishing the relative and absolute stereochemistry of canadensolide (51).

Although the unstable monolactone derivative (63) of canadensolide (51) had been prepared as discussed above, it was desirable to prepare stable monolactone derivatives with a view to establishing the absolute stereochemistry. This was finally achieved by treatment of the antibiotic with silver oxide and methyl iodide in dimethyl formamide to give a high yield of a less polar product, which was identical on t.l.c. to the by-product obtained on methanolysis of canadensolide (51) under forcing conditions referred to earlier. This compound was found to be an oil, $C_{12}H_{16}O_4$, whose u.v. spectrum showed strong absorption at 236 nm. (ϵ 8,500) attributed to the π system of a conjugated diene or a fumarate type chromophore. The i.r. spectrum (CCl_4) showed no hydroxyl absorption but had a single peak at 1768 cm.^{-1} corresponding to an α, β unsaturated



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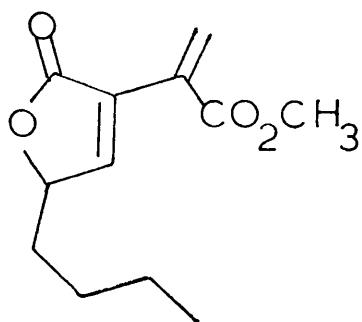
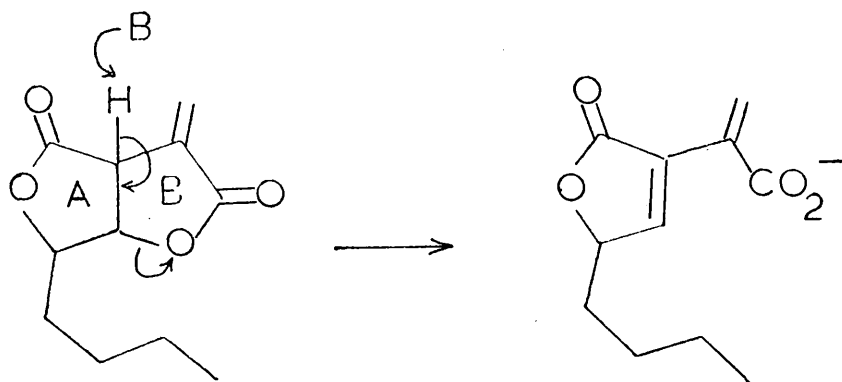


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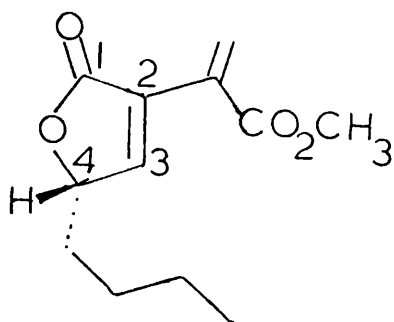
γ -lactone. The presence of an α, β unsaturated ester was deduced from absorption at 1732 cm.^{-1} , although no peak above 1600 cm.^{-1} attributable to a double bond appeared to be present. The structure would thus appear to be the diene (68) or (71).

The n.m.r. spectrum did not exhibit a vinyl methyl resonance and was consistent with the former diene structure. The olefinic proton of the lactone ring occurred at low field (2.07τ) in accordance with its being the proton of an α, β -unsaturated carbonyl system as well as part of a diene system. It was slightly coupled ($J = 1.5 \text{ Hz.}$) to the proton geminal to oxygen (H-4) which appeared as a multiplet at 5.03τ . The two protons of the terminal methylene group appeared as 1H singlets at 2.95 and 3.40τ , while the 3H singlet at 6.15τ confirmed the presence of the carbomethoxyl grouping. Like the various derivatives of canadensolide and dihydrocanadensolide to be described, this ester showed resonances of an n-butyl group similar to those found for canadensolide. The mass spectrum of the diene (68) was likewise in accord with its structure. In contrast to normal γ -lactones, cleavage at the γ -carbon atom was not particularly favoured, but loss of the methyl acrylate grouping could account for the ion at m/e 139 (21%), while charge

Diag. 2 Mode of formation of diene (68)



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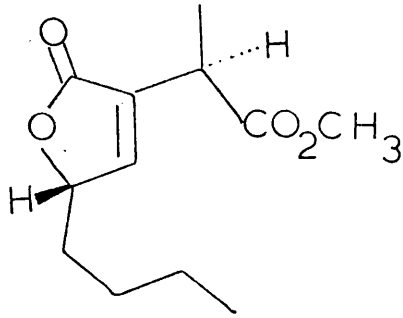
retention by this group would give the ion at m/e 85 (71%). Loss of butylene (56 mass units) from the m/e 139 ion could result in the ion at m/e 83, the base peak in the spectrum.

The formation of this diene (68) is explained by

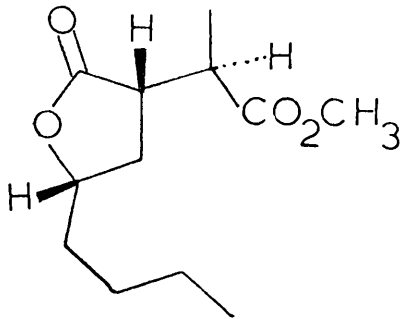
β -elimination of lactone ring B, initiated by proton abstraction adjacent to the carbonyl of lactone ring A as shown (diag. 2), the carboxylate anion acting as a good leaving group. Subsequent esterification by methyl iodide would give the isolated product. The traces of diene (68) formed in the methanolysis reaction can be explained by simple dehydration of the hydroxy ester (63) in the acidic medium. It was also formed in poorer yield on attempted acetylation of the hydroxy ester (63) in pyridine where a base catalysed elimination most probably occurs.

The diene (68) was important in that it contained only one of the asymmetric centres in canadensolide (51). The absolute stereochemistry at C-4 was determined from the c.d. and o.r.d. curves of the diene (68), which established it as the $4S$ configuration shown (cf. Chapter 5). This result was to prove important in determining the relative stereochemistry of C-3 and C-4 in canadensolide (51).

A similar β -elimination reaction could also be carried out on dihydrocanadensolide (54) using a large excess of silver



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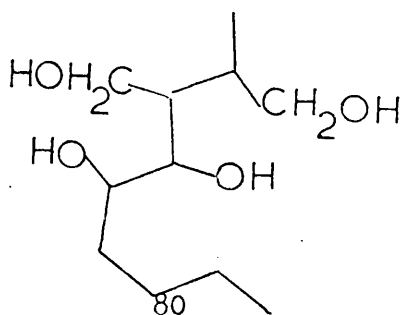
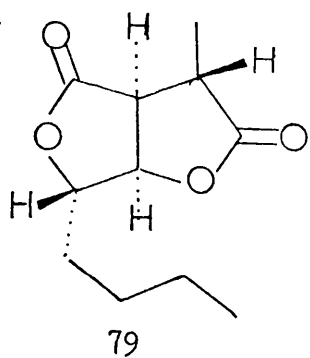


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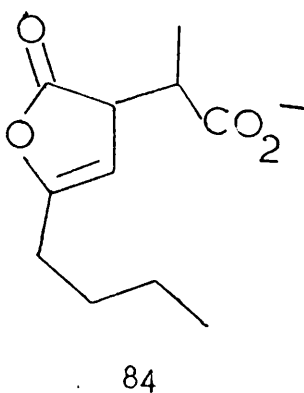
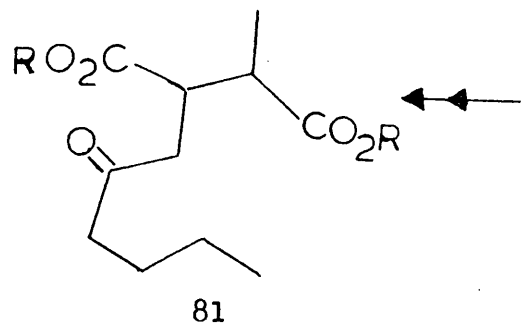
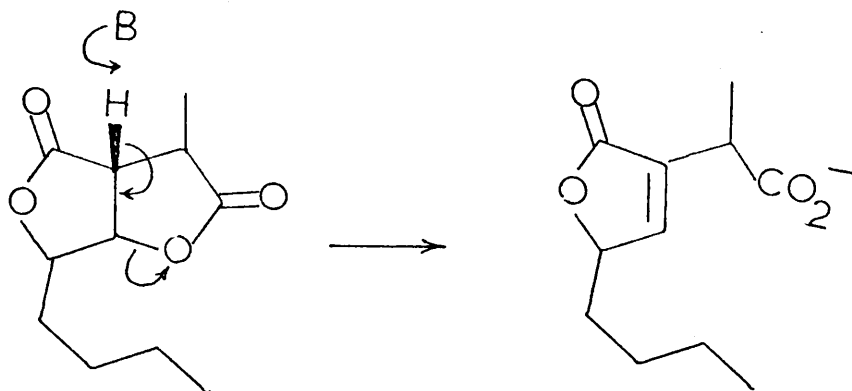
oxide in dimethyl formamide with methyl iodide to give a high yield of an oil consisting of the corresponding butenolide ester (69). Presumably due to the slight decrease in acidity of the proton on C-2 (as well as an increased steric hindrance to approach of the reagent) the reaction was much slower than in the case of canadensolide (51) and required overnight stirring. (Selective β -elimination of the lactone ring of the antibiotic could in fact be achieved if a 1:1 mixture of canadensolide (51) and dihydrocanadensolide (54) was subjected to these conditions). The butenolide ester structure (69) for this oil was supported by strong i.r. peaks (CCl_4) at 1770 and 1730 cm^{-1} assigned to an unsaturated γ -lactone and methyl ester group respectively. The u.v. spectrum showed strong absorption (ϵ 9,750) at 215 nm. corresponding to the butenolide chromophore. In the n.m.r. spectrum, the olefinic proton (H-3) appeared as a double doublet ($J_1=J_2=2.0\text{Hz.}$) at low field (2.80 τ), and was coupled to the proton geminal to oxygen (H-4) (dt at 5.07 τ), and the proton H-10 (dq at 6.47 τ), these assignments being confirmed by decoupling experiments. The methyl ester grouping was indicated by the 3H singlet at 6.30 τ , while the 3H doublet ($J=7.5\text{Hz.}$) at 8.57 τ confirmed the presence of the secondary methyl group

(3H-11). The mass spectrum was also in agreement with the postulated structure. Hydrogenation of the butenolide ester (69) gave the dihydro ester (70) as an oil, $C_{12}H_{20}O_4$. Saturation of the double bond of the butenolide (69) was confirmed by the u.v. spectrum, which had no absorption maximum above 200 nm. and the higher lactone carbonyl frequency (1775 cm.^{-1}) shown in the i.r. spectrum (thin film). The n.m.r. spectrum showed no resonance which could be assigned to an olefinic proton, and proton H-4 which is no longer deshielded by the double bond, appeared as a 1H multiplet at 5.62τ . The two protons, H-2 and H-10, which are on carbon atoms α to carbonyl, appeared as a 2H multiplet at 7.00τ . A 3H doublet ($J=7.0\text{Hz.}$) at 8.59τ confirmed the presence of the secondary methyl group (3H - 11). The mass spectrum showed significant intense ions at m/e 228 (M^+), 197 ($M^+-\text{OMe}$), 171 ($M^+-C_4H_9$), 142 ($M^+-\text{CH}_2=\text{CHCO}_2\text{Me}$ by McLafferty rearrangement⁶⁴), 139 (171^+-MeOH), 111 (139^+-CO) and 88 ($\text{MeCH}=\text{C}(\text{OH})\text{OMe}^+$, base peak, by McLafferty rearrangement⁶⁴).

This dihydro ester (70) is depicted as having the cis stereochemistry for protons H-2 and H-4 because adsorption of the butenolide ester (69) onto the catalyst surface must occur from the less hindered β side of the molecule. No trace of any diastereoisomer could be detected in the n.m.r. spectrum, and

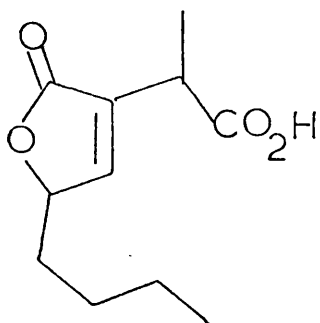


Scheme 1 Formation of keto acid (81, R=H)

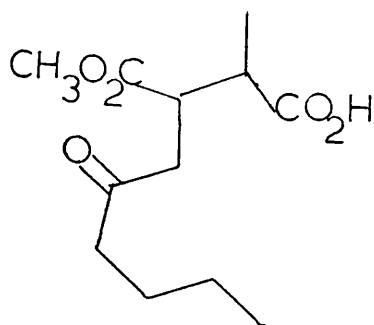


g.l.c. established that none of the 2R, 10S dihydro ester was present (cf. Chapter 3).

Following the publication of a preliminary communication⁶⁵ recording the findings of Dr. J.L.C. Wright on canadensolide (51) and dihydrocanadensolide (54), we learnt from Dr. R. Rickards that he and his co-workers had isolated a dilactone from the fungus Aspergillus indicus and comparison showed this compound to be identical to dihydrocanadensolide (54). They had arrived at the same tentative structure (79) independently⁶⁶. Proof of the presence of the a glycol structure and hence of the butyl side chain was provided in their case by lithium aluminium hydride reduction of dihydrocanadensolide (54) to a tetrol (80), followed by periodate cleavage to give n-valeraldehyde which was isolated as its 2,4-dinitrophenylhydrazone. In addition, they had found that an excess of sodium in methanol converted dihydrocanadensolide (54) into the keto dicarboxylic acid (81, R=H), $C_{11}H_{18}O_5$, ν_{max} . (in $CHCl_3$) 3,600 - 2,300 (CO_2H) and 1715 cm^{-1} ($C=O$ and CO_2H). Sublimation converted this into a keto anhydride, $C_{11}H_{16}O_4$, shown to be a substituted succinic anhydride by infrared absorption (in CCl_4) at 1863 and 1790 cm^{-1} in addition to ketonic absorption at 1720 cm^{-1} . Diazomethane treatment of the acid gave a dimethyl ester (81; R=Me), ($C_{13}H_{22}O_5$, ν_{max} . (in CCl_4) at 1745 cm^{-1} (CO_2Me) and 1723 cm^{-1} ($C=O$).



82



83

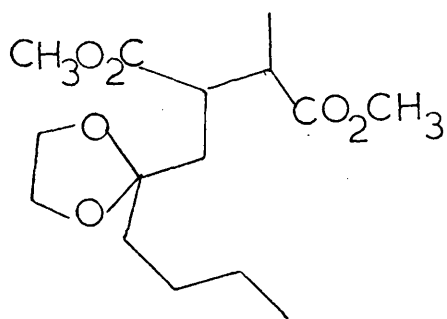
The keto acid (81, R=H) was envisaged as arising by a similar base catalysed β -elimination of ring B to that previously described for dihydrocanadensolide (54). The intermediate α, β butenolide acid was, however, converted by double bond isomerisation and hydrolysis to the isolated acid (81, R=H) (Scheme 1). An analogous reaction had been encountered by the same group in the base hydrolysis of antimycin A⁶⁷.

Since the α, β butenolide acid is thermodynamically more stable than its β, γ isomer and the reaction would appear to be driven to the side of the keto acid (81, R=H) by hydrolysis of the latter isomer, we considered that under controlled conditions it should be possible to prepare this α, β butenolide acid (82) using this reagent. Therefore, dihydrocanadensolide (54) was subsequently treated with approximately one equivalent of sodium methoxide in dry methanol. After careful work-up, this reaction was found to give an acidic product in moderate yield. This was not the keto dicarboxylic acid (81, R=H), since its i.r. spectrum showed not only absorptions at 3,500 - 2,600 cm.^{-1} and 1720 cm.^{-1} corresponding to a carboxylic acid, but also a peak at 1765 cm.^{-1} indicative of an unsaturated γ -lactone, together with weak double bond absorption at 1640 cm.^{-1} . The u.v. spectrum likewise showed strong absorption at 210 nm. (ϵ 9,340) suggesting that the desired butenolide acid (82) had been isolated. Its n.m.r. spectrum showed a 1H multiplet at 2.40 τ

assigned to the β proton (H-3) of the double bond, coupled to the 1H multiplet at 5.02 τ (H-4) and the 1H double quartet ($J = 6.0, 2.0$ Hz.) at 6.35 τ (H-10). The secondary methyl group appeared as a 3H doublet ($J = 6.0$ Hz.) at 8.55 τ

Esterification of this acid (82) using the methyl iodide-potassium carbonate-acetone procedure gave after p.l.c. the corresponding methyl ester as an oil, which was identical on t.l.c., and in its i.r. and n.m.r. spectra to the butenolide ester (69) obtained directly from dihydrocanadensolide using the silver oxide reagent. Having characterised the butenolide intermediate it was decided to repeat the above reaction on dihydrocanadensolide (54) using the large excess of sodium in methanol employed by Rickards and co-workers⁶⁶.

The acidic product obtained in high yield from this reaction was the keto half ester (83), an oil, whose i.r. spectrum (thin film) showed absorption at 3,400 - 2,600 cm.^{-1} (CO_2H) and a strong band centred at 1720 cm.^{-1} assigned to carbonyl absorption of the ketone, acid and ester groups. The n.m.r. spectrum confirmed the presence of the acid grouping by a 1H resonance at 1.90 τ which was removed on deuteration, and the methyl ester by a 3H singlet at 6.30 τ . A series of unresolved resonances between 6.40 - 7.60 τ (6H) were assigned to protons on carbon atoms α to carbonyl. The



Ethylene ketal from (81, R=Me)

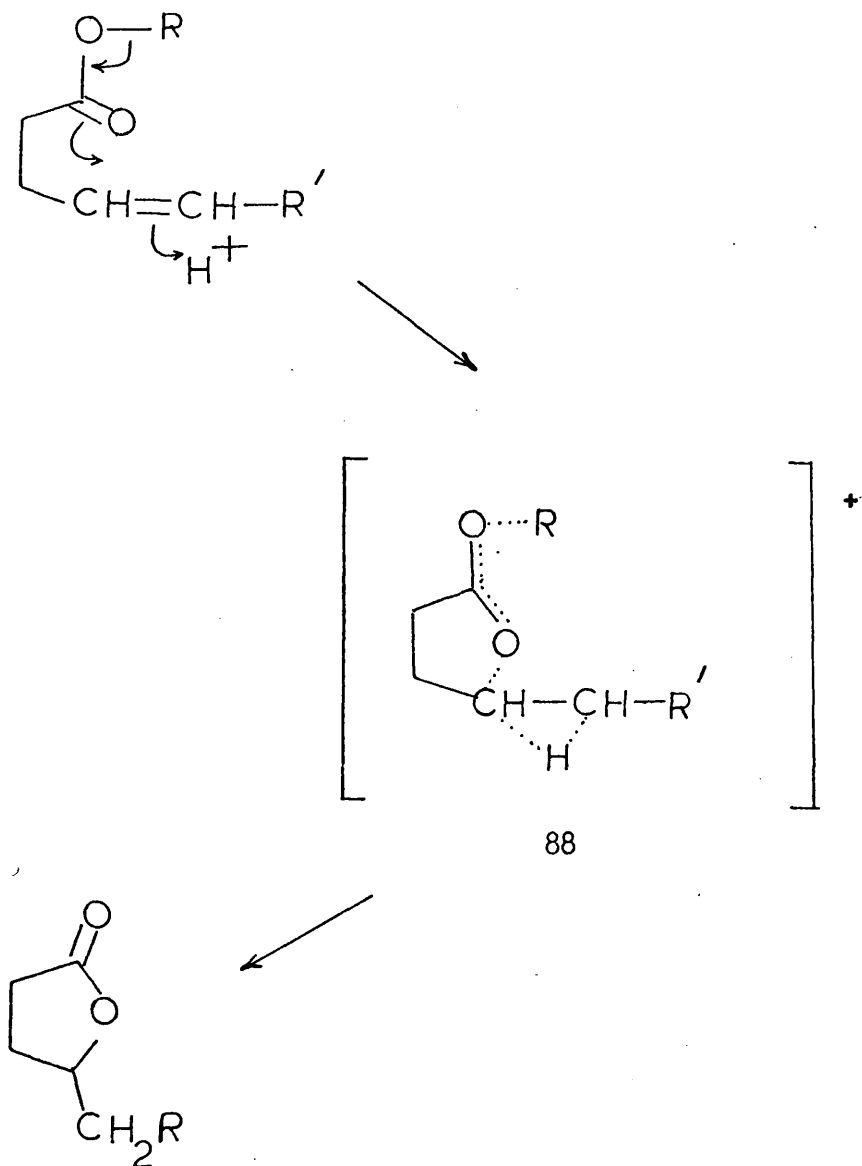
complexity of these proton singals was assumed to be due to higher order coupling and to the presence of C-2 epimers, which also resulted in the secondary methyl group appearing as a broadened 3H doublet ($J=7.0$ Hz.) at 8.80τ . The remaining n-propyl residue was confirmed by resonances at $8.50\tau(4H)$, and $9.10\tau(3H)$. This compound must be formed by methanolysis of the β, γ butenolide (84). Hydrolysis of this ketohalf-ester (83), either by nucleophilic attack by methoxide ion at the methoxyl carbon atom on prolonged reflux or by alkaline (or acidic) conditions on work-up could produce the keto dicarboxylic acid (81, R=H). Only traces of this latter compound were, however, formed in this reaction.

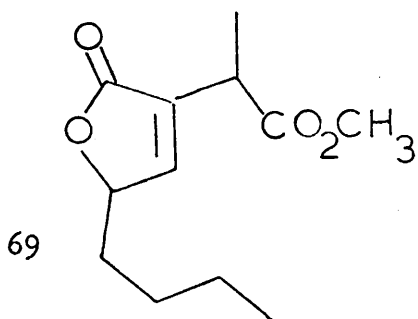
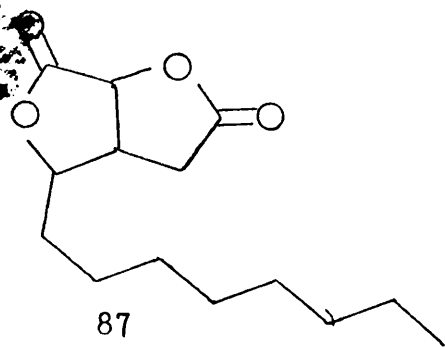
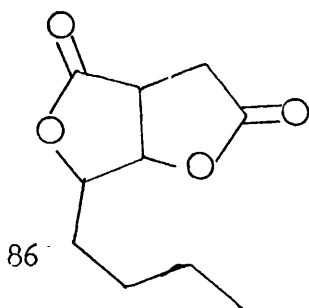
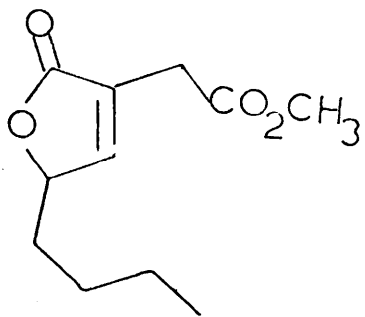
Esterification of the keto half-ester (83) using diazomethane produced the diester, $C_{13}H_{22}O_5$, $\nu_{max.}(CCl_4)$ 1745 cm.^{-1} (CO_2Me) and 1725 cm.^{-1} (ketone), whose n.m.r. spectrum showed similar resonances to that of the former compound (83) except for a 6H singlet due to methoxyl groups at 6.30τ . Comparison of the i.r. and mass spectra of this diester to those reported for the keto dicarboxylic ester (81, R=Me) by Rickards⁶⁶ suggested that they were identical, and this was confirmed by the identity of the fragmentation pattern of the derived ethylene ketal to that reported for the corresponding derivative of (81, R=Me).

During the chemical investigation of canadensolide (51) and dihydrocanadensolide (54) consideration was directed towards a

Diag. 3

Mechanism of cyclisation of unsaturated esters.

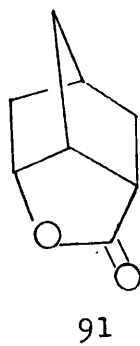
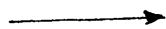
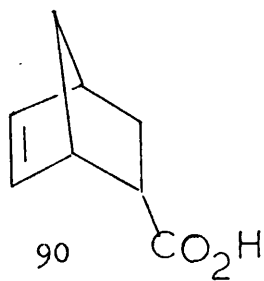
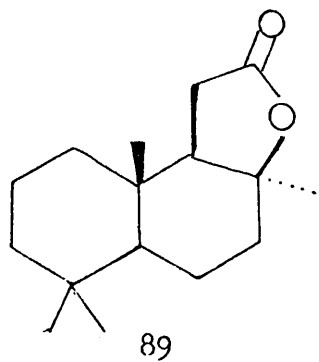
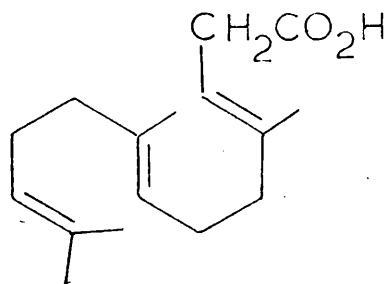




feasible partial synthesis of the antibiotic. The diene (68) or the structurally related butenolide (85) were considered possible synthetic intermediates, as cyclisation of these could produce canadensolide (51) or the dilactone (86). The introduction of an exocyclic methylene group at the requisite position in this latter compound appeared reasonable, since this grouping has been formed at the α carbon atom of unsubstituted δ -lactones⁶⁹. This technique has been successfully applied to the analogous dilactone (87) in the total synthesis⁷⁰ of avenaciolide (58). The butenolide ester (69) was chosen as a model compound for these cyclisation reactions.

The formation of δ -lactones from unsaturated acid and esters has been extensively examined⁷¹. The mechanism favoured for the cyclisation is a concerted attack⁷¹ of the oxygen of carbonyl on the double bond to give the intermediate (88) (diag. 3), which then forms a δ -lactone. Various protonating agents such as sulphuric acid, formic acid, trifluoroacetic acid, etc., have been used to carry out this transformation. Trifluoroacetic acid, however, failed to affect the butenolide acid (87) under various conditions.

Formic/sulphuric acid mixtures have been successfully used in the cyclisation of farnesylacetic acid to the sesquiterpene lactone (89)⁷³, and the unsaturated acid (90) to the endo lactone (91)⁷⁴. The butenolide acid, however, was unaffected by this reagent at or above



room temperature while the methyl ester eventually hydrolysed to the acid after several weeks. The expected product, dihydrocanadensolide, was found to be stable under the reaction conditions.

The failure of the butenolide to cyclise must be due to the oxygen of the acid carbonyl group being too far removed from the nucleophilic centre to enable attack to occur. Steric factors are important in that the two reactive centres must approach sufficiently close for bond formation to occur⁷¹. Examination of a model of the butenolide shows that considerable distortion of the bond angles is required to approach the transition state. The resultant energy barrier must be large enough to prevent this point being reached.

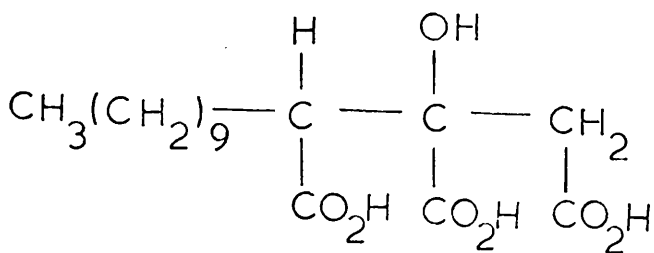
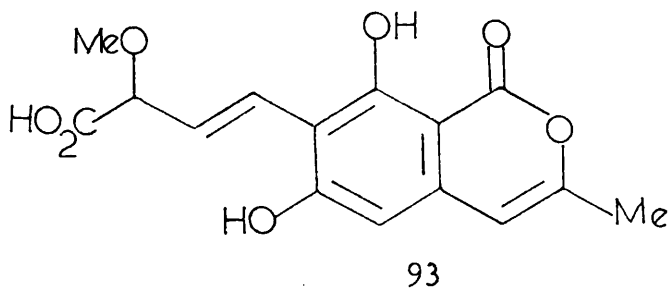
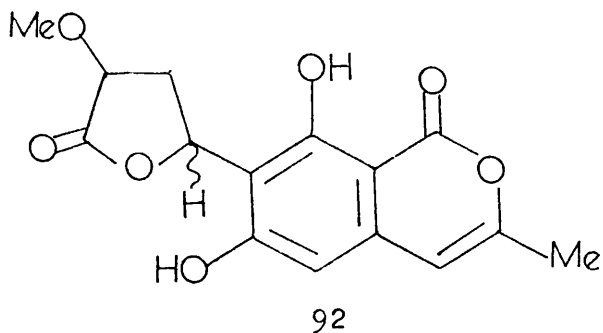
An attempt was also made to hydrate the olefinic bond. Since dihydrocanadensolide (54) is regenerated from a basic solution, the introduction of a hydroxyl group β to the lactone and cis to the acid grouping should lead to cyclisation to the required dilactone. Hydroboration was discounted as this results in an anti-Markownikoff addition which would create a hydroxyl group at C-2. The alternative to hydroboration is an oxymercuration-demercuration procedure which results in the usual Markownikoff hydration⁶⁸. This usually involves the desired trans addition of mercuric acetate to the double bond although in sterically

constrained systems cis addition can occur. The alkoxymercurial is then reduced to the alcohol by alkaline sodium borohydride. Confidence in this reaction stemmed from the fact that crotonic acid was known to form an oxymercurial with the oxygen group attached to the β carbon atom⁷². However, no dilactonic products could be obtained by application of this reaction to the butenolide ester (69). This may have been due to anti-Markownikoff addition as has recently been found in some 2-substituted acrylic esters⁷⁵.

A further attempt at direct hydration of the double bond was made by treating the butenolide ester (69) with dilute acid under various conditions, and also by attempting addition of hydrogen bromide to the double bond with subsequent replacement of bromine with hydroxide. No trace of dihydrocanadensolide (54) could be detected in these reactions.

Thus the butenolide system (69) does not appear to represent a good synthetic intermediate in a route to the dilactone systems of canadensolide (51) or dihydrocanadensolide (54). More promising studies are reported later.

CANADIAN ACID.



The occurrence of both canadensic acid C-2 epimers (55) was viewed at the start of the current investigation with a certain amount of scepticism. Very few secondary metabolites occur in pairs having an epimeric centre as enzymic reactions such as hydration, hydrogenation etc., are usually highly specific. It was, therefore, considered possible that partial epimerisation of the active centre C-2 could have occurred by chemical means, producing the canadensic acid C-2 epimer as an artefact. Some doubt also exists about the validity of canescins A and B (92)⁷⁶, the isomeric γ -lactones from Aspergillus malignus. These are believed to arise from a chemical rather than enzymic, cyclisation of the postulated intermediate (93) in the culture medium. On the other hand, both (+) - and (-) -decylcitric acids (94) have been found as genuine co-metabolites of the fungus Penicillium spiculisporum Lehman⁷⁷. As these latter compounds appear to be closely related biosynthetically to the canadensic acid epimers, the epimeric nature of these metabolites was acceptable.

However, it was found by Dr. W.B. Turner (I.C.I. Pharmaceuticals Limited), who was carrying out biological tests, that broth which had been treated with charcoal still afforded some acidic material upon extraction with ethyl acetate. This material crystallised from benzene - light petroleum as needles m.p. 114° (i.e. 16° lower than

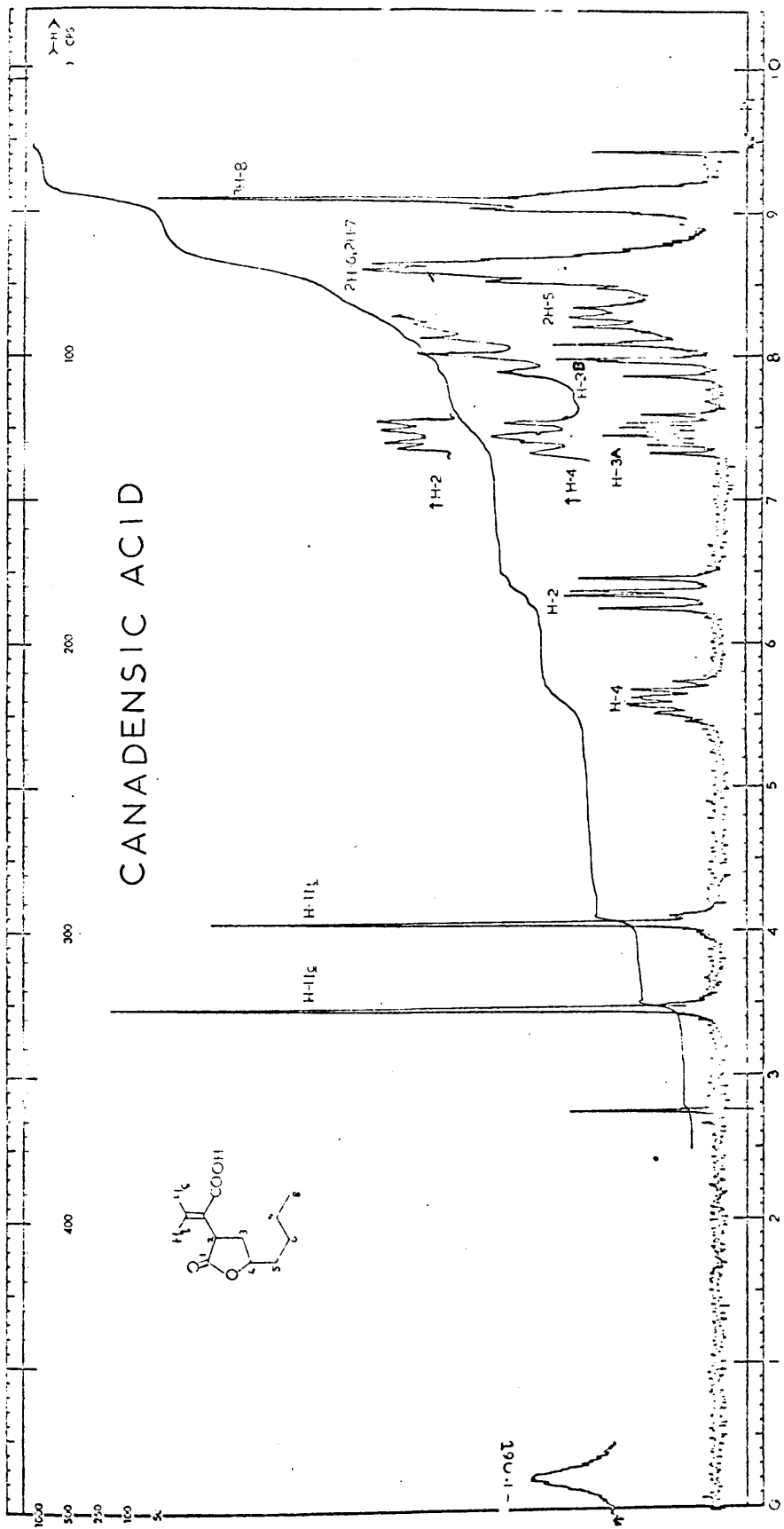
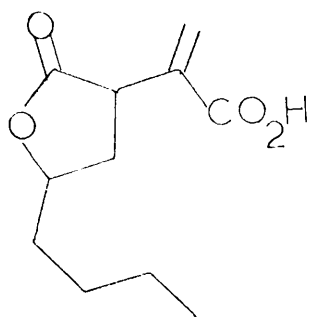


Fig. 6.

the mixture of canadensic acid epimers) and appeared to consist of one epimer. In view of this information, ethyl acetate extraction was used, henceforth, as the means of isolating the broth metabolites.

The acid isolated by the new procedure had molecular formula, $C_{11}H_{16}O_4$, supported by elemental analysis and mass spectrometry. The i.r. spectrum (KBr disc) had broad absorption at 3,200 - 2,600 cm^{-1} and at 1696 cm^{-1} indicative of an acid group. The presence of a γ -lactone was suggested by a peak at 1752 cm^{-1} , and of a double bond by absorption at 1624 cm^{-1} . This spectrum was similar to but not superimposable on that of the mixture of canadensic acid epimers (55). The u.v. spectrum exhibited high intensity end absorption indicating the conjugated nature of the metabolite (λ_{max} . 214nm., ϵ 2,000).

The structure of this metabolite was evident from its n.m.r. spectrum (fig.6). The olefinic protons of the double bond, by contrast with the 'doublets' in the spectrum of the mixture of canadensic acid epimers, appeared as singlets at 3.46 and 4.05 τ . This material is thus the naturally occurring C-2 epimer of 'canadensic acid' (95). The remainder of the spectrum was much less complex than in the mixture of epimers enabling decoupling experiments to be performed and assignments to individual protons to be made. The proton geminal to oxygen, H-4 appeared as a multiplet at 5.60 τ due to vicinal coupling with the methylene protons of the butyl group and of



95

the lactone ring. The proton on a carbon atom α to carbonyl, H-2 occurred as a doublet at 6.34 τ ($J_1 = 9.0$, $J_2 = 12.0$ Hz.,) due to coupling with the non-equivalent protons ⁷⁸ of the lactone methylene group (2H-3). These protons H-3A (7.46 τ) and H-3B (8.03 τ) from the AM part of an AMXY system which has H-2 and H-4 as the X and Y protons respectively.

H-3A appeared as a multiplet at 7.46 τ with $J_{2,3A} = 9.0$, $J_{3A,3B} = 12.0$ and $J_{3,4} = 6.0$ Hz., which upon irradiation at H-4 collapsed to a double doublet ($J_1 = 12.0$, $J_2 = 9.0$ Hz.), and irradiation at H-2, which transformed H-3A into another double doublet ($J_1 = 12.0$, 6.0 Hz.).

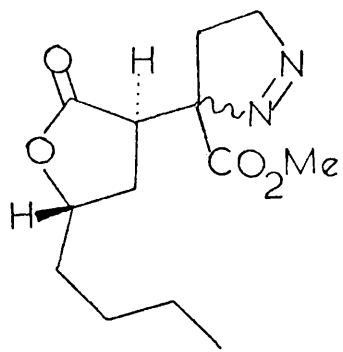
The proton H-3B occurred at higher field (8.03 τ) and appeared as a slightly distorted quartet indicating the approximately equal coupling constants, $J_{2,3B} = J_{3B,3A} = 12.0$, $J_{3B,4} = 12.5$ Hz. The remaining resonances which were assigned to the n-butyl group consisted of a 2H multiplet at 8.30 τ (2H-5), a 4H multiplet at 8.60 τ and a 3H triplet at 9.09 τ ($J = 6.0$ Hz.). The presence of this group was also supported by the mass spectrum which showed an intense peak at m/e 155 ($M^+ - 57$) due to the loss of the butyl side chain.

The 1:1 mixture of epimers of canadensic acid which had previously been isolated is produced by partial epimerisation of the centre C-2, by the active charcoal used in the isolation procedure.

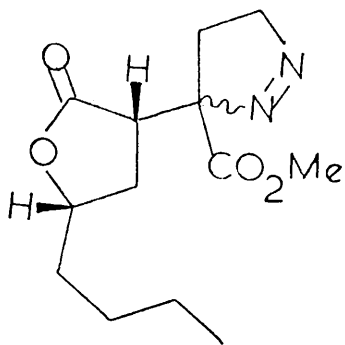
Whether 2-epicanadensic acid is a true metabolite is still open to question. Since even using the modified extraction procedure the crude canadensic acid isolated from column chromatography always contains some 2-epicanadensic acid (up to 10% by n.m.r.). Pure canadensic acid is obtained on crystallisation. As the culture is grown in an acidic medium (pH of broth is approximately 4 after 10 days) and workup is done under neutral conditions, it seemed unlikely that partial epimerisation should occur.

The relative configuration of canadensic acid remained to be determined i.e. whether the butyl group is cis or trans to the unsaturated ester grouping. It had been hoped that this might have been deduced from n.m.r. spectrum by application of the Karplus equation⁶⁰. However, this approach requires the accurate measurement of the corresponding dihedral angles, and as a model of canadensic acid (95) shows the ring system to be very flexible these angles cannot be predicted with any degree of certainty. The situation is further complicated by the recent finding⁷⁹ (using microwave spectroscopy) that the oxygen atom, carbonyl and C-2 carbon atom in γ -lactones do not lie in the same plane. In any case the similarity of $J_{2,3A}$ and $J_{2,3B}$ would have almost certainly prevented any possible distinction between the two isomers. Other evidence had therefore to be obtained in order to derive the relative stereochemistry of canadensic acid, and this involved the preparation of various derivatives.

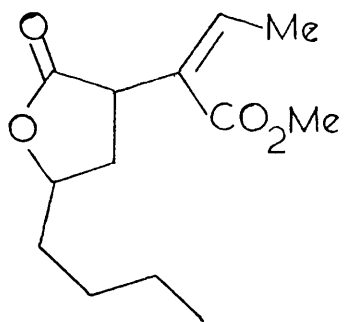
Attempted esterification of canadensic acid with diazomethane resulted in the formation of one major product less polar (R_f 0.15,



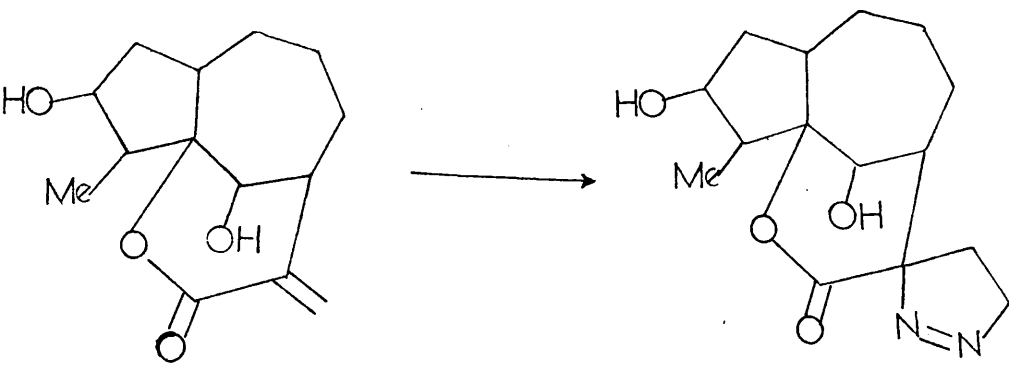
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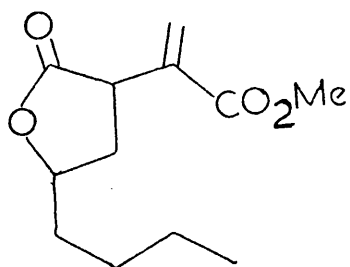
CHCl_3) than the starting material. P.l.c. enabled this material to be obtained pure. The i.r. spectrum (CCl_4) showed no absorption attributable to a double bond but showed δ -lactone absorption at 1778 cm.^{-1} and that attributable to a saturated ester (1745 cm.^{-1}). This latter frequency is too high for an unsaturated alkoxy carbonyl system. These facts were further confirmed by the n.m.r. which showed no resonance which could be ascribed to olefinic protons, although the resonance of a methoxyl group was present as a sharp 'singlet' at 6.30τ . The appearance of a slightly broadened triplet at 5.30τ ($J=5.0\text{Hz.}$) together with the absence of the double bond indicated by the i.r. spectrum suggested that diazomethane had not only esterified the carboxyl group but had added to the terminal methylene group to form the pyrazoline ring system (96). The complex multiplets from $6.7 - 8.0\tau$ (5H) coupled with the split methoxyl resonance at 6.30τ were in agreement with its being a mixture of C-10 epimers. The pyrazoline ring system was further confirmed by the i.r. spectrum (thin film) which showed weak absorption at 1550 cm.^{-1} characteristic of the $\text{N}=\text{N}$ group⁸⁰, and an ion at m/e 140 due to the loss of 28 mass units ($\text{N}=\text{N}$) from the molecular ion in its mass spectrum.

Many similar reactions involving the addition of diazomethane to double bonds activated by a suitable electron - withdrawing group have been reported, for example, that of Zaluzalin A (97)⁸¹. The reaction of canadensic acid (95) with diazomethane, however, was

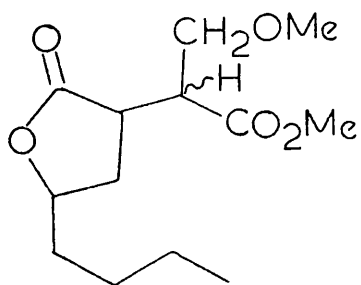
particularly facile. Treatment with an excess of diazomethane at ice temperature for one minute giving the same product with little trace of the less polar methyl canadensate(100).

A similar treatment of the mixture of canadensic acid C-2 epimers (55) had given rise to two main components of R_f s 0.15 and 0.25 (CHCl_3). Separation of these by p.l.c. using multiple elution proved that the more polar component had identical i.r. and n.m.r. spectra to the pyrazoline adducts (96) obtained from canadensic acid (55) and hence would appear to have the same relative configuration at C-2. The other component was an oil which had $\nu_{\text{max.}}$ (CCl_4) at 1768 cm.^{-1} (γ -lactone) and 1740 cm.^{-1} (CO_2Me) but did not correspond to the 2-epipyrazoline compound (98) since it showed weak but sharp i.r. absorption at 1680 cm.^{-1} together with a 3H narrow multiplet at 8.0τ in the n.m.r. This substance was not further characterised but could be a mixture of ethylidene esters e.g. (99) formed from pyrazoline intermediates. It was evident that this was not a suitable method of preparing the required methyl ester and the reaction was not pursued.

When canadensic acid was allowed to reflux in dry methanol with a drop of sulphuric acid as catalyst for several hours an oil was obtained, which appeared to be homogeneous by t.l.c. and to be less polar than canadensic acid. Its i.r. spectrum (thin film) showed absorption at 1765 cm.^{-1} , 1720 cm.^{-1} and 1635 cm.^{-1} corresponding



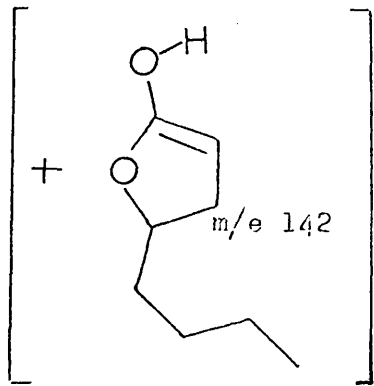
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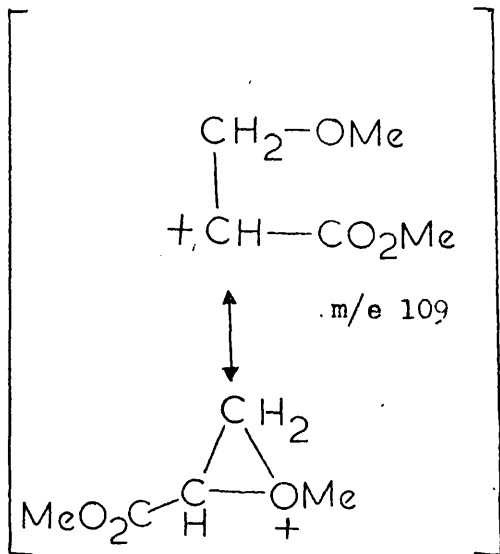
101

to a γ -lactone system, an unsaturated alkoxy carbonyl system and a terminal methylene group respectively. This was corroborated by its n.m.r. spectrum which showed a 3H singlet at 6.24 τ corresponding to a methoxyl group, and by the characteristic singlets at 3.60 τ and 4.17 τ which confirmed that epimerisation had not occurred in the acidic reaction medium. The remainder of the spectrum was similar to that of canadensic acid. The m.s. data showed a molecular ion at m/e 226 and ions at m/e 195 (M-31) and m/e 169 (M-57), corresponding to the anticipated losses of the methoxyl and butyl side chain groups. This data afforded the evidence that the product was the desired methyl canadensate (100).

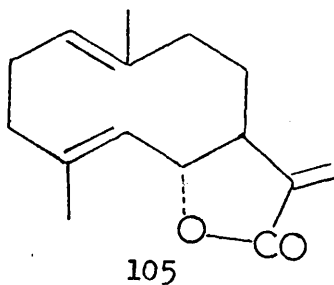
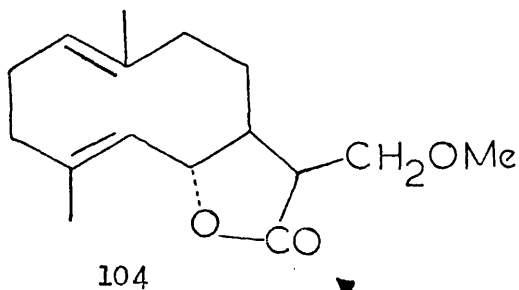
The methyl ester could also be prepared by allowing canadensic acid to stand at R.T. in methanol with a drop of sulphuric acid as catalyst. Refluxing the methanol solution for long periods, however, resulted in the formation of significant amounts of a slightly more polar compound in addition to the expected methyl canadensate (100). P.l.c. gave an oil, which analysed as $C_{13}H_{22}O_5$. The i.r. spectrum (thin film) showed absorption at 1768 cm^{-1} (γ -lactone) and 1734 cm^{-1} (CO_2Me) but no absorption which could be attributed to a double bond. Also the n.m.r. spectrum lacked any olefinic resonances. Instead of a single methoxyl resonance, however, there were two 3H singlets. The one at lower field 6.29 τ must be that of the carbomethoxy group, while the resonance at 6.63 τ was ascribed to an aliphatic ether.



102



103



The multiplets at 6.30 τ (2H) and 7.70 τ (2H) were assigned to the protons geminal to oxygen, 2H-11, and α to carbonyl, H-2 and H-10, respectively. The structure (101) for this compound was confirmed by its mass spectrum which showed a molecular ion at m/e 258, and ions at m/e 227 (M-31) and m/e 201 (M-57) due to the losses of methoxyl and the butyl side chain groups respectively. The major peaks now arise, however, from cleavage of the saturated ester side chain. This gives rise to the ion at m/e 142 (102), presumably by McLafferty rearrangement,⁶⁴ and, corresponding to charge retention by the side chain, the ion (103). The latter could be stabilised as shown by the methyl ether group and is the base peak in the spectrum.

The methyl ester (101) is formed by the addition of methanol to the terminal methylene group of methyl canadensate (100) by acid catalysis. The addition of methanol to α, β unsaturated ketones and esters under slightly basic conditions is well known⁸². This has been demonstrated in the case of 12-methoxydihydrocostunolide (104) which was shown to be an artefact formed from the interaction of methanol used in the work-up with constunolide (105)⁸³.

When canadensic acid (95) was refluxed in a slurry of potassium carbonate, acetone and methyl iodine for several hours an oil was obtained which was just separable into two components by analytical t.l.c. (R_f 0.62, 0.61 CHCl_3). The i.r. spectrum showed absorption at 1767 cm^{-1} (δ -lactone) and 1721 cm^{-1} (unsaturated ester)

Table 4.

G.l.c.c. Retention data of methyl canadenstate epimers.

Column	Temp.	R _t (mins.)	Compound
1% P.E.G.A	125°	17.0	methyl canadenstate epimers
		18.0 (overlapping)	
	150°	13.0	"
		14.0 (overlapping)	
	150°	14.0	methyl canadenstate
	175°	7.7	methyl canadenstate epimers
1% OV17	125°	31.5	"
		34.0 (broad overlapping)	
	150°	10.0	"
		11.5 (overlapping)	
	150°	11.5	methyl canadenstate
	1% N.G.S.	150°	6.7 (shoulder)
7.1			
150°		7.1	methyl canadenstate

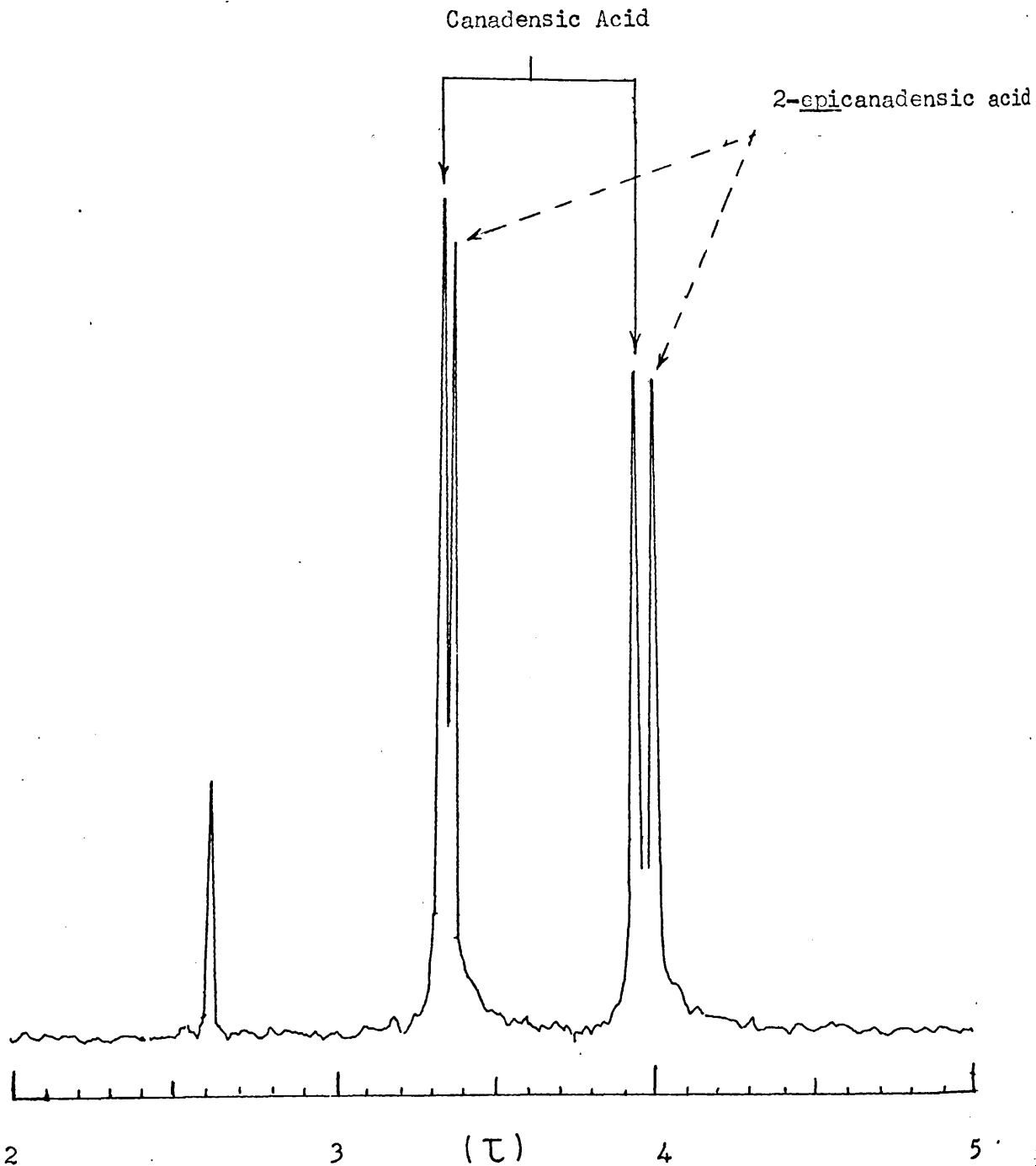
in the carbonyl region while terminal methylene group absorption was present at 1633 cm^{-1} . The n.m.r. spectrum showed, apart from the methoxyl resonance at 6.23τ , the characteristic pairs of doubles at ca. 3.60 and 4.17τ , indicating that this oil was a 1:1 mixture of methyl canadensate (100) with its C-2 epimer, identical to the esters prepared from the mixture of canadensic acid C-2 epimers. This result was perhaps not surprising in view of the basic catalyst used and the known acidity of H-2.

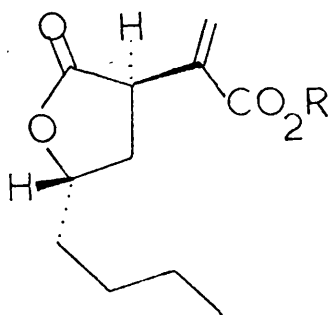
Although the methyl canadensate epimers (57) could be just differentiated on t.l.c., they were not readily separated on g.l.c. No separation was achieved at all on non selective columns when only a single sharp peak was obtained; 1% SE 30 ($125^{\circ}\text{Rt } 7.0\text{ min}$), 1% QF 1 ($120^{\circ}\text{Rt } 9.0\text{ mins}$) and 5% APL ($150^{\circ}\text{Rt } 11.0\text{ min.}$).

Columns containing a more polar stationary phase and which tend to interact more with the functional groups of the epimeric esters, gave better results and enabled a partial separation to be obtained (Table 4).

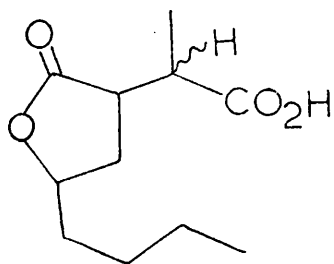
From the retention data it can be seen that methyl canadensate (100) is slightly more polar than methyl 2-epicanadensate in keeping with the t.l.c. deductions. It was noted that although the peaks corresponding to the two isomers could not be completely separated preventing integration of the peak areas, the peak due to methyl 2-epicanadensate was always smaller in height than that of

Fig. 7. Olefinic proton resonances in the n.m.r. spectrum of the mixture of canadensis acid C-2 epimers.





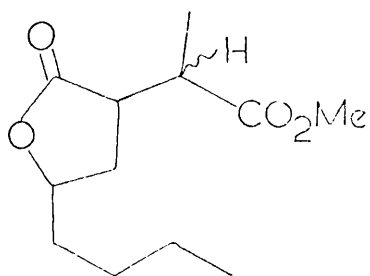
106



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methyl canadensate (100). Since the mixture of methyl canadensate C-2 epimers (57) are formed by equilibration of the proton α to the lactone carbonyl then the thermodynamically more stable trans compound (106) should be formed in the slightly greater amount. If methyl canadensate (100) corresponds to the major epimer as suggested by the g.l.c. data, then this means that the butyl and acrylic ester groupings are probably trans to one another (106). A similar conclusion could be drawn from the olefinic proton resonances in the n.m.r. spectrum of the canadensic acid C-2 epimers where the resonances of greatest intensity in the two "doublet" (Fig. 7) corresponded to the olefinic protons of canadensic acid (95). This pattern might alternatively be explained, however, by slight coupling of the olefinic protons of 2-epicanadensic acid. Confirmation of this stereochemical assignment was hence desirable and was obtained as follows.

Hydrogenation of canadensic acid (95) for one hour using platinum oxide as catalyst gave a crystalline dihydro derivative (107). The i.r. spectrum (KBr) showed absorption at 1695 cm.^{-1} (COOH) and 1758 cm.^{-1} (γ -lactone). The lack of double bond absorption in the i.r. spectrum was corroborated by its u.v. spectrum which showed no absorption above 200 nm. The n.m.r. spectrum similarly showed no olefinic resonances, but a doublet at 8.62τ (3H, $J=8.0\text{ Hz.}$)

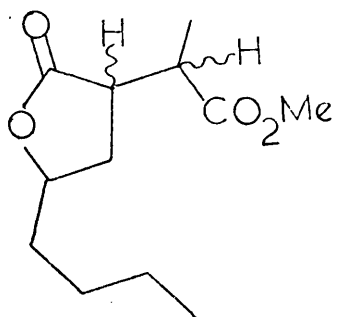


10³

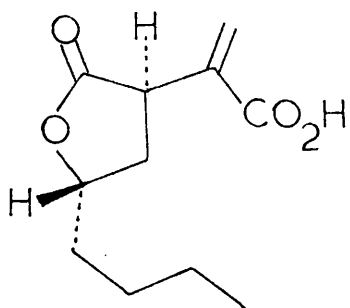
indicated the presence of a secondary methyl group β to a carbonyl function, confirming the saturation of the terminal methylene group. The proton on carbon atoms α to carbonyl functions likewise gave rise to 2H multiplet centred at 6.90 τ , while the ring methylene protons 2H β , occurred as another 2H multiplet at 7.62 τ .

The above reduction product was methylated using ethereal diazomethane which could now be successfully employed since the terminal methylene group had been reduced. The resulting dihydro esters again failed to crystallise, although analytically pure. The i.r. spectrum, (thin film) showed absorption at 1765 and 1730 cm^{-1} consistent with a γ -lactone system and saturated ester group. The n.m.r. spectrum now clearly showed that this product was a mixture of epimers. It exhibited a split methoxyl resonance at 6.28 τ (3H), and two doublets at 8.60 τ ($1\frac{1}{2}$ H, $J = 7.0$ Hz.) and 8.74 τ ($1\frac{1}{2}$ H, $J = 7.0$ Hz.) due to the secondary methyl groupings in the two epimers. The remainder of the spectrum was in accord with its structure, the protons on carbon atoms α to carbonyl functions appearing as a 2H multiplet at 6.96 τ . Irradiation at this centre caused the doublets due to secondary methyl groups to collapse to singlets.

The mass spectrum of the mixture of these epimeric γ -lactones was in good accord with their structure (108), significant ions occurring at m/e 228 (M^+), 197 ($M^+ - \text{OMe}$), 171 (90% base peak, $M^+ - \text{C}_4\text{H}_9$), 142 ($M^+ - \text{CH}_2 = \text{CHCO}_2\text{Me}$ by McLafferty rearrangement⁶⁴), 139 ($171^+ - \text{MeOH}$),



109



110

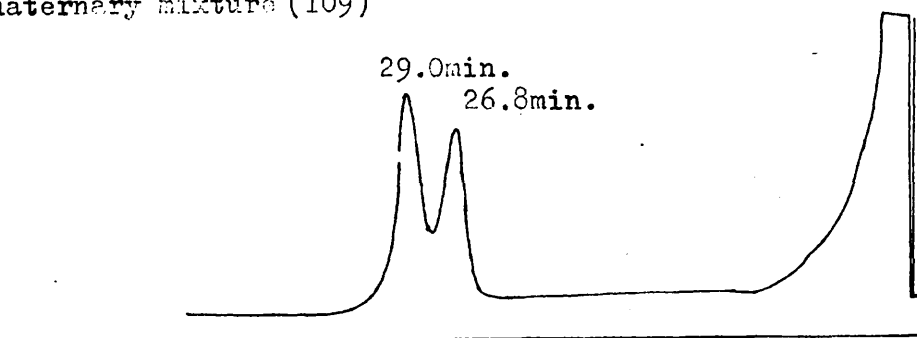
111 ($139^+ - CO$), and 88 ($Me.CH = C(OH)OMe^+$ base peak by McLafferty rearrangement ⁶⁴).

Although the presence of the epimeric centre at C-10 could be deduced from the n.m.r. spectrum, attempts to detect two components by t.l.c. or g.l.c. were completely unsuccessful. It was therefore, decided to prepare the mixture containing the four methyl dihydrocanadensates having all the possible configurations at C-2 and C-10 to determine if separation of these could be achieved. This was prepared by hydrogenation of the mixture of canadensic acid and 2-epicanadensic acid followed by methylation using ethereal diazomethane. The quaternary mixture (109) was obtained as an oil, $C_{12}H_{20}O_4$, ν_{max} . (thin film) 1770 cm^{-1} (γ -lactone) and 1725 cm^{-1} (CO_2Me). Although the methoxyl resonance at 6.22τ (3H) in the n.m.r. spectrum was only partially split, that corresponding to the secondary methyl group now appeared as three doublets at 8.56τ ($1\frac{1}{2}H$, $J=7.0\text{ Hz.}$), 8.67τ ($\frac{3}{2}H$, $J=7.0\text{ Hz.}$) and 8.69τ ($\frac{3}{2}H$, $J=7.0\text{ Hz.}$). The remaining proton resonances, although extremely complex, were similar in chemical shift to those of the methyl dihydrocanadensate C-10 epimeric mixture prepared previously. The mass spectra of binary and quaternary mixtures were found to be almost identical.

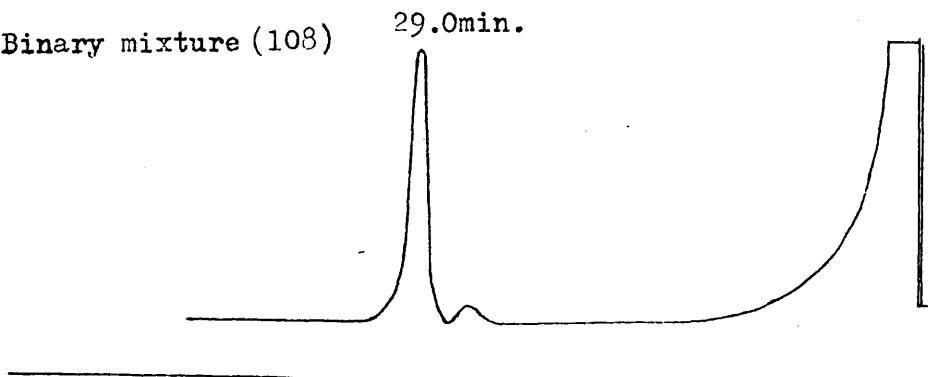
If we assume that canadensic acid (95) has its substituents in a trans relationship and the absolute stereochemistry as shown in (110) (the elucidation of this will be discussed later),

Fig. 8 G.l.c. traces of methyl dihydrocanadensates on a 10% APL column at 163°.

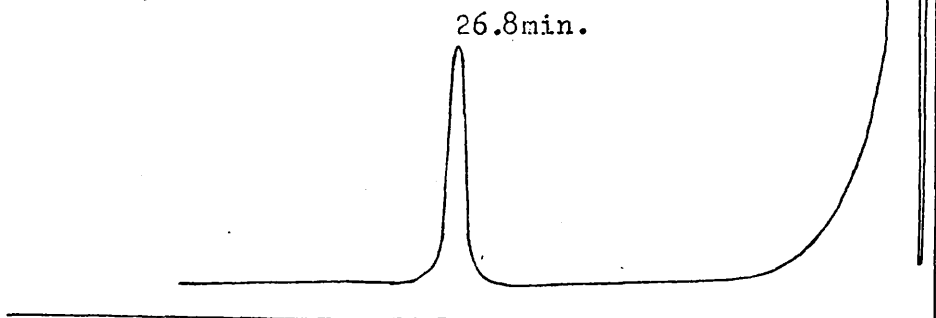
Quaternary mixture (109)



Binary mixture (108)



Methyl C-2epidihydrocanadensate (70)



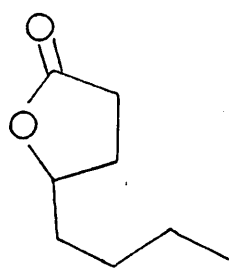
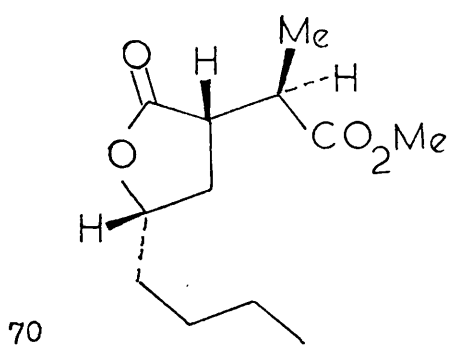
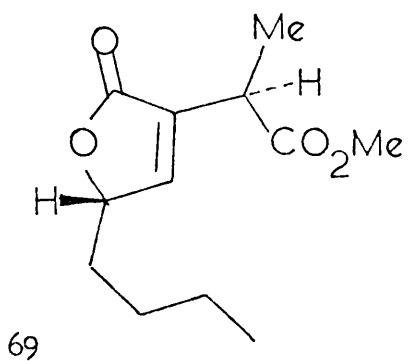
	<u>Component(s)</u>	<u>Retention Time(min.)</u>
Quaternary mixture	(2S 10R)(2S 10S) (2R 10R)(2R 10S)	26.8 29.0
Binary mixture	(2R 10R)(2R 10S)	29.0
Methyl C-2 <u>epi</u> dihydro- canadensate	(2S 10S)	26.8

(Peaks of identical retention time were confirmed by combined injection).

then the two epimeric methyl dihydrocanadensates can be represented as (2R, 10R) and (2R, 10S). The four diastereoisomers which form the quaternary mixture are thus represented as (2R, 10R), (2R, 10S), (2S, 10R), and (2S, 10S).

The best resolution of the four methyl dihydrocanadensate diastereoisomers was obtained on g.l.c. using a 10% APL column at 163^o. This enabled two peaks of approximately the same area to be obtained (R_t 26.8, 29.0 min.). (10R + 10S) methyl dihydrocanadensate on the same column gave a single peak (R_t 29.0 min.), identical to the one of longer retention time given by the quaternary mixture. This data (Fig. 8) shows that the g.l.c. column used can distinguish the dihydroesters only by their configuration at C-2. The configuration at C-10 is not differentiated. This would appear reasonable since the rigidity imparted by the lactone ring means that a molecule with a 2R absolute configuration will have a trans relative configuration of the butyl and saturated ester groups while a molecule with the 2S absolute configuration will have a cis configuration. This results in the molecules having entirely different shapes which is more likely to affect their interactions with the stationary phase than in the case of the absolute configuration at C-10, since free rotation can occur about the C-2 - C-10 bond resulting in molecules with 10R or 10S absolute configurations having substantially the same shape.

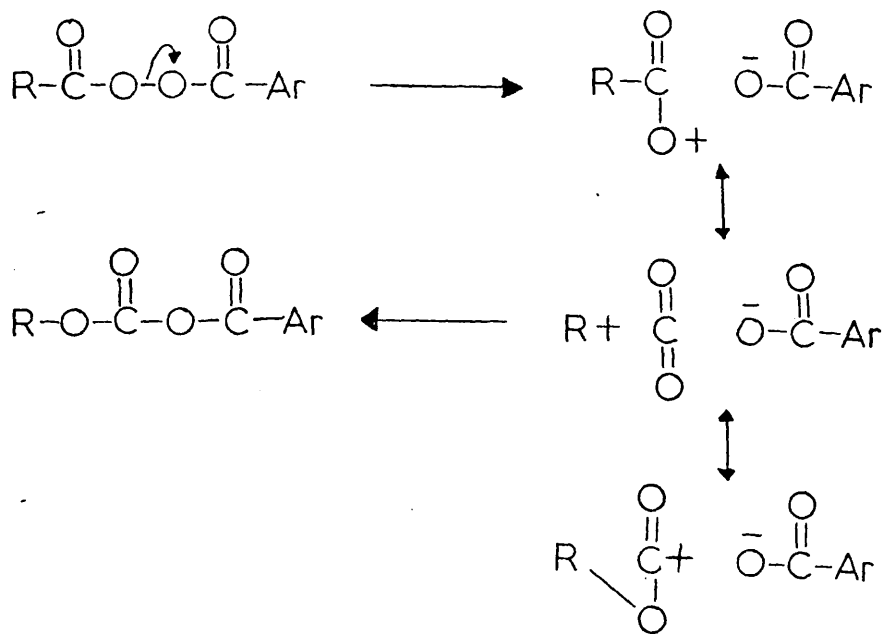
A further indication that canadensic acid has a trans



configuration can be obtained from the relative peak areas (Fig. 8). The peak (R_t 29.0 min.) corresponding to the 2R methyl dihydrocanadensates was found to be slightly larger than that of the 2S dihydroesters in the g.l.c. trace of the quaternary mixture. Since hydrogenation does not affect the configuration at C-2, this supports the earlier suggestion that methyl canadensate might be the thermodynamically more stable of the two methyl canadensic epimers and hence have the trans relative configuration.

Although g.l.c. enabled the dihydroesters to be distinguished according to whether they have a 2R (trans relationship of butyl and propionate ester groups) or a 2S (cis) configuration, the original assignments were somewhat tentative. We had, however, prepared during the chemical investigation of dihydrocanadensolide (54), a methyl dihydrocanadensate of known relative configuration (70). This was synthesised from the butenolide ester (69) by catalytic hydrogenation, a process which would be expected to give mainly a cis configuration of the butyl and propionate ester groups and hence a 2S absolute configuration. This ester was, therefore, subjected to g.l.c. on the 10% APL column previously described, and found to give only a single peak (R_t 26.8 min), which was superimposable with the one of lower retention in the g.l.c. of the quaternary mixture of methyl dihydrocanadensates. This result establishes that the dihydroesters of R_t 26.8 min. have a 2S or cis configuration of butyl and propionate ester groups, and conversely

Fig. 9 Rearrangement of an acyl-aroyl peroxide.



that the dihydroesters of R_t 29.0 min. have a 2R or trans configuration. This thus establishes the 2R configuration of canadensic acid and hence its postulated structure (110).

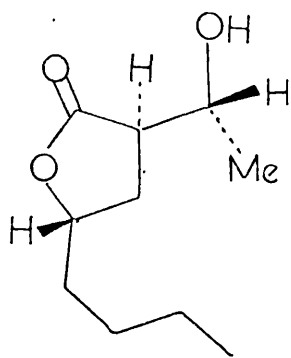
With this solution to the relative stereochemistry of canadensic acid (95) finally achieved, attention was focussed on determining the absolute configuration of the molecule. As this could not be derived from the acid directly (cf. chapter 5), a suitable degradation product was required, the ultimate aim being caprylolactone (111) which would contain only the asymmetric centre C-4 present in canadensic acid (95). A secondary objective was the possible establishment of a degradation scheme suitable for biosynthetic labelling studies.

Application of the Schmidt reaction and of an elimination-decarboxylation procedure gave similar results to those described for the canadensic acid C-2 epimers (55) (discussed in chapter 6). The most promising approach proved to be one in which one of the mixtures of dihydrocanadensic acid epimers was subjected to a sequence of reactions involving "carboxy-inversion"⁸². This latter reaction represents a means of degrading an acid to an alcohol, and involves the rearrangement of an acyl-royl peroxide to the corresponding acyl-royl carbonate (Fig. 9). It is known⁸² from consideration of experimental data that the peroxide linkage breaks to give the potentially more stable carboxylate anion which in the case of acyl-royl peroxides is the aroyl carboxylate anion (see opposite). It is

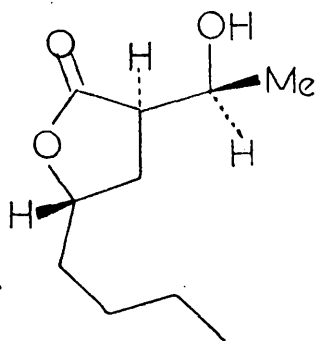
thought that the partial positive charge is distributed over the triad of atoms involved in the 1,2 shift from carbon to oxygen, as illustrated by the resonance structures shown.

(10R + 10S) dihydrocanadensic acid chloride was prepared in high yield by the reaction of thionyl chloride on the corresponding acid. On treatment with metachloroperbenzoic acid and pyridine in hexane at ice temperature the acid chloride was converted to the mixed peroxide. It is known that the rates of the rearrangement of the mixed peroxides varies ⁸² according to the nature of the alkyl group present, the peroxides with primary alkyl groups being the slowest. Since the mixed peroxide prepared above involved the migration of a secondary carbon atom, a convenient rate was found to occur stirring in hexane at R.T. A qualitative test for peroxide with starch-iodide paper proved negative after 24 hours, proving that the rearrangement had gone to completion. Hydrolysis of the carbonate was achieved by adding methanol with a trace of acid catalyst and refluxing under nitrogen. The liberated carbon dioxide was trapped as barium carbonate (50% yield).

The non-acidic products of this reaction were found to consist mainly of two components (R_f 0.31 and 0.45, 1% MeOH in CHCl_3), which were separated by p.l.c. The less polar and major component, an oil which failed to crystallise, was assigned the molecular formula, $\text{C}_{10}\text{H}_{18}\text{O}_3$, on the basis of elemental analysis and mass spectrometry.



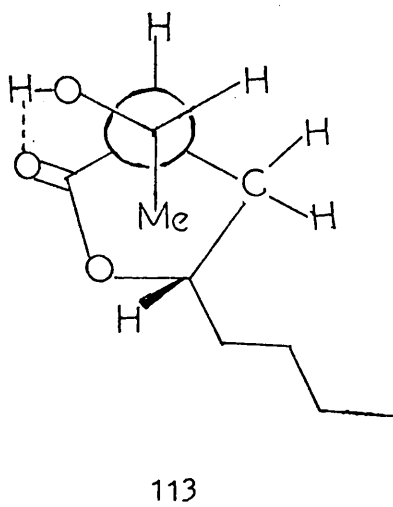
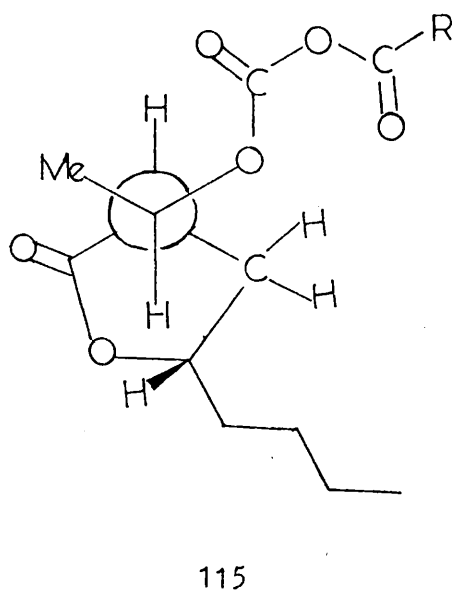
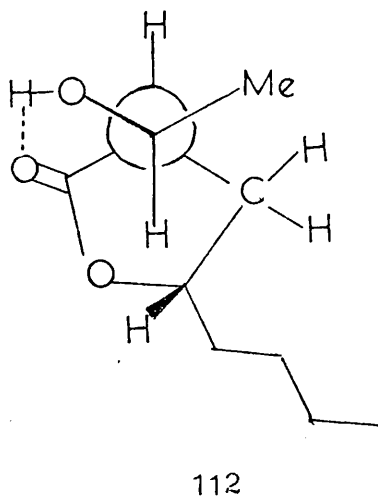
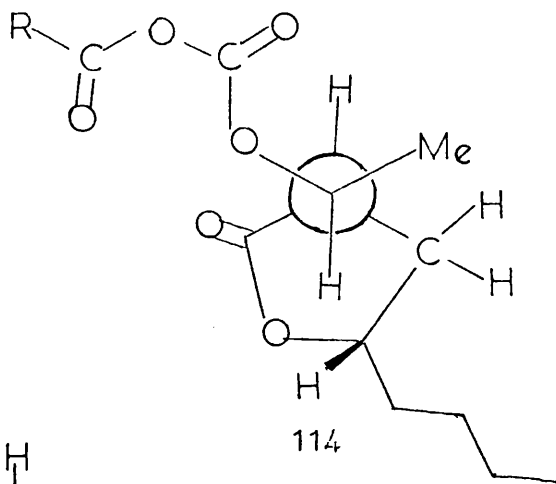
112



113

Its i.r. spectrum (CCl_4) showed absorption due to a hydroxyl group ($3,500 \text{ cm.}^{-1}$) and a γ -lactone (1758 cm.^{-1}), confirming that it is one of lactone alcohols expected from this reaction. This compound, named norcanadensol A is depicted as having structure (112) as its corresponding carbonate might be predicted to be thermodynamically more stable than its C-10 epimer as discussed below. Of particular importance in the n.m.r. spectrum of this alcohol was the 3H doublet ($J = 6.2 \text{ Hz.}$) at 8.67τ due to the secondary methyl group, and a 1H double quartet ($J = 6.2, 3.0 \text{ Hz.}$) at 6.10τ assigned to the proton geminal to the hydroxyl group. Irradiation at the doublet resulted in this double quartet collapsing to the expected doublet. The remaining resonances in the n.m.r. spectrum were as expected for the postulated structure. In accord with the recorded spectra of most secondary alcohols, the parent peak was extremely weak and overshadowed by the prominent ($M^+ - 1$) ion at m/e 165. The mass spectral breakdown was in complete agreement with the structure (112), significant ions occurring at 171 ($M^+ - 15$), 142 ($M^+ - \text{CH}_2 = \text{CH}(\text{OH})$, by McLafferty rearrangement), 129 ($M^+ - 57$), 100 ($129^+ - \text{CHO}$), and 85 (base peak, $129^+ - \text{CO}_2$).

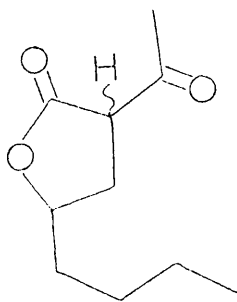
The more polar and minor component of the mixture of epimeric alcohols, norcanadensol B (113), was again obtained as an oil, $\text{C}_{10}\text{H}_{18}\text{O}_3$, $\nu_{\text{max.}}$ (in CCl_4), $3,590 \text{ cm.}^{-1}$ (-OH) and 1765 cm.^{-1} (γ -lactone). The n.m.r. spectrum was similar to that of its C-10 epimer, and also



exhibited the expected secondary methyl doublet (8.80 τ , $J=6.0\text{Hz.}$) The resonance of the proton geminal to the hydroxyl group was, however, now obscured by that of the proton H-4 resulting in a ^2H multiplet at 5.80 τ . The mass spectral breakdown was also parallel, the same intense ions being recorded in the spectra of both alcohols.

The two alcohols discussed above had been assigned the respective structure, epimeric at C-10, on the basis that only two possible alcohols could be formed from the carboxy-inversion reaction. That they differed at this centre and not C-2 was demonstrated by treatment of the mixture of alcohols with methanolic potassium hydroxide. Epimerisation of the centre α to carbonyl occurred, resulting in a mixture with four components (R_f 0.45, 0.43 and 0.31, 0.27, 1% MeOH/99% CHCl_3). These were t.l.c. identical to the products of a carboxy-inversion reaction on the quaternary mixture of dihydrocanadensic acid (C-2, C-10) diastereoisomers.

The 10R structure (112) had been postulated for norcanadensol A, the thermodynamically more stable isomer, on the basis that in the two possible anti conformers about the C-2, C-10 bond, there appeared to be less steric interaction in the 10R carbonate (114), than in the corresponding 10S carbonate (115). The important interaction was taken to be that between carbonate ester (or methyl)

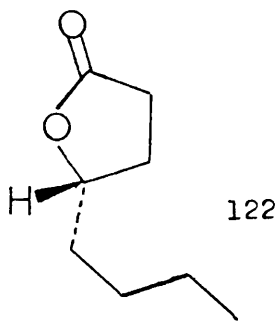
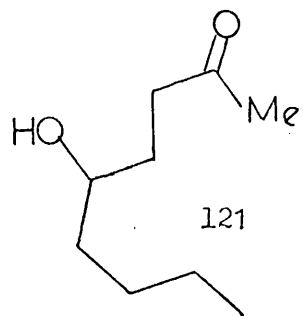
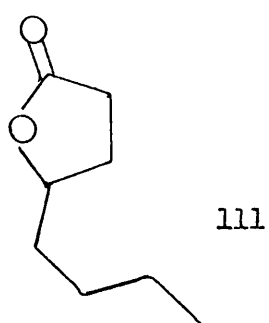
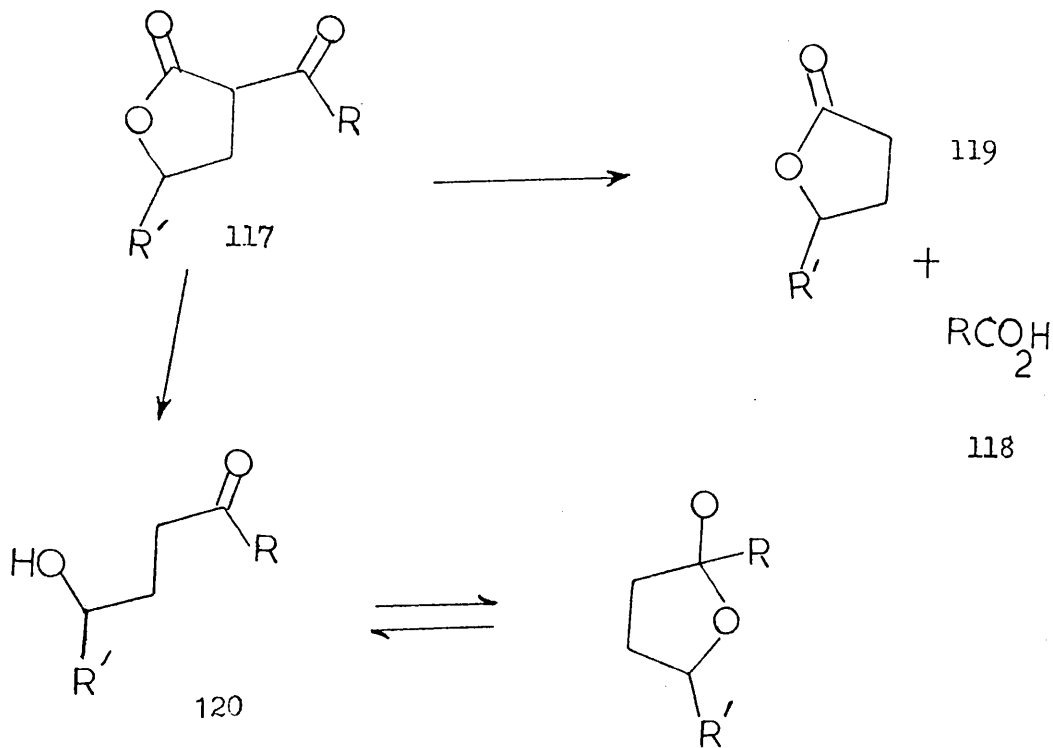


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grouping and the 3β proton. This tentative assignment appeared to be confirmed by the lower hydroxyl ($3,500\text{ cm.}^{-1}$) and lactone carbonyl (1753 cm.^{-1}) absorptions shown in the i.r. spectrum of norcanadensol A, which can intramolecular hydrogen bond in a more stable anti conformation. Norcanadensol B (113), which has corresponding i.r. absorption at $3,590\text{ cm.}^{-1}$ ($-\text{OH}$) and 1765 cm.^{-1} (γ -lactone) would be expected to exhibit weaker intramolecular hydrogen bonding because of the interactions in the gauche conformation required.

Jones oxidation of norcanadensol A readily formed the acyl-lactone (116), easily recognised on t.l.c. by its purple colouration with methanolic ferric chloride. Its i.r. spectrum (CCl_4) showed strong absorption at 1780 and 1728 cm.^{-1} in the carbonyl region attributed to the lactone and ketone groups respectively. No detectable amount of enol tautomer was present in the i.r. spectrum, unlike the case of acetyl- δ -lactones which exist mainly in the enol form⁸⁴. This is presumably due to the greater ring strain produced by the exocyclic double bond in the enol form of acetyl- γ -lactones.

The n.m.r. confirmed that epimerisation of the active centre, C-2, had occurred since the 3H resonance of the methyl group attached to carbonyl was a finely split singlet at 7.40τ . A complex multiplet was also obtained for the proton, H-2, at the expected low field value of 6.27τ . The mass spectrum exhibited a molecular ion at m/e 184 and a breakdown pattern containing ions common with the



norcanadensols A and B. Prominent ions at 142 ($M^+ - H_2C=C=O$) by McLafferty rearrangement), 127 ($M^+ - 57$), 83 ($127^+ - CO_2$) and 43 (base peak, $CH_3.C\equiv O^+$) helped substantiate the postulated structure.

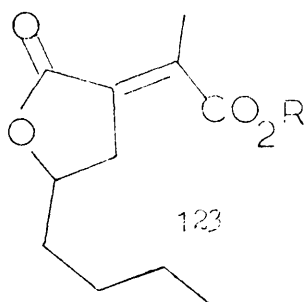
Oxidation of norcanadensol B with Jones reagent did not occur readily and a poor yield of ketone (116) was obtained. The mildly basic Sarett reagent was found to be equally unsatisfactory, little reaction having occurred after several days at R.T. A further attempt to effect a clean oxidation was made using the silver carbonate on celite method⁸⁶. This reagent, unfortunately, was found to affect neither norcanadensol B nor norcanadensol A (112) even on prolonged reflux. It has been shown, however, that β -diols cannot be oxidised beyond the ketol stage using this technique⁸⁶, and hence, presumably β -hydroxylactones are equally inert.

Acyl- γ -lactones (117), like β -ketoesters can be further degraded in two distinct manners. Attack by strong bases at the ketonic carbonyl group causes cleavage producing an acid (118), and a γ -lactone (119), while dilute acid induces decarboxylation⁸⁷ with the formation of a δ -hydroxyketone (120), which exists in equilibrium with its ring opened form. The application of these reactions to the ketone (116) would not only enable carbon atoms C-1 (as CO_2) and C-10, C-11 (as acetic acid) to be isolated, but moreover the degradation product from the base treatment would be the required

caprylolactone (111), which should enable the absolute configuration at C-4 to be determined unequivocally.

When the ketone (116) was refluxed in dioxane-dilute hydrochloric acid there did result a significant amount of carbon dioxide, which was trapped as barium carbonate in the usual way. The expected product (121), however, could not be isolated from the complex reaction mixture. Attempts to separate it as its dinitrophenylhydrazone likewise failed, and this reaction was not pursued further.

Cleavage of the ketone (116) was achieved by refluxing in methanolic potassium hydroxide for one hour. The major product (R_F 0.43, CHCl_3), did not stain on t.l.c. with ferric chloride and was slightly more polar than the ketone (R_F 0.46, CHCl_3). Purification by p.l.c. gave an oil, whose i.r. (thin film) showed a single carbonyl absorption at 1768 cm^{-1} , attributable to a saturated γ -lactone. The n.m.r. exhibited a 1H multiplet at 5.50τ due to the proton geminal to oxygen, and a multiplet at 7.56τ (2H) assigned to the protons α to carbonyl. Resonances between 7.6 and 8.0τ (2H) were ascribed to the protons β to carbonyl. The presence of the n-butyl group could again be inferred from the 6H multiplet at ca 8.5τ and the 3H triplet at 9.06τ . That this compound was indeed caprylolactone was confirmed by comparison of its i.r. spectrum with that of an authentic sample, when the two were found to be identical. The absolute configuration of the caprylolactone, and hence C-4 in canadensic acid (95), was shown to be



as in (122) from its o.r.d spectrum (cf. chapter 5).

Interrelation of canadensic acid (95) with canadensolide (51) could have proven important in determining the relative stereochemistry of the antibiotic. It was hoped that this might be achieved via isomerisation of the double bond of canadensic acid (95). Acidic conditions would appear to be unhelpful since no isomeric product could be detected from the treatment of canadensic acid (95) with methanol and concentrated sulphuric acid. A similar result was obtained from attempted isomerisations using dry hydrogen chloride in various solvents (such as ether, chloroform), conditions which have been found effective in double bond isomerisation⁸⁸. This lack of reactivity may be due to the increased ring strain of the desired product (123, R=H) which has an exocyclic double bond, although it was hoped this compound would be sufficiently stabilised by the resultant conjugation of the two carbonyl systems. A further reason could be that acid catalysed protonation of the double bond of canadensic acid (95) will tend to give a primary carbonium ion since the alternative carbonium ion would be α to a carbonyl group and hence less stable. Deprotonation would thus merely regenerate canadensic acid (95).

Although base catalysis would seem more likely to effect the desired change, treatment with triethylamine at least was unsuccessful. It had been found during n.m.r. studies, however, that

on storing the mixture of canadensic acid C-2 epimers (55) in deuterated dimethylsulphoxide (DMSO) for 24 hours, isomerisation seemed to have occurred. The n.m.r. spectrum, which showed no further change after storing for one week, now exhibited the resonance of a vinyl methyl group at 7.93τ (3H) while the resonances of the terminal methylene protons appeared as singlets at 3.76 and 4.15τ (each 1H). The rest of the spectrum was similar to that of canadensic acid (55). It was tempting to interpret this result as isomerisation of one of the canadensic acid C-2 epimers (55). Although this proved correct, it was found that in dry DMSO (in which no isomerisation was found to occur), the terminal methylene protons of the two C-2 epimers resonated at the same frequency. The above observation merely indicated that half of the mixture had been transformed.

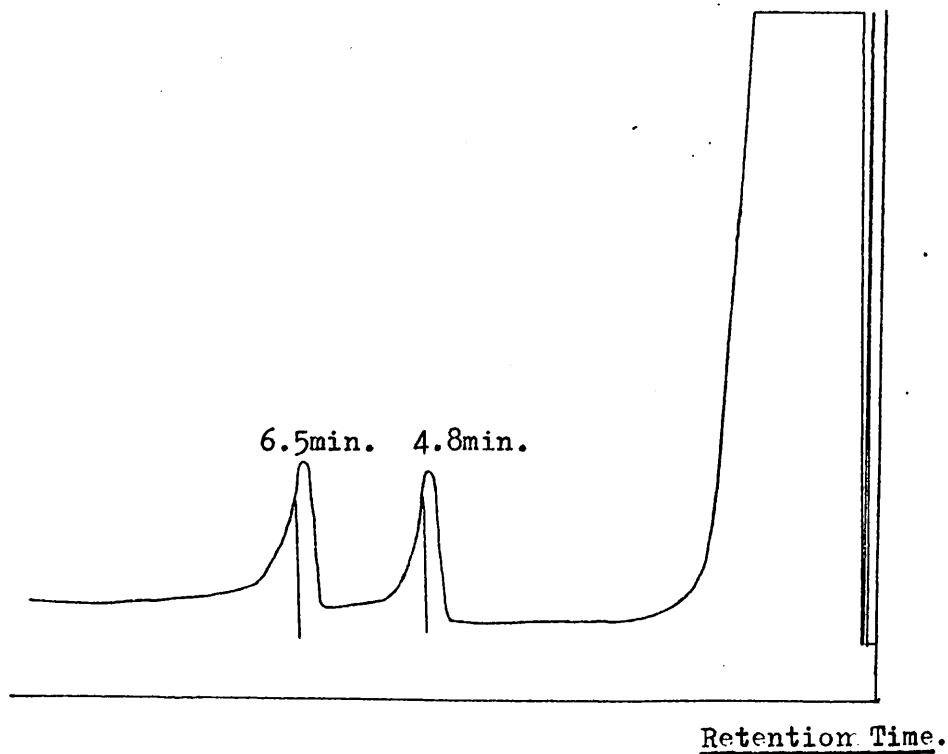
In an attempt to substantiate this result, the mixture of canadensic acid C-2 epimers (55) was stored in DMSO for 24 hours. The DMSO was subsequently removed by extracting a chloroform solution of the reactants several times with water. The resulting solid was esterified by the methyl iodide/potassium carbonate/analar acetone procedure to give an oil which was homogeneous and indistinguishable from the mixture of methyl canadensate C-2 epimers (57) on t.l.c. The i.r. spectrum (thin films), however, was not identical to that of the mixed epimers. The carbonyl region was broader in absorption, a new peak occurring at 1730 cm.^{-1} in addition to the normal absorptions

Table 10.

G.c.m.s. Mass Spectral Data.

Derivation	Peak	Methyl canadensate C-2 epimers		Methyl ester of isomeric acid	
		Rel Abundance (%)	Rel. Abundance (%)		
M ⁺	212	0.7		2.0	
M ⁺ - 15	211	-		0.6	
- M ⁺ - OMe	195	7.5		6.0	
M ⁺ - 57	169	36		100	
M ⁺ - 85	141	47		30	
M ⁺ - 88	138	-		53	
M ⁺ - (57+44)	125	100		-	

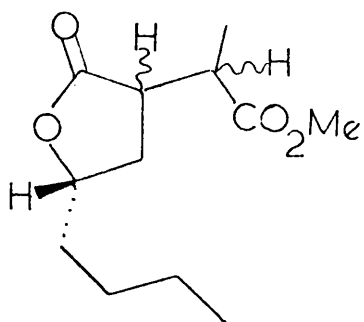
Fig. 10 G.l.c. trace of isomeric mixture on a 1% SE 30 column at 141°.



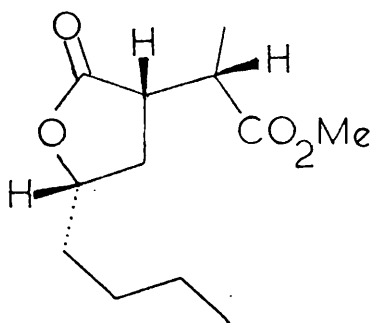
	<u>Retention Time.</u>
Methyl canadensate C-2 epimers (57)	4.8 min.
Methyl ester of isomeric acid (123, R=Me)	6.5 min.

at 1760 cm.^{-1} (γ -lactone) and 1707 cm.^{-1} (CO_2Me) found in the i.r. spectrum of the methyl canadensate C-2 epimers (57). A new double bond absorption was also apparent at 1668 cm.^{-1} , as well as that of the terminal methylene group at 1625 cm.^{-1} . The remainder of the spectrum was likewise similar but not identical to that of the epimeric esters. This evidence was consistent with the formation of some isomeric acid methyl ester (123, R=Me), and this was confirmed by the n.m.r. spectrum which exhibited two distinct methoxyl resonances, namely at 6.15 and 6.25τ ($1\frac{1}{2}\text{H}$ each). In addition a slightly coupled singlet at 7.98τ ($1\frac{1}{2}\text{H}$) indicated the presence of a vinyl methyl group, while the "doublets" of the terminal methylene protons were still present at 3.59 and 4.17τ (each $\frac{1}{2}\text{H}$). The remainder of the spectrum was again similar to that of methyl canadensate C-2 epimers (57). The product would thus appear to consist of a mixture of one part of each of the methyl canadensate epimers (57) and two parts of the methyl ester of the isomeric acid (123, R=Me).

Although p.l.c. had failed to separate this mixture of isomeric compounds, g.l.c. (using a 1% SE30 column at 141°) readily separated the methyl canadensate C-2 epimers (57) (R_t 4.8 min) from the methyl ester of the isomeric acid (123) (R_t 6.5 min.). The corresponding peak areas were found to be identical (Fig.10), confirming the proportions deduced from n.m.r. Combined gas liquid chromatography - mass spectrometry (g.c.m.s.) enabled the mass spectra of the two isomers to be recorded (Table 6). Both compounds exhibited



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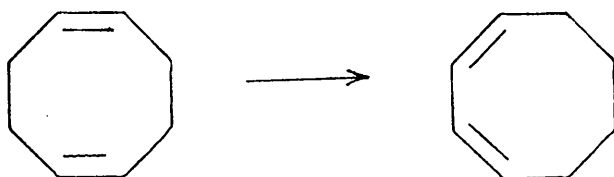
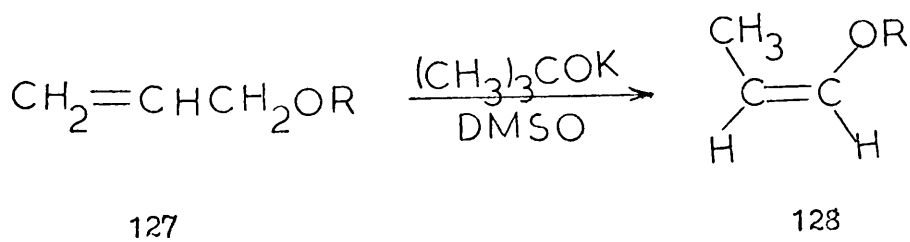
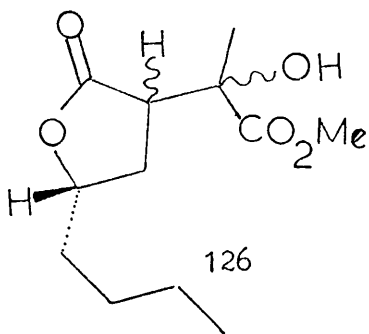
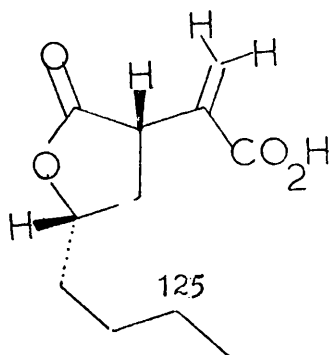


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relatively weak molecular ions at m/e 212, confirming that they were isomers. The mass spectra were found to possess many common ions, which differed only in relative abundance thus defying structural distinctions. Exceptions were the lack of an $M^+ - 15$ ion in the spectrum of the methyl canadensate C-2 epimers (57), and a strong ion at m/e 138 (loss of butene + MeOH) in the mass spectrum of the isomeric ester (123, R=Me).

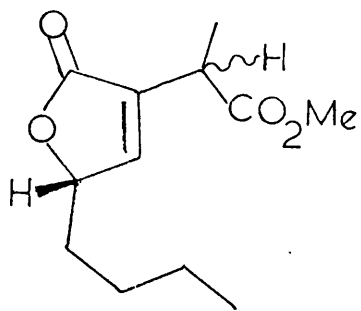
Further proof was obtained from hydrogenation of the above mixture of isomers. Saturation of both double bonds was confirmed by the absence of any olefinic absorptions in the i.r. spectrum which exhibited ν_{\max} . (thin film) at 1765 cm.^{-1} (γ -lactone) and 1735 cm.^{-1} (CO_2Me). Comparison of the i.r. spectrum with that of the quaternary mixture of methyl dihydrocanadensate (C-2, C-10) diastereoisomers showed that they were essentially superimposable. From hydrogenation of the methyl canadensate epimers (57) present in the mixture of isomers a 1:1 ratio of dihydroesters having either a 2S or 2R absolute configuration would be expected. From the isomeric methyl ester (123), however, only one dihydroester (124), having a 2S absolute configuration, will be obtained if hydrogenation is stereoselective due to the steric effect of the butyl group. This explains the observed g.l.c. trace which corresponded to a mixture of dihydroesters having a ratio of 3:1 of those with a 2S to those with a 2R absolute configuration.

The above result had established that half of the mixture of canadensic acid C-2 epimers (55) was isomerised. It was further shown that in fact 2-epicanadensic acid(125) was completely transformed while

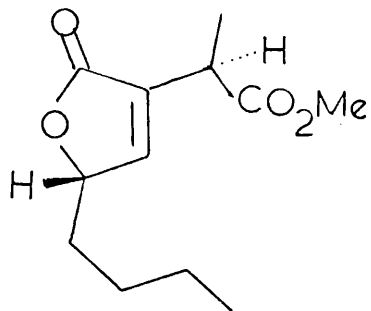


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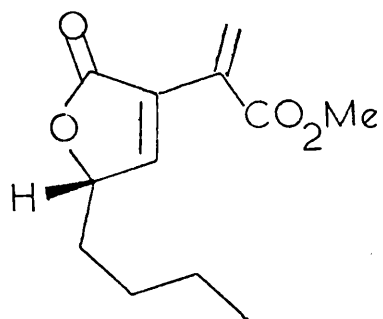
canadensic acid (95) itself was unaffected. In order to do this it was necessary to remove the DMSO in the manner previously described before the n.m.r. spectra were run. Time studies showed the gradual disappearance of the two higher field signals of the two "doublets" which correspond to the terminal methylene protons of 2-epicanadensic acid, the thermodynamically less stable isomer. However, instead of a vinyl methyl group at 7.93 τ , a 3H singlet at 8.62 τ appeared. This must be assigned to a tertiary methyl attached to a carbon carrying an oxygen as in the compound (126) which would arise from hydration of the conjugated double bond of the isomeric acid (123, R=H) on work-up. Esterification of this acid using methyl iodide/potassium carbonate/acetone will result in dehydration to the isolated methyl ester (123, R=Me). DMSO is of course a very potent and versatile solvent ⁸⁹ and has been found to promote reactions not otherwise realisable. Striking solvent effects found using this medium have been attributed to the greatly enhanced reactivity of anions in DMSO in contrast to the reduced activity of hydrogen-bonded anions in hydroxylic solvents of comparable dielectric constant. Although no reports of double bond migration in DMSO alone have been reported, the solvent has been found to greatly accelerate prototropic rearrangements under catalysis by potassium t-butoxide. Price and Snyder ⁹⁰ used this reagent to isomerise alkyl ethers (127) to cis-propenyl ethers (128), while the same base-solvent combination has been used extensively for the



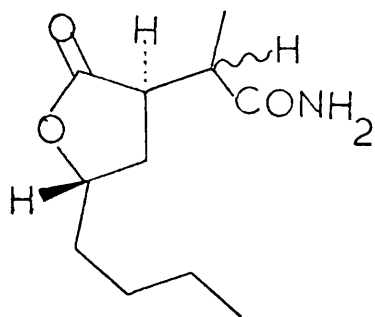
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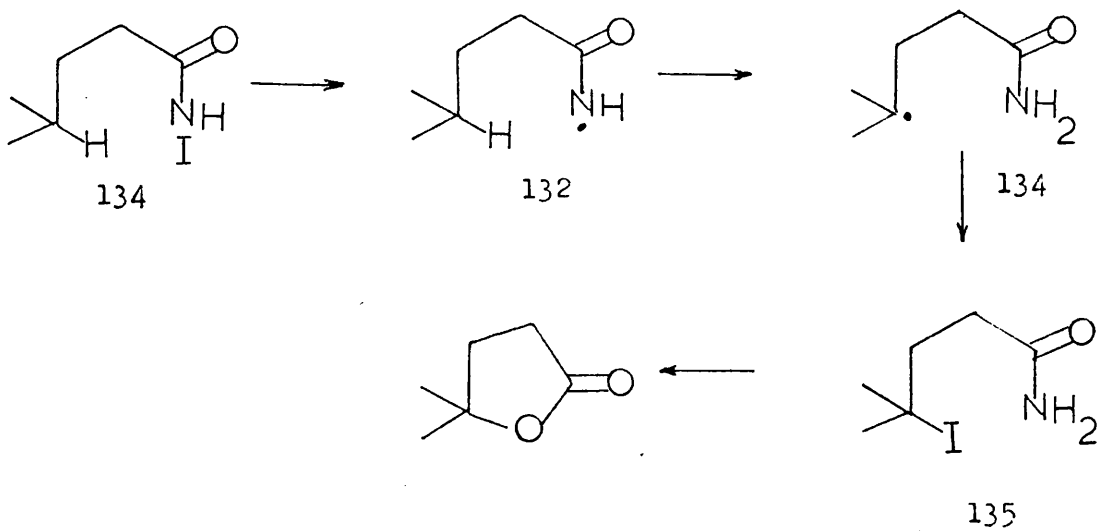
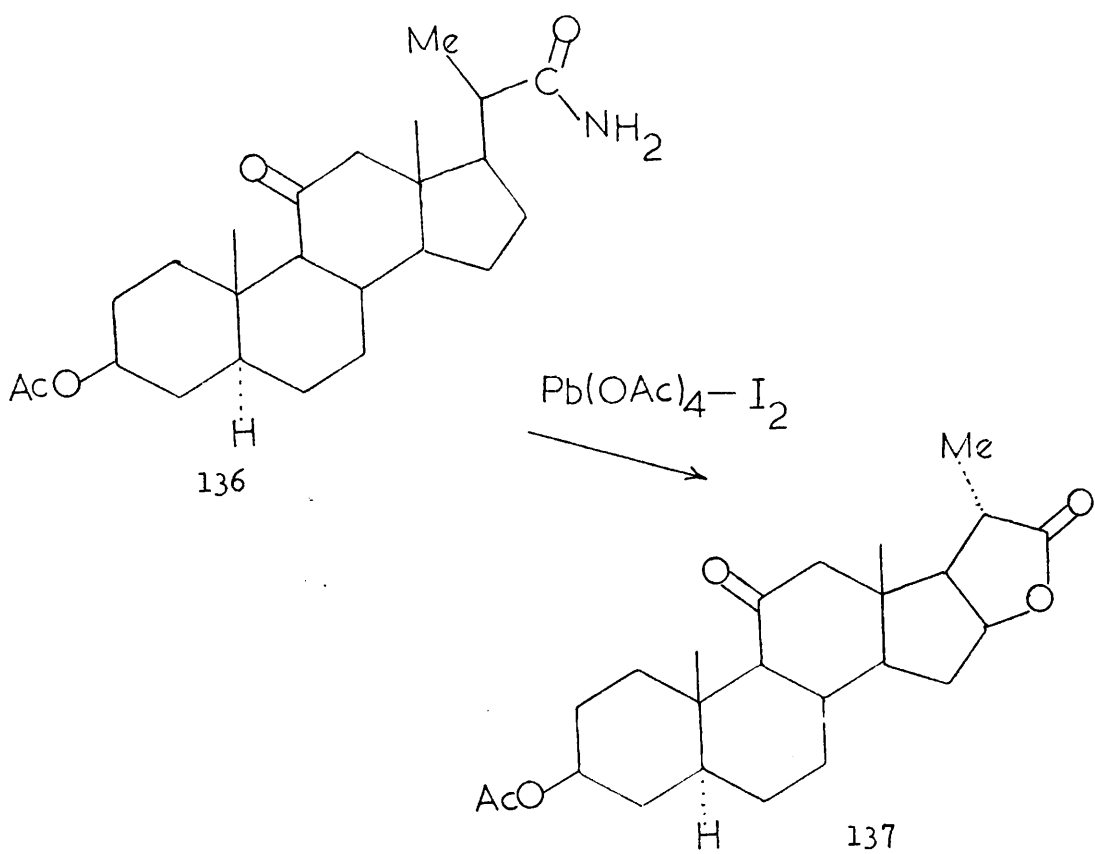


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isomerisation of olefins, cyclohexadienes, cyclooctadienes etc., under mild conditions, for example, 1,5-cyclooctadiene (129)⁹¹. The mechanism of isomerisation in the case of the canadensic acid C-2 epimers (55) must involve proton abstraction by a DMSO molecule from the epicanadensic acid (125) molecule, followed by a double bond migration to the isomeric acid (123, R=H). One possible explanation why this only occurs with the one epimer, is that the proton H-2 is more accessible in the epicanadensic acid molecule where H-2 and H-4 are cis than in that of canadensic acid (95) where approach to H-2 is restricted by the bulky butyl group. It is unlikely that the isomerisation can be explained only on thermodynamic grounds as both epimers appear to be one of the same order of stability.

Although isomerisation of one epimer of canadensic acid had been achieved, attempts to further isomerise the resulting isomeric ester (123, R=Me) to give a butenolide (130) by treatment with acid were unsuccessful. Attempts to isomerise the butenolide (69) derived from dihydrocanadensolide (54) by basic treatment were also unsuccessful.

Reaction of the isomeric acid (123, R=H) with selenium dioxide might have opened a route to the dihydrocanadensolide system but this was not investigated. It was also hoped that conversion of canadensic acid (55) into the diene (68) obtainable from canadensolide (51) (see p. 34), could be achieved using dichlorodicyanobenzoquinone (D.D.Q.) which is known to convert δ -lactones into their α , β unsaturated



derivatives⁹². However, dehydrogenation of methyl canadensate (100) could not be affected with this reagent.

It seemed possible that interconversion of canadensic acid (95) and canadensolide (51) might have been effected by means of a "Barton reaction"⁹³, which enables γ -lactones to be synthesised from the amide of the corresponding saturated acid. The mechanism involves photolysis of the N-iodo amide (131) to the radical (132) which undergoes intramolecular hydrogen atom transfer. Coupling of the resulting radical (134) with iodine generates the δ -lactone via an intermediate γ -imino lactone. By means of this reaction the steroid 20 - carboxamide (136) was converted into the compound (137), which has a γ -lactone ring cis fused to a five membered ring⁹⁴. This appeared to be a reasonable analogy to the dilactone ring system of canadensolide (51). It may be noted that synthesis of canadensolide (51) from canadensic acid (95) could not have been achieved without inversion of the configuration at C-2, since the antibiotic has a cis orientation of the protons H-2 and H-4. However, it was decided to use dihydrocanadensic acid amide (C-10 epimers) (139) as a model system to explore this reaction.

Accordingly the mixture of dihydrocanadensic acid C-10 epimers (107) was converted to its amide (139) by treatment of the corresponding acid chloride with ammonia. The mixture of dihydrocanadensic acid amide C-10 epimers was obtained as an

oil, ν_{\max} . (thin film) 3,400 and 3,210 cm^{-1} (CO.NH_2) 1750 cm^{-1} (γ -lactone), 1660 cm^{-1} (broad) (amide). Its structure was also confirmed by the n.m.r. spectrum, which showed the broad 2H multiplet of the amide protons at 3.86 τ (non-exchangeable with D_2O). The remainder of the spectrum was, as expected, similar to that of the methyl dihydrocanadensate C-10 epimers (108). Irradiation of these amide epimers was carried out under Barton reaction conditions. Hydrolysis of the reaction mixture, however, produced the crystalline mixture of C-10 epimers of dihydrocanadensic acid (107). An attempt to detect any dihydrocanadensolide (54) (or 10-epidihydrocanadensolide) or the C-2 isomers was made by methylating a part of the reaction mixture using diazomethane. G.l.c. of the resulting oil showed only methyl dihydrocanadensate C-10 epimers (108) and none of the required dilactone compounds. It has been reported⁹⁵ that the Barton reaction was also unsuccessful as a synthetic approach to the related dilactone structure of avenaciolide (58). Once again it would appear that the reactive centre is too far removed from the required site of attack.

As will be shown later, interconversion of canadensolide with another metabolite, hydroxyisocanadensic acid (148), was to prove more useful in establishing the absolute and relative stereochemistry of the antibiotic.

HYDROXYISOCANADINIC ACID.

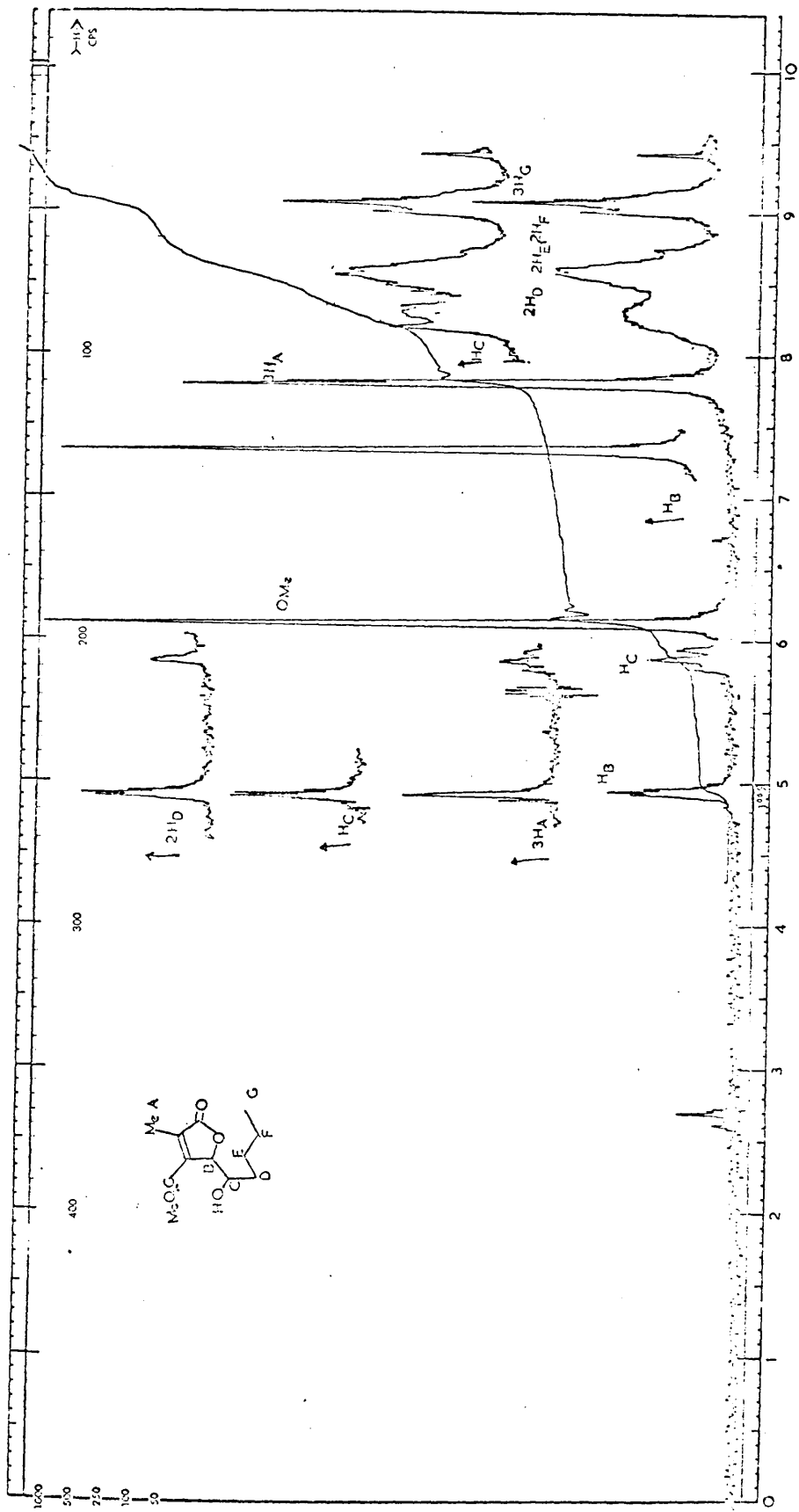
As indicated previously, monolactonic derivatives of canadensolide (51) were useful in establishing the absolute stereochemistry of the antibiotic using o.r.d. and c.d. More careful examination of the broth of P. canadense revealed the presence of a number of lactonic acids, present in small amounts. If the relationship of any of these to canadensolide could be demonstrated then these could afford complementary evidence about the stereochemistry of the antibiotic.

One of these acids was more polar than canadensic acid (95) and was obtained crystalline rather than as a jelly only if highly pure. The molecular formula, $C_{11}H_{16}O_5$, was assigned to this metabolite on the basis of elemental analysis and mass spectrometry. The polarity of this compound relative to canadensic acid (95) was explained by the presence of an extra hydroxyl group as indicated by i.r. absorption at $3,420\text{ cm.}^{-1}$. A carboxylic acid group was also suggested by broad absorption at $3,300 - 2,600\text{ cm.}^{-1}$ and 1708 cm.^{-1} in the carbonyl region. A strong band at 1759 cm.^{-1} was assigned to a γ -lactone system, and weak but sharp absorption at 1670 cm.^{-1} together with strong absorption at 229 nm. ($\epsilon 7,900$) in the u.v. spectrum was consistent with the double bond being in conjugation with both the lactone and carbomethoxyl groupings.

The n.m.r. spectrum indicated that the double bond must be tetrasubstituted since no resonance attributable to an olefinic proton

was apparent. A broadened singlet at 7.76τ , however, indicated the presence of a vinyl methyl group ($3H_A$). After addition of D_2O , the only other resonance below 7.76τ were two 1H multiplets at 4.91τ (H_B) and 5.70τ (H_C). It was assumed that these corresponded to protons geminal to oxygen. The remaining multiplets at 8.3τ (2H), 8.6τ (4H) and a triplet (3H) at 9.04τ exhibited a pattern comparable to that of the n-butyl group of canadensolide. Due to the low solubility of the metabolite in chloroform, the spectrum was fairly weak and unsuitable for decoupling experiments. The mass spectrum, however, showed an abundant ion at m/e 229 corresponding to an $M+1$ ion. This latter type of ion is typical of an acid ⁹⁶ and is normally obtained under relatively high gas pressures when the parent molecular ion is weak. Significant peaks at m/e 210 ($M^+ - H_2O$) and m/e 192 ($210^+ - H_2O$) indicated the presence of the hydroxyl group, while evidence for a butyl moiety was provided by a strong ion at m/e 171 ($M^+ - 57$).

Since derivatives of acids generally give cleaner n.m.r. spectra, the crystalline methyl ester ($C_{12}H_{18}O_5$) was prepared by treatment of the acid with ethereal diazomethane, no pyrazoline being formed in this case presumably because of the sterically crowded nature of the double bond. In addition to hydroxyl absorption at $3,590 \text{ cm.}^{-1}$, the i.r. spectrum ($CHCl_3$) now showed strong absorption at 1715 cm.^{-1} due to the carbomethoxyl group. The low ester frequency cannot be taken as



METHYL HYDROXYISOCADENATE

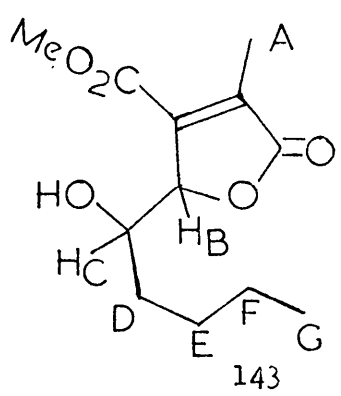
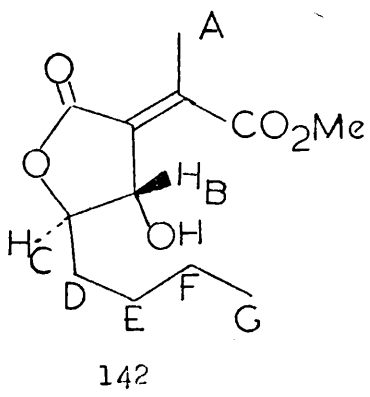
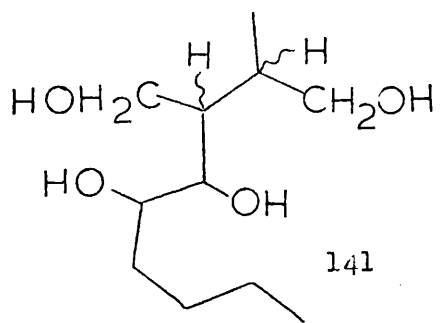
Fig. 11

evidence of conjugation since dilution studies demonstrated the presence of intermolecular hydrogen bonding. As indicated later, upon acetylation or oxidation of the hydroxyl group the ester frequency was found to be ca. 1735 cm.^{-1} . However, the conjugated nature of the compound was again stressed by the high intensity absorption at 230 nm. ($\epsilon 11,800$) in its u.v. spectrum.

Confirmation of the presence of the carbomethoxyl grouping was provided by the 3H singlet at 6.12τ in the n.m.r. spectrum (fig. 11). The vinyl methyl group ($3H_A$) was apparent as a 3H doublet ($J = 2.0 \text{ Hz.}$) at 7.80τ , which collapsed to a singlet on irradiation at a finely split 1H multiplet at 4.94τ (H_B), assigned to an allylic proton geminal to oxygen. This proton (H_B) was also coupled to a proton H_C which appeared as a slightly broadened triplet at 5.86τ again ascribed to a proton geminal to oxygen. The small value of J_{BC} (1.5 Hz.) was shown by irradiation at H_C when H_B collapsed to a fine quartet ($J = 2.0 \text{ Hz.}$). Likewise irradiation at a 2H multiplet ($2H_D$) centred at 8.2τ , and assigned to the protons of a methylene group geminal to an oxygen substituent, caused H_C to collapse to a broad singlet. The remaining high field resonances at 8.6τ (4H) and 9.06τ (3H) were again indicative of the n-propyl part of a n-butyl group.

Confirmation that the unit $\text{n-Bu} - \underset{\text{O}}{\underset{|}{\text{CH}}} - \underset{\text{O}}{\underset{|}{\text{CH}}} -$ was present was

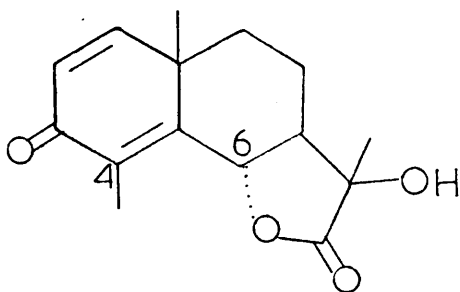
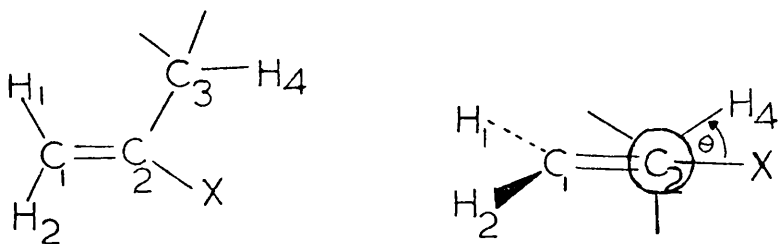
provided by treating this hydroxy compound with an excess



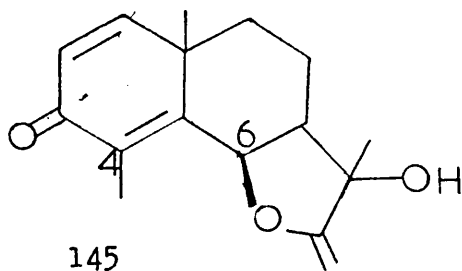
of lithium aluminium hydride to give a polar oil which showed no carbonyl absorption in its i.r. spectrum, but had a strong band at $3,450 \text{ cm.}^{-1}$ due to hydroxylic functions. The tetrol structure (141) assigned to this compound was supported by the n.m.r. spectrum which lacked any vinyl methyl resonance. Oxidation of the tetrol (141) with periodate gave n-valeraldehyde which was characterised as its 2,4-dinitrophenylhydrazone.

If we assume the small coupling between $3H_A$ and H_B is due to homoallylic coupling⁹⁸ then two possible structures (142) and (143), incorporating a carbomethoxyl group, a hydroxyl group and an α, β unsaturated γ -lactone system, can be postulated. These differ as to whether H_B or H_C is the proton geminal to the hydroxyl group. It is difficult to make a decision between these structures purely on the basis of chemical shift values. Thus the typical value for the proton geminal to oxygen of a γ -lactone (found for canadensic acid and its derivatives) would appear to be ca. 5.5τ . The signal at H_B (4.91τ) was proposed to be of this type if the butenolide structure (143) was correct, and the signal at H_C (5.86τ) if the isomeric lactone (142) was correct. The lower value of this proton, H_B (4.91τ), in the butenolide structure (143) would be consistent with its position α to the β carbon of an α, β unsaturated carbonyl system. It is more difficult to explain why the corresponding proton H_C (5.86τ)

Fig.12.



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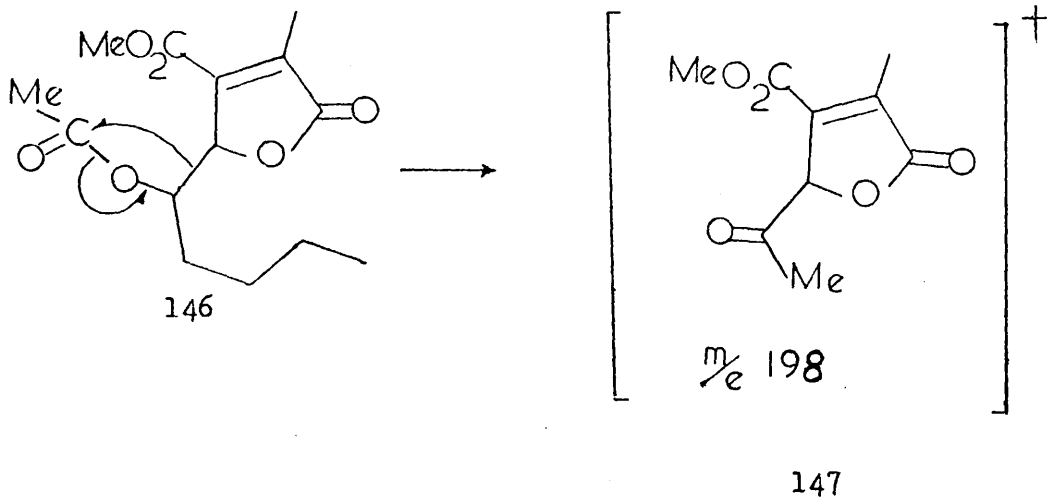
in the isomeric lactone compound (142) should appear at higher field than the 'normal' value, especially since it would be vicinal to the hydroxyl group. A similar argument can be applied to the proton geminal to the hydroxyl group where a typical value is ca. 6.3τ . The signal at H_C (5.86τ) was proposed to be of this type if the butenolide structure (143) was correct, and the signal at H_B (4.91τ) if the isomeric lactone (142) was correct. Arguments can be found to account for the deshielding effects which would be operative on the basis of either structure.

The observed coupling constants between the various protons, on the other hand, seem to favour structure (142). The homoallylic coupling between $3H_A$ and H_B ($J_{AB} = 2.0 \text{ Hz.}$) is explained by both models. This type of coupling through four bonds has been shown to depend on the magnitude of the angle θ^{98} (as shown in fig. 12) between the allylic C-H and the plane of the rest of the system, reaching a maximum of 2-3 Hz. as θ tends to 90° . In the santonin derivative (144), for example, the signal at 7.88τ assigned to the C-4 methyl group was observed as a doublet ($J = 1.4 \text{ Hz.}$) due to homoallylic coupling with H_6^{98} . Models of this compound with ring B in the favoured chair conformation indicated a value of approximately 115° for θ . In the 6-epi derivative (145), the C-4 methyl resonance appeared as a singlet at 7.93τ and in this case the value of θ is approximately 25° . In our case the observed coupling constant suggests a value of θ of close to 90° . Examination of models of the two postulated structures

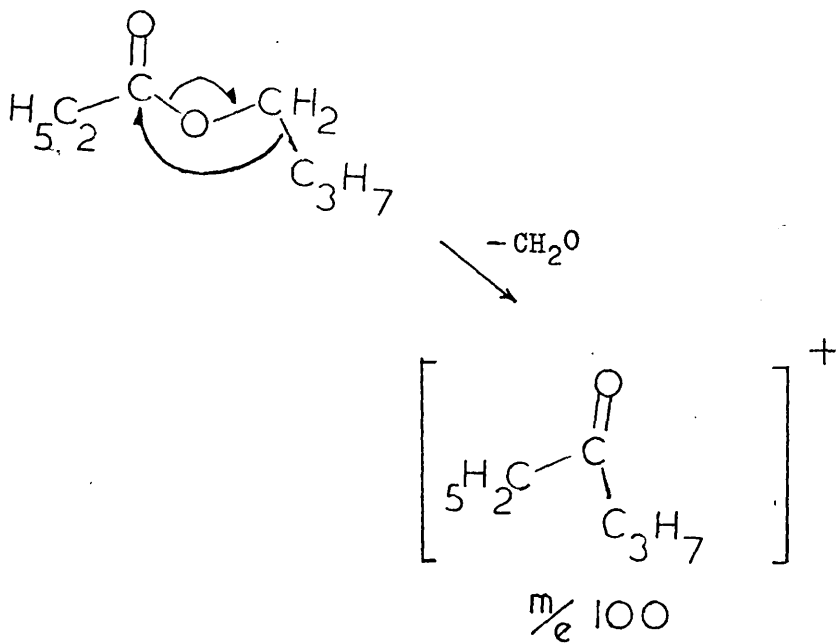
indicated that they both had values of θ ca. 70° , in good agreement with J_{obs} . If, however, we consider J_{BC} (1.5 Hz.) then differentiation of the structures would appear to be obtained. The small value of this coupling constant can be explained by a trans orientation of H_B and H_C in the isomeric lactone (142), when the dihedral angle (115°) from a model predicts a coupling constant of ca. 3.0 Hz. by the Karplus equation. The observed coupling constant, however, would appear to be too small for the butenolide structure (143), where free rotation about the C-C bond would be expected to furnish a much larger coupling constant, in the order of 6-8 Hz.⁹⁹.

However, conflicting with this, the fragmentation pattern of this metabolite clearly favoured the butenolide structure (143) rather than the isomeric γ -lactone (142). Thus the most favoured cleavage¹³¹ would be expected to be at the lactone γ -carbonyl atom and this evidently gives rise to the ion at m/e 156 (the base peak of the spectrum) corresponding to a loss of $C_5H_{10}O$ as would be expected from the butenolide structure (143). On the other hand, the ion at m/e 185 corresponding to a loss of C_4H_9 was of low abundance (1.7%) which would not be expected if the isomeric γ -lactone structure were correct.

Acetylation of this alcohol was carried out in an attempt to clarify this situation. The corresponding acetate, $C_{14}H_{20}O_6$, was obtained as a colourless oil. Its i.r. spectrum (thin film) showed a strong band at 1765 cm.^{-1} assigned to the unsaturated lactone and



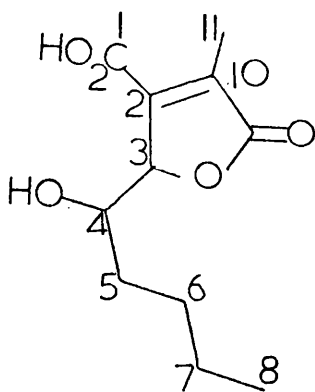
Diag. 4. Mass spectral rearrangement of butyl propionate.



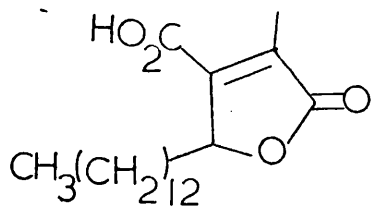
acetate groups while the peak at 1740 cm.^{-1} indicated the presence of the carbomethoxyl grouping. Weak absorption at 1670 cm.^{-1} was again assigned to the tetrasubstituted double bond, while the u.v. spectrum exhibited the typical absorption at $228\text{ nm.}(\epsilon\ 9,760)$ of the expected chromophore.

The presence of the acetate group was confirmed by the 3H singlet occurring at 8.10τ in the n.m.r. The remainder of the spectrum was similar to that of the corresponding alcohol, previously described (cf. fig. 11), except that the proton H_C which had appeared at 5.86τ now occurred as a 1H slightly broadened triplet ($J = 6.0\text{ Hz.}$) at 4.68τ , and was now partly superimposed on the 1H quartet of the allylic proton H_B (4.84τ). This shift can only be interpreted as the deshielding of the proton geminal to an acetoxy group relative to that in the corresponding hydroxy compound (the so called acetylation shift⁹⁷). This result hence was consistent only with the butenolide structure (143) for the original alcohol and makes untenable the isomeric structure (142) where H_C is ascribed to the proton geminal to the oxygen of the lactone.

Of particular interest was the mass spectrum of the acetate, (146) which showed in addition to the molecular ion at $m/e\ 284$ and the strong ion at $m/e\ 224$ ($M^+ - \text{CH}_3\text{CO}_2\text{H}$), an ion at $m/e\ 198$ (147), the base peak in the spectrum. This latter ion was postulated to arise from the molecular ion by the molecular rearrangement shown. Support for



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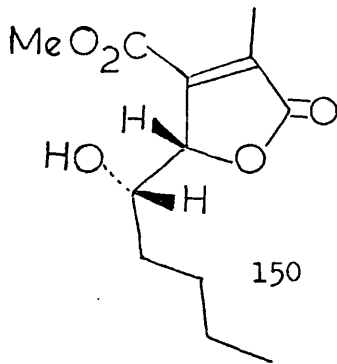
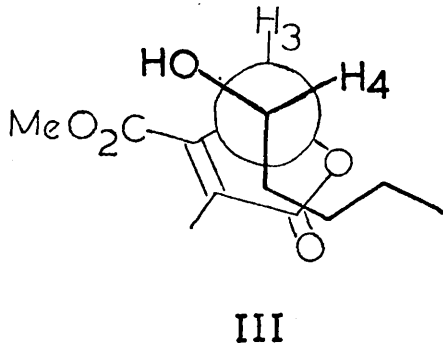
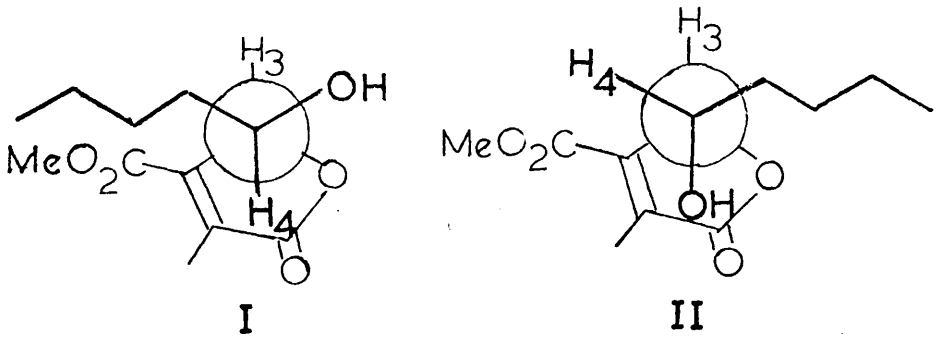
149

its structure was provided by a metastable peak at m/e 123, corresponding to the loss of ketene (42 mass units) from this ion. Moreover, a similar type of mechanism has been used to explain the loss of formaldehyde from the molecular ion of butyl propionate¹⁰⁰ (diag.4), and the corresponding loss of acetaldehyde from the molecular ions of 2-butyl esters of aliphatic acids.¹⁰¹

The original metabolite thus has structure (148) and was named hydroxyisocanadensic acid. It would appear to be derived biogenetically in a similar manner to the other isolated metabolites, and was numbered accordingly. It differs structurally from canadensic acid (95) not only in the additional hydroxyl group but also by apparently having lactone ring B of canadensolid (51) as part of its skeleton. It is similar in structure to many lichen metabolites e.g. lichesterinic acid (149)¹⁰², and possesses the α, β butenolide moiety found in the metabolites of several micro-organisms,¹⁰³ as in tetric acid derivatives some of which display antibacterial activity.

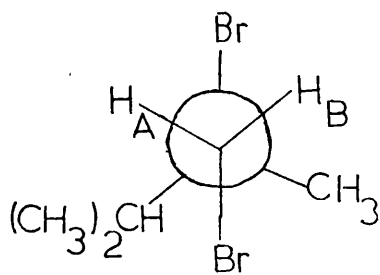
An anomaly in the n.m.r. spectrum of methyl hydroxyisocanadensate (143) was however, the small coupling constant (1.5 Hz.) between the protons H-3 and H-4, which appeared abnormally low for this open chain compound. A possible explanation could have been intramolecular hydrogen bonding between the hydroxyl group and the carbonyl of ester grouping resulting in a fixed conformation having the requisite

Fig. 13 Conformers about (C-3) — (C-4) bond in methyl hydroxyisocanadensate.

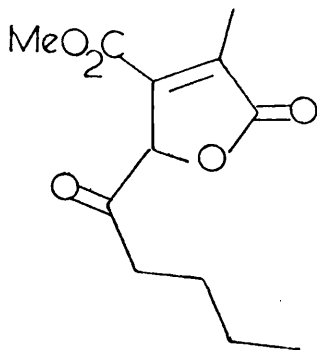


dihedral angle (ca. 70°) and hence small coupling constant. I.r. dilution studies, however, showed that there was essentially no intramolecular hydrogen bonding in the molecule. It was possible that in the concentrated n.m.r. solution intermolecular hydrogen bonding might have caused dimer formation etc., and hence again produce a fixed conformation with the appropriate small coupling $J_{3,4}$. However, when the n.m.r. spectrum of the ester (143) was recorded in deuterated D.M.S.O., a solvent of high dielectric constant (ϵ 48.9 at 20°), which should prevent any self-association, no change in $J_{3,4}$ was observed although the hydroxyl proton appeared as a doublet ($J = 7.0$ Hz.) at 5.30τ due to coupling with H-4. D.M.S.O has, of course, been recommended as a good solvent when it is desirable to see the hydroxyl resonance ¹⁶¹, as the proton exchange rate is invariably quite slow in this solvent. Strong hydrogen bonding occurs with the solvent accounting for the low chemical shift recorded above. This result did confirm the presence of a secondary hydroxyl grouping.

Temperature studies on both methyl hydroxyisocanadensate (143) and methyl acetoxyisocanadensate (146) showed that the coupling constant $J_{3,4}$ did not noticeably increase in magnitude even at 160° (in tetrachloroethylene), thus apparently excluding restricted rotation about the (C-3)-(C-4) bond as a possible explanation. If however, the three conformers obtained by rotation about this bond are considered (fig. 13), assuming for the moment the absolute stereochemistry of the alcohol to be that given in (150) (as will be shown later), it would appear that



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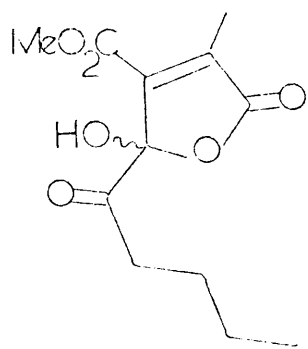
152

considerable steric interaction occurs between the butyl and carbomethoxyl groups in conformers I and III. This suggests that conformer II has the lowest energy of the three conformers, and hence a greater population. Since fast rotation about the (C-3)—(C-4) bond occurs, the observed coupling constant ($J_{3,4}$) is the average of the coupling constants of the three conformers weighed according to their populations. If conformer II is a highly preferred one, then its coupling constant will be similar to that observed ($J_{3,4}$). In fact the coupling constant calculated for conformer II is ca. 2Hz. (for a dihedral angle of 60°), which is similar to $J_{3,4}$ obs. Raising the temperature would not necessarily equalise the populations, and hence increase $J_{3,4}$ obs.¹⁰⁴ A relevant case is the gauche conformer of sym-tetrachloroethane¹⁰⁵ whose inter-proton coupling constant has been calculated to be 2.0 Hz. Moreover, similar 'locked' conformations have recently been postulated¹⁰⁶ for 1,2 dihalo -, 1,3 dihalo -, and 1,2,3 trihalo-2,3-dimethylbutanes on the basis of their n.m.r. spectra. Thus conformation (151) of 1,2 - dibromo-2,3 - dimethylbutane appeared to highly favoured over all the others, and was utilised to explain the observed spectral features.

Further confirmation of the postulated structure for this metabolite was obtained by oxidation of methyl hydroxyisocanadensate (143) using Jones reagent. By rapid work-up and exclusion of air as much

as possible, an unstable, yellow oil, $C_{12}H_{16}O_5$, was obtained. Its i.r. spectrum (CCl_4) showed absorption at 1780 cm.^{-1} typical of the butenolide group, while a strong band at 1732 cm.^{-1} suggested the presence not only of the expected carbomethoxyl group but also of an (α -polar substituted) ketone ¹⁶⁷. The ketone function was also suggested by the u.v. spectrum which in addition to absorption at $240\text{ nm.} (\epsilon 5,190)$ exhibited a weaker band at $382\text{ nm.} (\epsilon 721)$ attributed to the conjugated chromophore produced on enolisation, although no enol form was apparent in the i.r. and n.m.r. spectra. This assignment was confirmed on basification when the u.v. spectrum showed only strong absorption at $382\text{ nm.} (\epsilon 14,850)$.

The n.m.r. spectrum provided confirmation that the ketone (152) had been formed, as the resonance attributed to the proton geminal to hydroxyl group (H-4) of methyl hydroxyisocanadensate (143) was absent. The only low field resonance was the 1H quartet ($J = 2.0\text{ Hz.}$) of the allylic proton, H-3, which was shifted downfield by 0.3 ppm. from its previous value under the effect of the ketonic grouping. It was coupled to the characteristic 3H doublet of the vinyl methyl group at 7.77τ . An important feature was a 2H multiplet centred at 7.37τ which appeared to be the AB protons of an ABX_2 system, and was consistent with the protons of a methylene group α to the ketone. It was evident in the mass spectrum that the ketone group now directed fission, resulting in



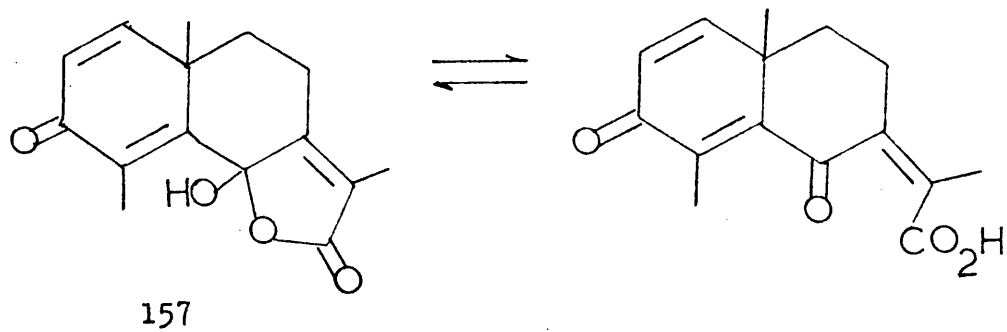
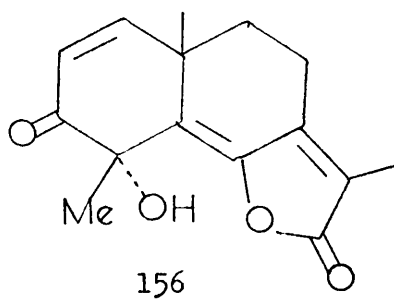
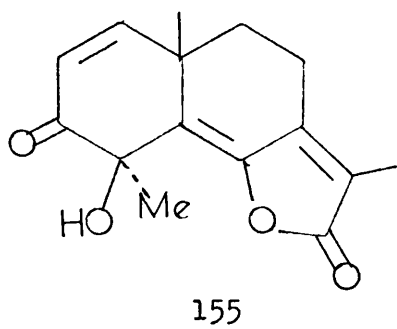
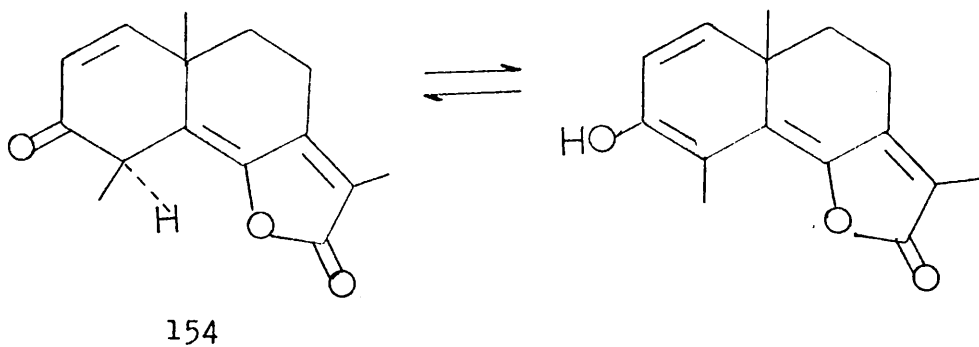
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the intense ions at m/e 85 ($\text{CH}_3\text{CH}_2\text{CH}_2\text{C}\equiv\text{O}^+$) and m/e 57 ($\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2^+$); ions which were not present in the mass spectra of previous derivatives.

As indicated above, care was necessary in the work-up of this oxidation reaction since the product was found to be sensitive to air and attempted purification on p.l.c. converted it into a more polar compound, which analysed as $\text{C}_{12}\text{H}_{16}\text{O}_6$. The i.r. spectrum (CHCl_3) of this compound showed the presence of a hydroxyl group by absorption at $3,430\text{ cm.}^{-1}$. In the carbonyl region, absorption at 1790 cm.^{-1} (ϵ 521, $\Delta\nu_{\frac{1}{2}}$ 20 cm.^{-1}) again typified the butenolide group while the peak at 1739 cm.^{-1} (ϵ 509, $\Delta\nu_{\frac{1}{2}}$ 25 cm.^{-1}) was assigned to the combined absorption of the carbomethoxyl and ketone functions. The u.v. spectrum, however, did not show the previously described base shift.

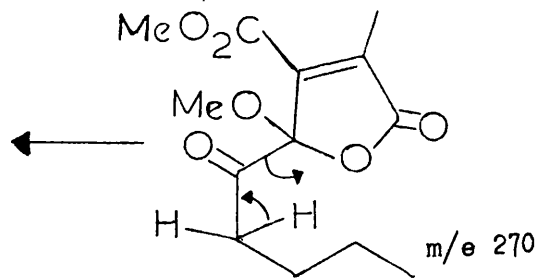
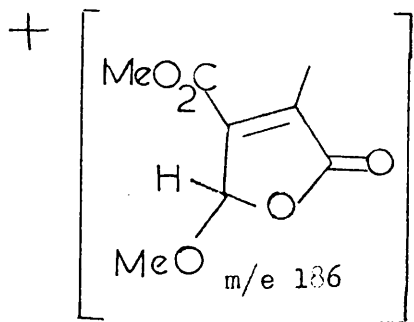
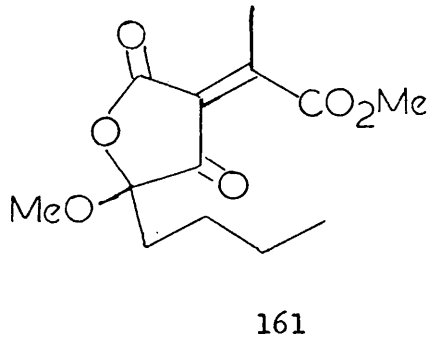
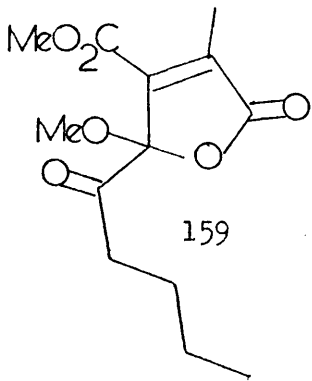
In the n.m.r. spectrum the absence of the 1H resonance of the allylic proton (H-3) found in ketone (152) suggested that C-3 now bore a hydroxyl substituent. This was supported by the absence of any coupling of the vinyl methyl group, which appeared as a sharp singlet at 7.70τ . The similarity of the remainder of the n.m.r. spectrum to that of ketone (152) suggested that the pseudo-acid (153) had been formed.

Formation of this lactol (153) must involve the ready aerial oxidation of the ketone (152) on chromatography. An obvious analogy



can be drawn with the reported¹⁰⁸ autoxidation of santonene (154), which possesses a system vinylogous to that postulated for the ketone (152). When santonene (154) was set aside in ethanol solution with access to the air then the two neutral compounds (155) and (156), and the 'acid' (157) were isolated. (In the case of ketone (152), of course, only the pseudo acid (153) is possible). This autoxidation would appear to involve the enol form. Keto-enol tautomerism studies¹⁰⁹ on santonene (154) showed that no enol form was apparent by i.r., n.m.r., or u.v. measurements in chloroform solution, but an estimated 39% enol form was present in methanol solution. Similar conclusions were reached about the enolisation of the ketone (152) from its spectral data (see above).

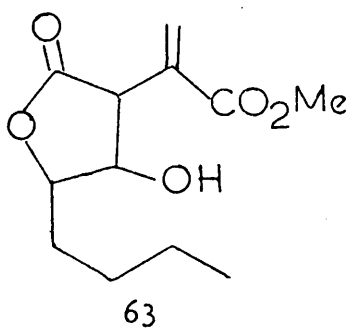
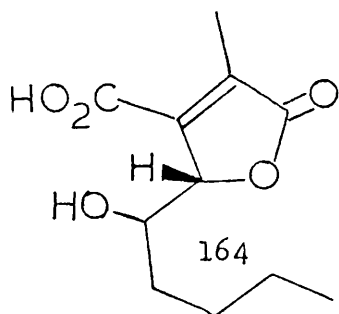
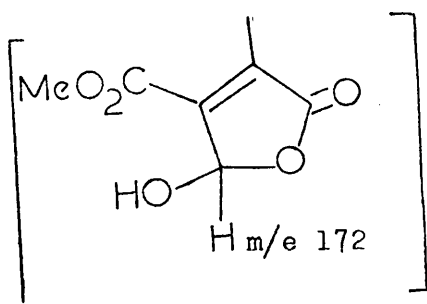
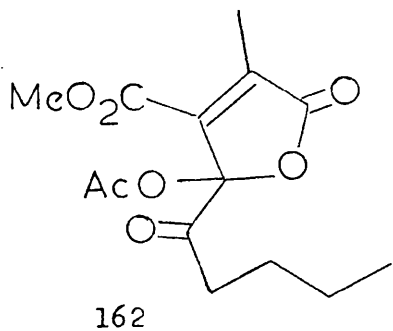
The progress of this oxidation could easily be followed visually since the yellow colour of the starting material, presumably due to its enol form, gradually disappeared with oxidation, for example when bubbling oxygen through its methanol solution (under these conditions the pseudo-acid (153) was obtainable in high yield after about 2 hours). Although the pseudo-acid (153) was readily soluble in dilute alkali, it did not appear to exist in a detectable state of equilibrium (CHCl_3 solution) with its keto-acid form. It is hence unlike the corresponding pseudo-acid (157) of santonene (154), whose keto-acid form (158) was apparent in its n.m.r. spectrum and which was readily soluble in aqueous sodium hydrogen carbonate.¹⁰⁸



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Further proof of the pseudo-acid structure (153) was obtained by its brief treatment with methanol containing sulphuric acid as catalyst when the lactol ether (159) was obtained as an oil, $C_{13}H_{18}O_6$. Its i.r. spectrum (CCl_4) lacked any hydroxyl absorption but showed peaks at 1790 cm.^{-1} (γ -lactone), 1735 cm.^{-1} (CO_2Me and $C=O$). Support for its structure was provided by the n.m.r. spectrum which exhibited a new methoxyl resonance at 6.67τ (3H) in addition to the resonances found previously for the pseudo-acid (153). Although the molecular ion at m/e 270 in the mass spectrum was weak, cleavage α to the ketone function resulted in significant, intense ions at m/e 186 (160), and m/e 85 ($CH_3\cdot CH_2\cdot CH_2\cdot CH_2\cdot C\equiv O^+$) in agreement with the structure. It may be noted that the m/e 186 ion would have been more difficult to account for on the basis of a product (161) derived from the discredited isomeric lactone structure (142).

The tertiary hydroxyl group, although unaffected by acetic anhydride and pyridine at room temperature, was acetylated by refluxing in acetic anhydride with *p*-toluensulphonic acid to give an oil, $C_{14}H_{18}O_7$. Strong absorption at 1800 cm.^{-1} with a shoulder at 1790 cm.^{-1} in its i.r. spectrum (CCl_4) was attributed to the unusual gem acetoxy lactone system. (Gem diesters are known to show abnormally high carbonyl frequencies ¹¹⁰). The remaining absorption in the carbonyl region occurred at 1742 cm.^{-1} and was again assigned to the combined absorption of carbomethoxyl and ketone functions. In agreement



with structure (162) the n.m.r. spectrum showed a 3H singlet at 7.80 τ corresponding to the acetate group, and the mass spectrum showed a molecular ion at m/e 298, and major ions, corresponding to cleavage directed by the ketone group, at m/e 256 ($M^+ - CH_3 \cdot CH = CH_2$ by McLafferty rearrangement ⁶⁴), m/e 172 (163), and m/e 85 ($CH_3 \cdot CH_2 \cdot CH_2 \cdot C \equiv O^+$), the base peak in the spectrum.

With the butenolide structure (148) of hydroxyisocanadensic acid substantiated, its absolute configuration at C-3 was readily determined from the o.r.d. and c.d. curves of the acid and its methyl ester (143) to be that shown (164).

The similarity of structure between hydroxyisocanadensic acid (164) and canadensolide (51) suggests that the two might be readily interconverted, and in fact the acid might be an artefact arising from hydrolysis of ring A of the antibiotic followed by double bond isomerisation. Attempts to demonstrate this by alkaline treatment of canadensolide (51), however, were unsuccessful as no product corresponding to hydroxyisocanadensic acid (148) could be obtained. Besides, as the culture medium is acidic, any hydrolysis would occur at ring B as has been demonstrated for methanolysis of canadensolide (51). Since the hydroxy ester (63) from this latter reaction cyclises, then the corresponding acid would be expected to cyclise even more readily.

The presence of hydroxyisocanadensic acid (148) in the ethyl

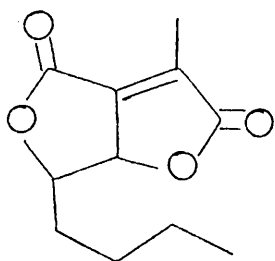
Table 7

Relative acidities of various carboxylic acids.

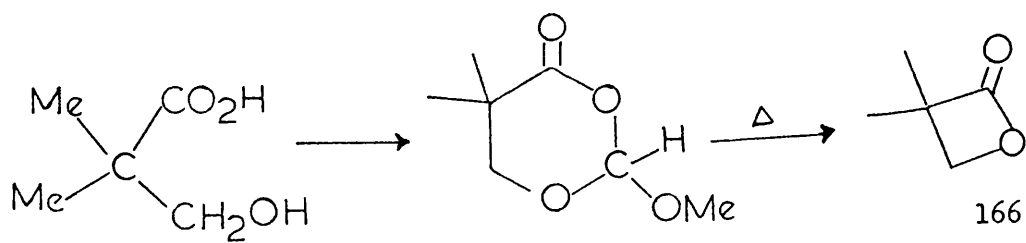
<u>Acid</u>	<u>pKa</u>
Formic	3.77
acetic	4.76
propionic	} 4.85 ± 0.03
<u>n</u> butyric	
isobutyric	
<u>canadensic</u> (95)	4.30
acrylic	4.26
lactic	3.87
<u>hydroxyisocandensic</u> (148)	3.45
fumaric	3.10
pyruvic	2.46

acetate extract of the broth was demonstrated by dissolving the gum obtained on evaporation of the organic solvent in the minimum amount of chloroform, and extracting once with a small volume of aqueous sodium hydrogen carbonate. Acidification of this aqueous extract produced highly pure hydroxyisocanadensic acid (148). This preferential extraction of the acid must be attributed partly to its low solubility in chloroform (shown by its weak n.m.r. spectrum), and also its greater acidity (pK_a 3.45) relative to canadensic acid (pK_a 4.30). This difference in acidity is in agreement with their structures, since hydroxyisocanadensic acid (148) has a stronger electron withdrawing group (a vinylogous lactone carbonyl) than canadensic acid (which has a double bond). A comparison with various related acids is given in (Table 7). The extra hydroxyl group present in hydroxyisocanadensic acid (148) should increase its water solubility relative to canadensic acid (95) and thus might also facilitate this simple method of isolation.

Since canadensolide (51) did not seem to be readily converted into the hydroxyisocanadensic acid (148) the latter seemed extremely unlikely to be an artefact. Their interconversion, however, was still important since the relative stereochemistry of H-3 and H-4 in canadensolide (51) was as yet undetermined. Since the absolute configuration at C-4 in the antibiotic and at C-3 in hydroxyisocanadensic acid (95) had been determined, then an unambiguous interconversion of these two metabolites would establish the absolute configurations of

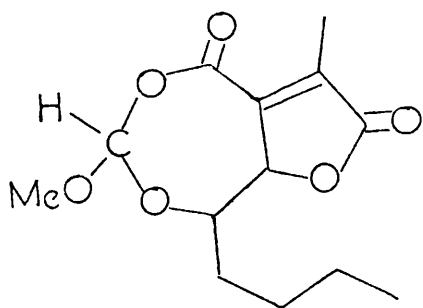


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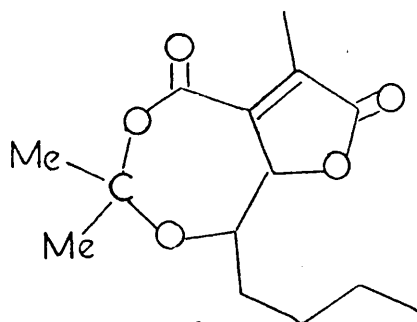


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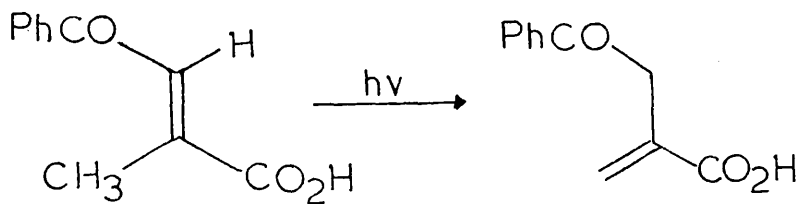
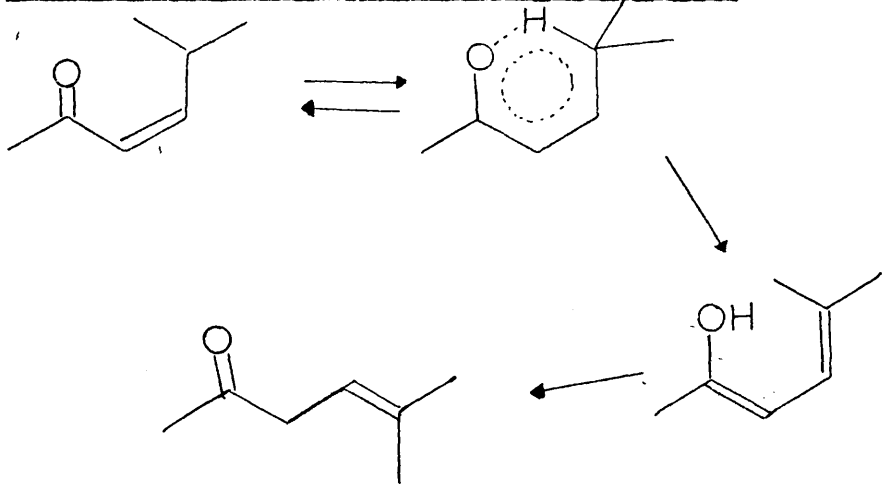
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two centres in both metabolites.

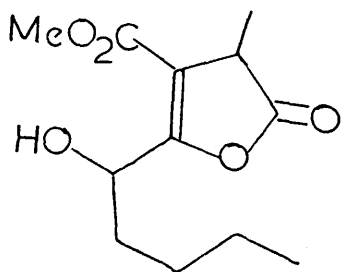
As no success had been achieved in selectively opening ring A of canadensolide (51), chemical modifications of hydroxyisocanadensic acid (148) were attempted in the hope that this would be more readily interrelated with the antibiotic. Although cyclisation of hydroxyisocanadensic acid (148) to the highly strained compound (165) did not appear a likely possibility, dehydrating reagents were employed in the hope that double bond migration might occur with concomitant cyclisation. However, acidic reagents, such as *p*-toluenesulphonic acid, concentrated sulphuric acid and polyphosphoric acid were completely unsuccessful in effecting this transformation.

The recent preparation¹¹¹ of the highly strained β -lactone system (166), involved pyrolysis of the corresponding 4-oxo-1,3-dioxane (167) prepared from the corresponding hydroxy acid by treatment with methyl orthoformate. It was hoped that pyrolysis of the analogous compound (168) of hydroxyisocanadensic acid (148) might result in lactonisation to canadensolide (51). However, attempts to form this seven membered ring compound (168) were unsuccessful, and only traces of methyl hydroxyisocanadensate produced through transesterification were isolated. Similarly, the lactol ether (169) was not produced even on prolonged treatment in acetone with a dehydrating agent.

Diag. 5. Mechanism of double bond migration



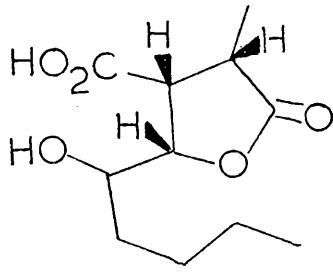
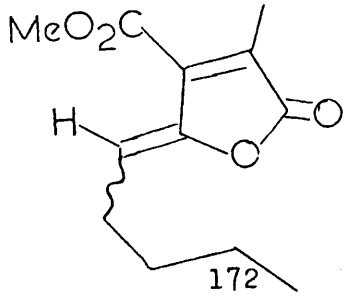
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Thus, from the results indicated above it became obvious that for the required conversion of hydroxyisocanadensic acid (148) into canadensolide (51), double bond isomerisation was a prerequisite. As this involves a tetrasubstituted fully conjugated double bond migrating to a less stable terminal methylene group an energy input was required. Double bond migration is known¹¹² to occur under the influence of u.v. light. The process involves enolisation and takes place through a six membered transition state involving γ -hydrogen abstraction (diag. 5). A particularly relevant example is the reported¹¹³ transformation of the keto acid (170) to its terminal methylene isomer. It was hoped that a similar "optical pumping" of the high absorbing chromophore of hydroxyisocanadensic acid (148) or its methyl ester (143) would produce the required isomerisation. Although irradiation of the methyl ester (143) in ether solution using a mercury lamp did result in the disappearance of this chromophore, canadensolide (51) was not produced. From the n.m.r. spectrum of the product, it was apparent that double bond migration had unfortunately resulted in compounds of type (171).

In a further attempt to effect cyclisation via a 4-chloro-derivative, methyl hydroxyisocanadensate was treated with thionyl chloride or phosphorus pentachloride when a non-polar product was obtained and later shown to be the diene (172). The best preparation of this highly unstable compound was by base catalysed elimination of acetic acid from

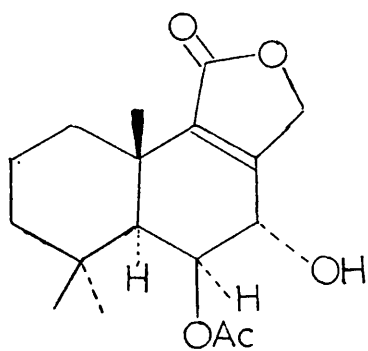


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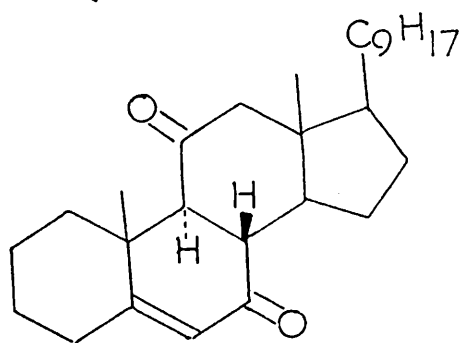
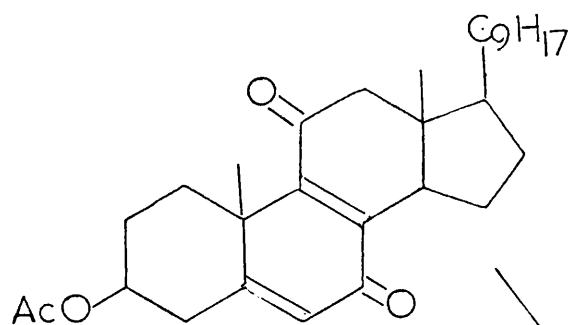
methyl acetoxyisocanadensate (146) using sodium hydride when it was obtained as an oil, which solidified on storing at ca. -20° . Bands at 1735 cm.^{-1} and 1730 cm.^{-1} in the i.r. spectrum (CCl_4) corresponded to the butenolide system and carbomethoxyl groups respectively, and the diene system was suggested by bands at 1660 and 1610 cm.^{-1} , and by intense u.v. absorption at 308 nm. ($\epsilon 9,550$).

In addition to the expected singlets for the carbomethoxyl (6.10τ) and vinyl methyl (7.77τ) groups, the n.m.r. exhibited a 1H triplet ($J = 6.0\text{ Hz.}$) at 4.03τ assigned to the olefinic proton (H-4). It was shown by double irradiation to be coupled to a 2H multiplet at 7.60τ , assigned to the allylic methylene protons (2H-5). The remaining n-propyl group appeared as multiplets at 8.5τ (4H) and 9.08τ (3H). At one stage it was hoped that irradiation of the diene (172) might lead to double bond migration but its rapid decomposition at room temperature made it unsuitable for the proposed photochemical experiments.

It had been previously realised that saturation of the double bond of hydroxyisocanadensic acid (148) would remove the difficulties encountered in the attempted cyclisations described above, and make the saturated dilactone system of the dihydrocanadensolide (54) or its C-10 epimer the ultimate goal. Hydrogenation of the acid (148) was expected to occur from the less hindered side of the molecule to produce the dihydrocompound (173), cyclisation of which would give the required



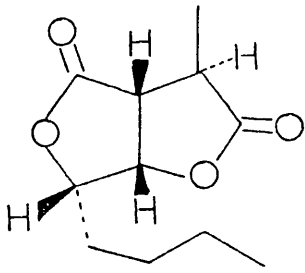
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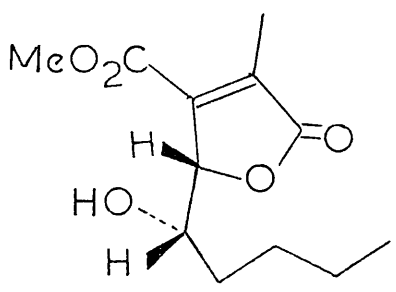
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cis fused dilactone structure of 10-epidihydrocanadensolide (61) or its C-4 epimer. However, it had been found that hydrogenation of this acid could not be effected even under 5 atmospheres pressure with acetic acid as solvent, and added perchloric acid. It is known that hindered tetrasubstituted double bonds are not readily reduced and similar difficulties were encountered¹¹⁴ with attempted reductions of the butenolide double bond of the sesquiterpene ugandensolide (174).

The reduction of the double bond of conjugated dienones, however, has been reported to occur using zinc dust and acetic acid. Thus the ergosterol-derived diene-7,11-dione was reduced selectively at the double bond flanked by the two carbonyls to give (175)¹¹⁵. It was concluded that a similar reduction might occur with the conjugated system of methyl hydroxyisocanadensate (143). In fact, by using more forcing conditions (zinc dust/acetic acid/concentrated hydrochloric acid) two dilactones were produced in moderate yield. Separation of these by p.l.c. proved that they were identical to authentic samples of dihydrocanadensolide (54) and epidihydrocanadensolide (61) by t.l.c., i.r., m.p. and mixed m.p. Moreover, the less polar product also had a similar value for its specific rotation to that recorded for dihydrocanadensolide (54), while the more polar one had an identical o.r.d. curve to that of 10-epidihydrocanadensolide. This confirmed that the synthesised samples had the same absolute and relative stereochemistry as the natural products, and disproved any possibility of their being racemic or



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optical antipodes. This reduction is believed to involve the cis addition¹¹⁶ of hydrogen to the double bond and isomerisation through the enol. No plausible means of affecting the absolute stereochemistry at C-3 or C-4 in methyl hydroxyisocanadensate (143) by this reduction can be envisaged. This means that dihydrocanadensolide (54) and 10-epidihydrocanadensolide (61) must possess the same absolute stereochemistry at C-3 and C-4 as methyl hydroxyisocanadensate (143). As the absolute stereochemistry at these positions have been shown independently to be 4S in dihydrocanadensolide (54) and 3S in methyl hydroxyisocanadensate (143) this means that dihydrocanadensolide has the structure (176) with a cis relation of H-3 and H-4, and methyl hydroxyisocanadensate the structure (177). The antibiotic canadensolide (51) has, of course, the same absolute stereochemistry and cis relationship of these protons.

Since optical rotatory dispersion was carried out in
this work, it was found that the optical activity of
various carbohydrates is not in general proportional to
concentration, and is dependent on the nature of the
medium in which they are dissolved.

The optical activity of various carbohydrates is
also dependent on the nature of the light used, and
on the wavelength of the light. This is especially
true in the case of the optical activity of the
various carbohydrates in solution.

DETERMINATION OF ABSOLUTE CONFIGURATIONS.

The determination of the absolute configuration of a
chiral substance is a problem of great importance in
chemistry. This is because the absolute configuration
of a chiral substance is a property which is
independent of the nature of the medium in which it
is dissolved, and is a property which is
independent of the wavelength of the light used.
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independent of the wavelength of the light used.

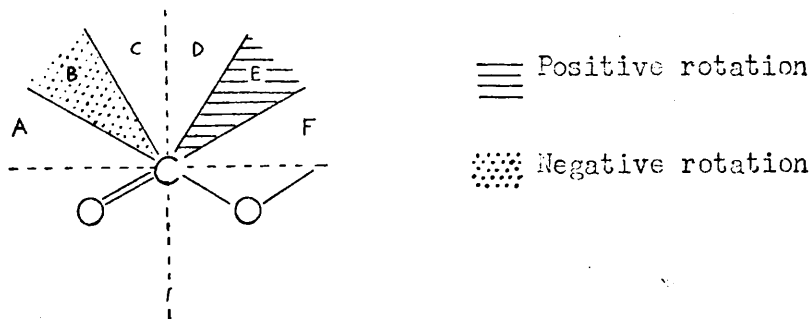
Of particular importance in this work, was the
determination of the absolute configuration of the
various carbohydrates. This was done by the
method of M. Szwarc and J. Drenth, which is
based on the measurement of the optical activity
of the various carbohydrates in solution.

Since optical rotary dispersion and circular dichroism measurements were found to play important roles in determining various stereochemical features of the foregoing metabolites these results are conveniently discussed together.

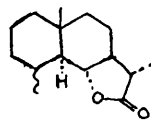
Any molecule which exhibits circular birefringence also shows circular dichroism, both effects occurring simultaneously in a molecule which has active absorption bands¹¹⁷. This combination of unequal absorption and unequal velocity of transmission of right and left circularly polarized light in the region in which optically active absorption bands occur results in the phenomenon of a "Cotton effect"¹²⁰. This effect arises because the inherently symmetric electronic distribution of the chromophore is asymmetrically perturbed by the rest of the molecule in such a way that the symmetry of the electron distribution is lowered sufficiently to permit optical activity. This optical activity usually requires the presence of an asymmetric carbon atom, and the associated Cotton effect can be used as a means of determining the extra chromophoric geometry, as has been elucidated by Djerassi et al¹¹⁸ for ketones using "The Octant Rule".

Of particular importance in our case, were rules relating the stereochemical environment of the carbonyl group of a lactone with its observed Cotton effect. Lactones had in fact been found to be a

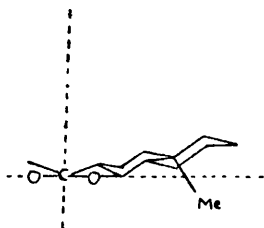
Lactone Sector Quadrants.



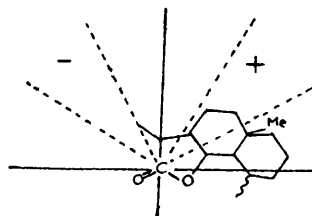
Applications of the lactone sector rule.



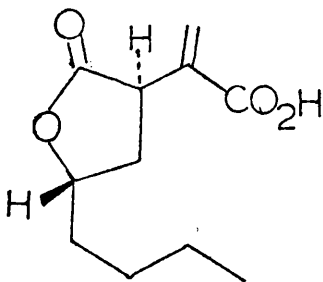
178



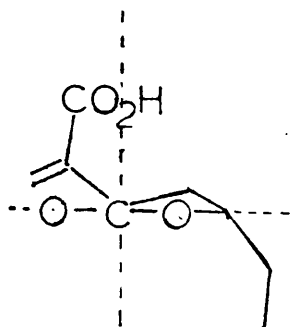
178(a)



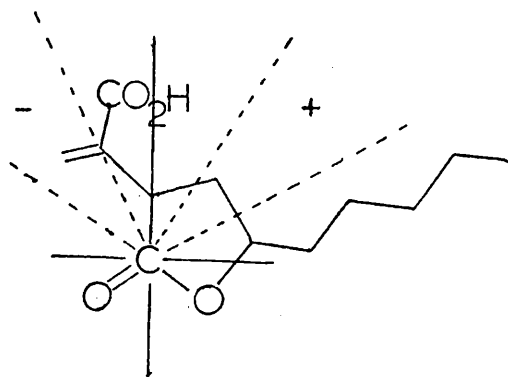
178(b)



95



95(a)



95(b)

useful group for this purpose¹¹⁹, in that the carboxyl group is held in a more or less fixed conformation in a ring, instead of rotating freely (as in a carboxylic acid). Lactones have a weak absorption band at 215 nm. giving rise to a Cotton effect with a first extremum about 225 nm. The "Lactone Sector Rule" established by Klyne relates the absolute configuration of α and δ -lactones to their observed Cotton effects.¹¹⁹ It was evolved by considering both carbon-oxygen bonds as having some double bond character and applying the Ketone Octant Rule to each double bond. Superimposition resulted in the signs of the contributions in some sectors cancelling in varying degrees while in other sectors the contributions reinforce one another, giving on balance a positive contribution in the back upper right sector E, and a negative contribution in the back upper sector B, the boundaries being provisional.

In order to predict the Cotton effect of a molecule from the lactone sector rule, two views of each molecule must be considered. These are (a) the view along the bisectrix of the O-C-O angle (the usual octant projection) and (b) the view of the molecule from above, projected onto the plane of the lactone ring (the sector projection). This is demonstrated by the compound (178) opposite. The signs given are those of the back upper sectors. The lower ones are necessarily opposite to those of the upper sectors, as the lactone group lies in a true symmetry plane. This rule was essential for the determination

Fig. 14 O.r.d. curves of 'canadensic acid.'

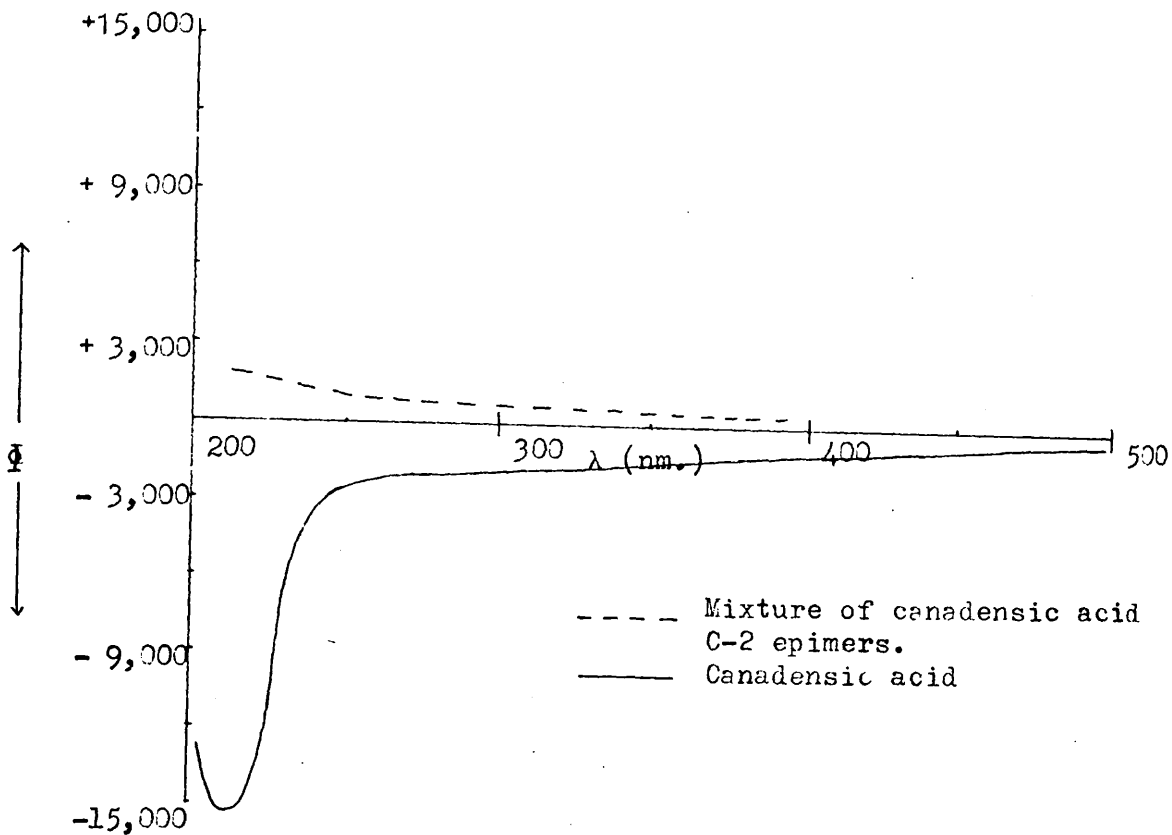
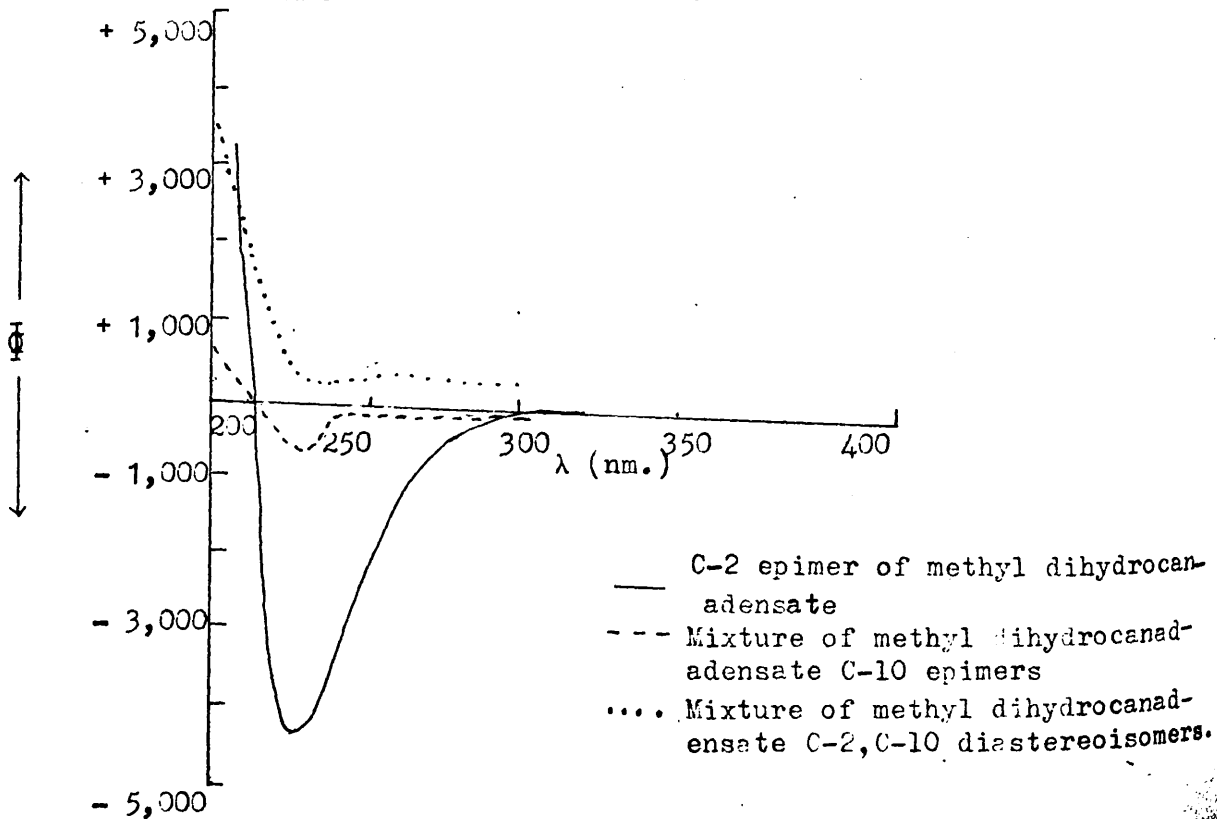


Fig. 15 O.r.d. curves of dihydrocanadensate esters.



of the absolute configurations of the three metabolites, canadensic acid (95), canadensolide (51) and hydroxyisocanadensic acid (168).

Absolute Configuration at C-2 and C-4 in Canadensic Acid.

Canadensic acid (95) exhibits a trough at 222 nm. in its o.r.d. curve (fig. 14), indicating the first extremum of a negative Cotton effect. If the lactone sector rule is applied to this molecule, contribution to the Cotton effect appears to come mainly from the substituent at C-2. This appeared to be confirmed by the o.r.d. curve of the mixture of canadensic acid C-2 epimers when essentially a plain positive curve was obtained. If the source of the Cotton effect is indeed the lactone and not the acrylic acid moiety, then the sector rule would appear to lead to assignment of the stereochemistry at C-2 as 2R. However, the o.r.d. spectra of isomers of methyl dihydrocanadensate differing in configuration at C-2 did not inspire confidence in this interpretation. Although the reduction product of canadensic acid (fig. 15) gave a negative Cotton effect and that of 2R + 2S canadensic acid an essentially plain curve, the C-2 epimer of dihydrocanadensic acid obtained from the butenolide (69) also showed a negative Cotton effect. The possibility that this was in fact due to traces of unreduced butenolide (69) cannot at present be discounted.

However, confirmation of the 2R assignment was obtained by unequivocal determination of the absolute configuration at C-4

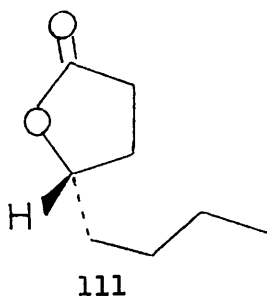
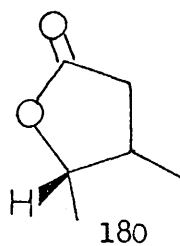
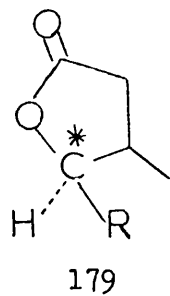
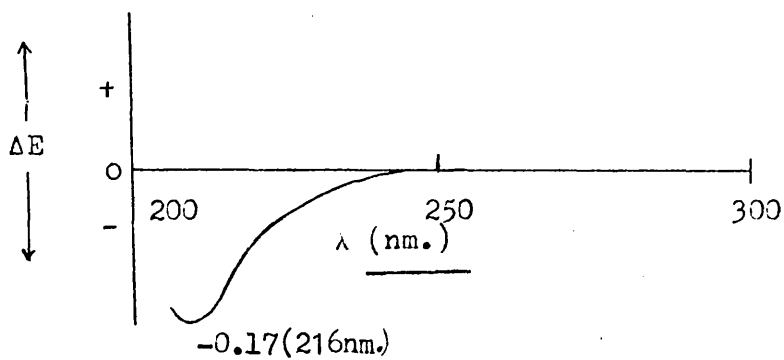


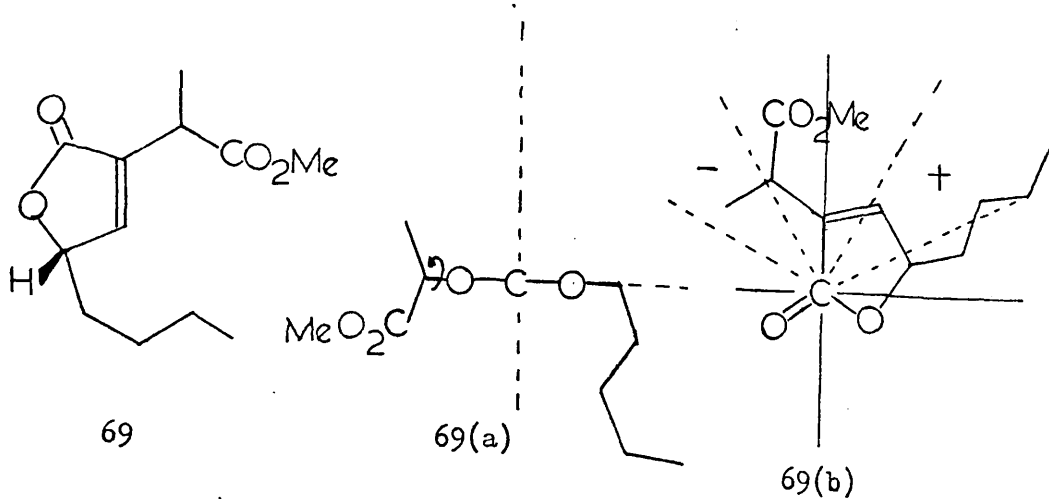
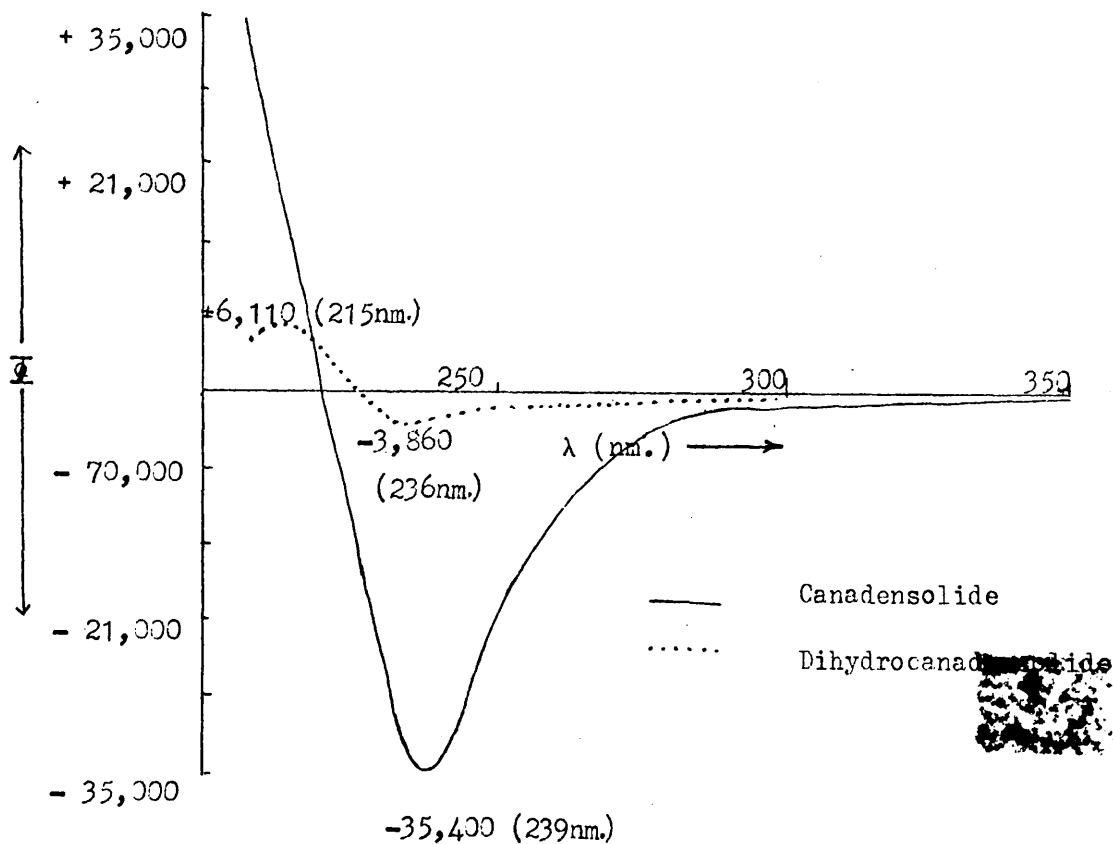
Fig. 16. C.d. of caprylolactone derived from canadensis acid.

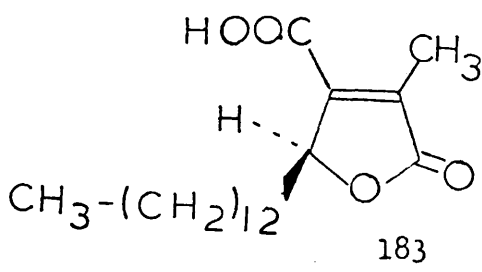
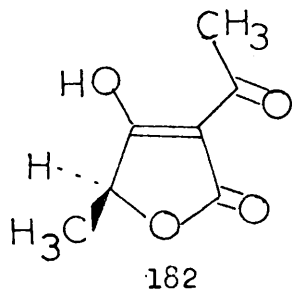
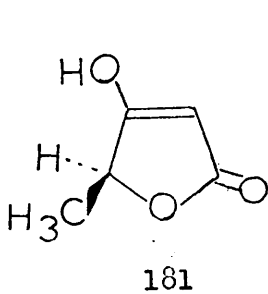
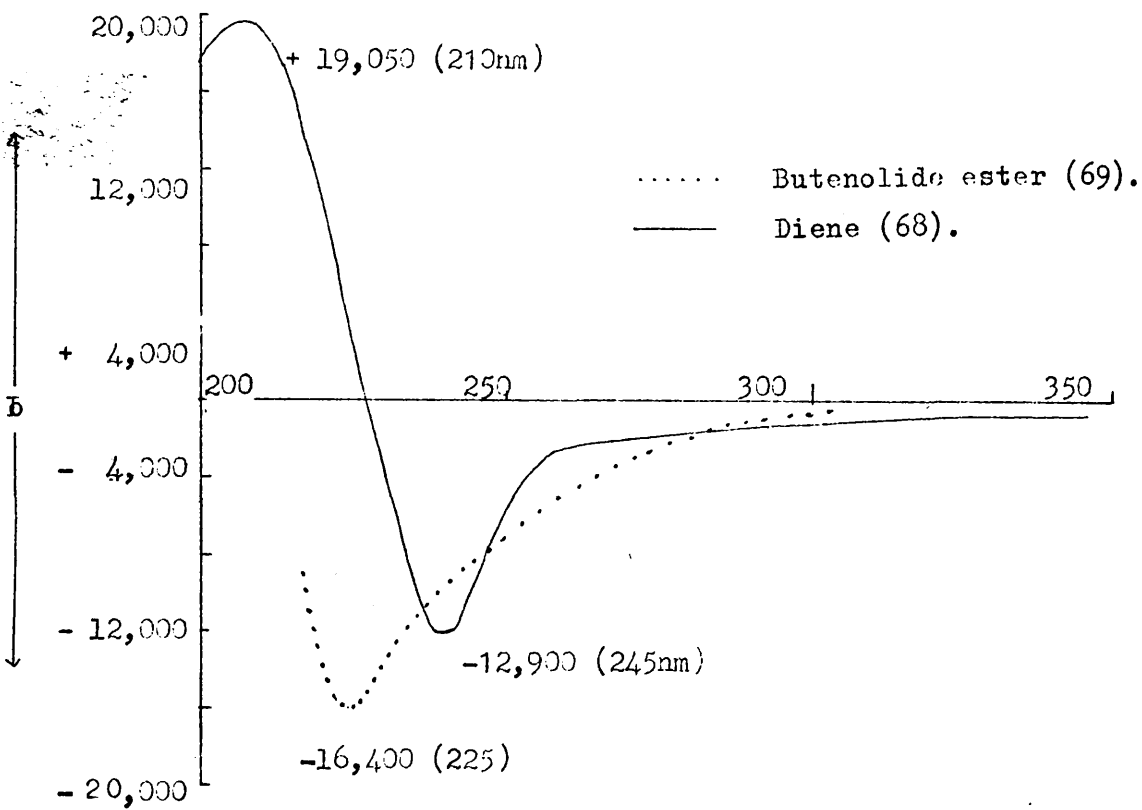


and using the fact that a trans relationship of H-2 and H-4 had been established (cf. chapter 3). This was achieved by the degradation of canadensic acid (95) to caprylolactone (111), which does not contain a C-2 substituent or ester chromophore, and was hence more suitable for o.r.d. studies than the previous compounds. An early attempt to relate the stereochemistry of a lactone, of type (179) to its optical rotation had been made by Hudson¹²¹ on the basis of studies on five and six membered lactones of the sugar series.

Hudson's lactone rule may be stated as follows, "If the hydrogen atom at the alkoxy-carbon (C*) in (179) lies below the plane of the lactone ring then the molecule is dextro-rotatory. Conversely if the hydrogen atom lies above the plane of the ring, the compound will have a negative rotation". In order to apply this rule to complex lactones with several asymmetric centres it was necessary to subtract from the lactone rotation that of a suitable reference compound, containing the same asymmetric centres as the parent but without the lactone ring¹²², such as the corresponding hydroxy acid or ester. However, Klyne subsequently showed¹²³ that the difference curve between the rotation of the lactone and that of its reference compound was of the same sign as the lactone Cotton effect at 225 nm. Thus compounds of type (180) were found to give negative Cotton effects. Hence, since the caprylolactone from the degradation was found to exhibit a negative Cotton effect at 216 nm. in its c.d. curve (fig.16) it must have the Δ

Fig. 17 O.R.D. curves of canadensolide, dihydrocanadensolide



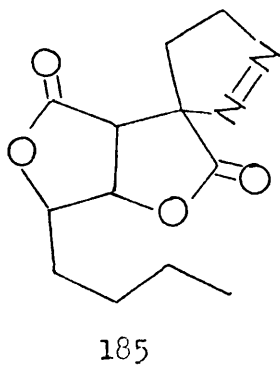
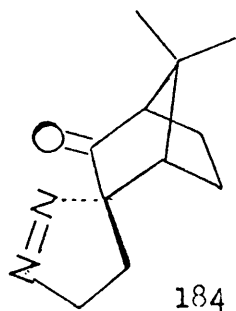


absolute configuration shown.

Absolute Configuration at C-4 in Canadensolide and Dihydrocanadensolide.

In the case of canadensolide (51) and dihydrocanadensolide (54) no attempt could be made at elucidating their absolute stereochemistry from their o.r.d. curves (fig. 17) since the lactone sector rule cannot be applied to hydroxy lactones¹²³. Their monolactone derivatives, however, appeared more suitable. Thus the butenolide ester (69) exhibited a negative Cotton effect (fig. 18) with its first extremum at 225 nm. Application of the lactone sector rule to this compound (see opposite) shows that this effect is due only to the configuration at C-4. Since the side chain at C-2 must lie in the symmetry plane of the lactone group then any small contribution which, for example, the methyl group attached to C-10 would be expected to make by lying in a lower positive quadrant will be cancelled out by free rotation about the (C-2)-(C-10) bond resulting in it also lying in an uppernegative quadrant. In fact the Cotton effects of butenolides have been used to assign the absolute configuration at C-4.¹²⁴ Thus (S) - γ -methyltetronic acid (181) and (S) - α -acetyl (-)-methyltetronic acid (182) exhibited negative Cotton effects while (-)-lichesterinic acid (183) was similarly assigned a 4S configuration on this basis.

Confirmation of the 4S configuration postulated for canadensolide (51) was obtained from the diene (68), which possesses only the desired asymmetric centre. The o.r.d. curve for this compound

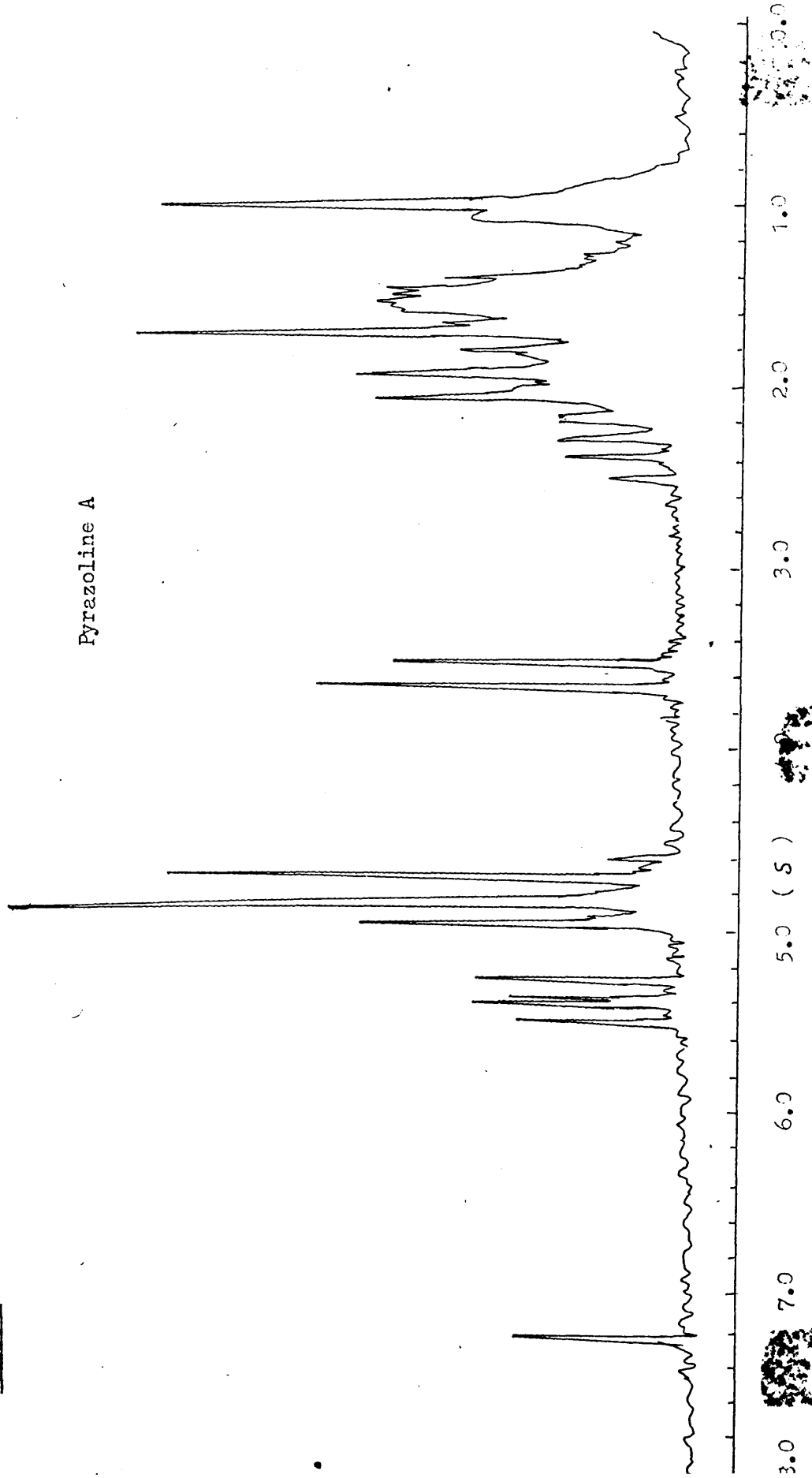


(fig. 18) showed a strong negative Cotton effect of molecular amplitude - 318.5, while its c.d. curve likewise showed a negative Cotton effect at 230 nm. ($[\Theta] = + 27,600$), corresponding to the position of maximum absorption in its u.v. spectrum.

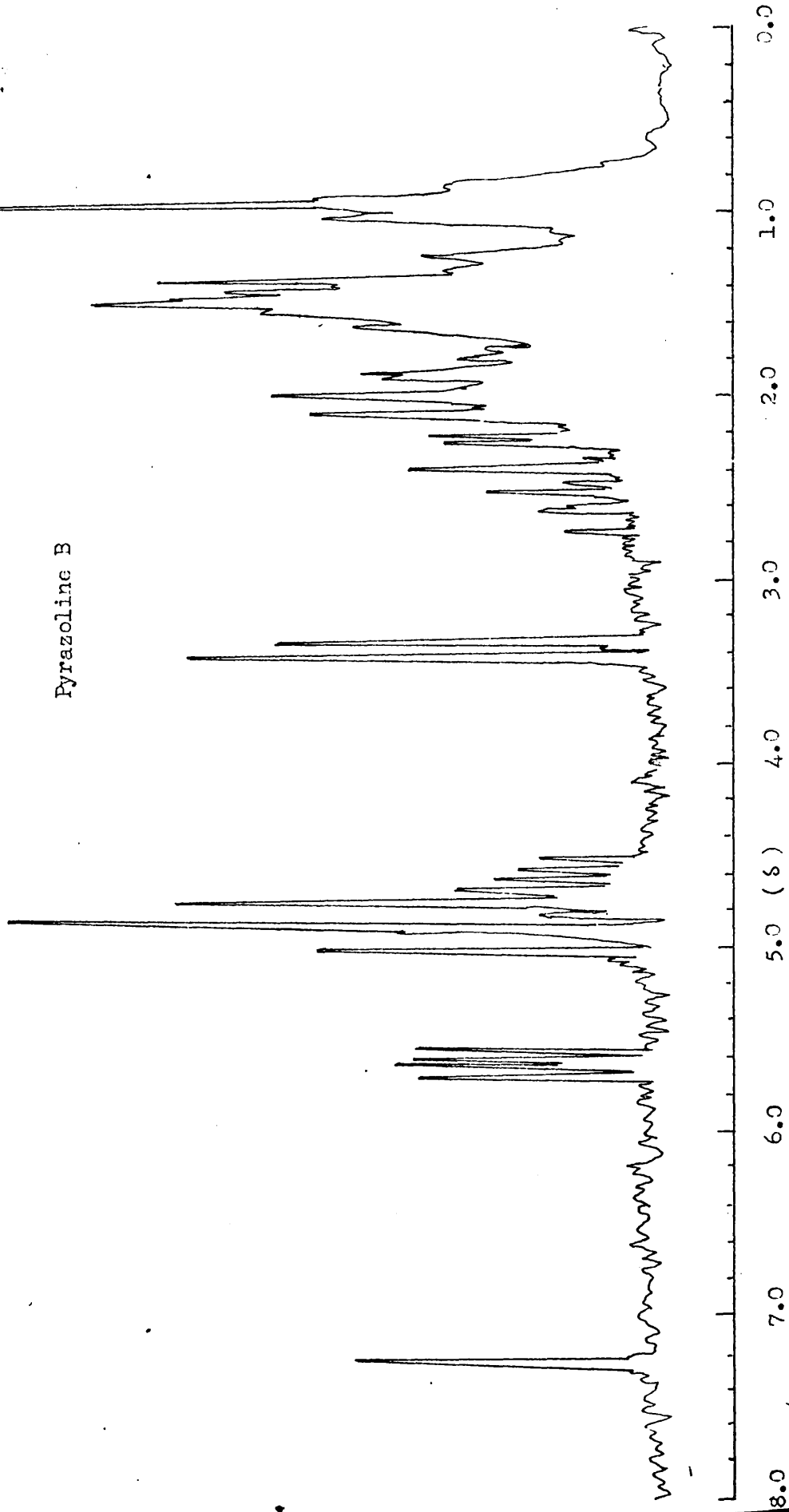
Absolute Configuration of C-2 and C-3 in Canadensolide, and Dihydrocanadensolide.

Since the relative stereochemistry of C-3 and C-4 in canadensolide was still uncertain, this could now be established if the absolute stereochemistry at C-3 were established independently. In view of the established cis ring fusion, establishment of the absolute configuration at C-2 would be equivalent. Since it has been shown¹²⁸ that the absorption of the azo group of spiro-pyrazoline camphor (184) and its corresponding alcohol were optically active with a negative Cotton effect at 330 nm., the most promising means of establishing the required absolute stereochemistry appeared to be formation of the corresponding pyrazoline compound from canadensolide (51). In view of the specificity observed in hydrogenation of the antibiotic, it was anticipated that one pyrazoline (185) would be formed preferentially.

Pyrazoline A



Pyrazoline B



Treatment of canadensolide (51) with excess ethereal diazomethane at ice temperature overnight, however, gave two pyrazoline compounds, isomeric at C-10, in approximately equal quantities (by n.m.r.). Separation was achieved by p.l.c. and the two compounds, named pyrazoline A and pyrazoline B, analysed as $C_{12}H_{16}N_2O_4$. Pyrazoline A (m.p. 107°) exhibited carbonyl absorption at 1798 and 1775 cm^{-1} attributed to the two α -lactone rings, in its i.r. spectrum while pyrazoline B (m.p. 100°) showed similar absorption at 1790 and 1775 cm^{-1} in its i.r. spectrum. Both exhibited weak absorption at 1550 cm^{-1} , characteristic of the N=N grouping^{186.1}. This group was also indicated in the mass spectra of both pyrazoline compounds by the loss of 28 mass units from the weak molecular ion at m/e 252.

Before any conclusion about the absolute configuration at C-2 can be made from the c.d. curves of these molecules, the stereochemistry of this centre relative to the azo grouping at C-10 must be determined i.e. which pyrazoline compound has the azo group cis to H-2. It was hoped that this might be deduced from a comparison of their n.m.r. spectra (fig. 15). Both these spectra show the features expected of structure (185). Thus the 1H double doublet around 4.5 τ was assigned to the proton

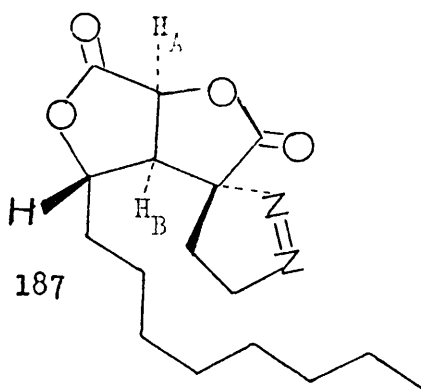
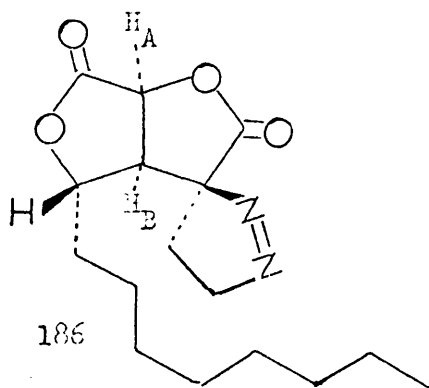
Table 8

Compound	H-2 (τ)	H-3 (τ)
- Pyrazoline A	6.43	4.63
Pyrazoline B	6.63	4.37
$\tau_B - \tau_A$	+ 0.20	- 0.26

Compound	H _B (τ)	H _A (τ)
Major <u>t</u> pyrazoline (186)	6.83	4.57
Minor <u>c</u> pyrazoline (187)	6.90	4.83
$\tau_c - \tau_t$	+ 0.07	+ 0.26

geminal to oxygen H-3. The 2H "triplet" at 5.2 τ is due to the resonance of the methylene protons α to the N=N group, which must form the AB part of an ABXY system. The other proton geminal to oxygen (H-4) occurs as a broad multiplet in the same region. The 1H doublet around 6.5 τ was assigned to the proton α to carbonyl (H-2), while the 2H multiplet around 7.9 τ was ascribed to the resonance of the XY protons of the ABXY system. The remaining resonances were attributed to the *n*-butyl side chain.

Of particular importance in these two n.m.r. spectra are the protons H-2 and H-3, which appear to be most affected by the formation of this 1-pyrazoline (Table 8). Both these protons are at lower field (ca. 0.5 ppm) than in the case of dihydrocanadensolide (54). This would be expected from the greater electron-withdrawing effect of the azo group at C-10. There are, however, significant differences in chemical shift between the corresponding protons of the two pyrazoline compounds. Thus H-2 in pyrazoline B appears at 0.20 ppm. higher than in pyrazoline A, while H-3 in pyrazoline B appears at 0.26 ppm. lower than in pyrazoline A (cf. Table 8). At first it was thought that this higher value of H-2 in pyrazoline B could be explained by H-2 being cis to the azo group. Models suggest that it might lie in the shielding zone of this group. However, this could likewise



be due to an unspecified deshielding of H-2 in pyrazoline A. It is difficult to account for the significant difference in τ values of H-3 in the two compounds.

It was hence decided to compare the results obtained above with the values observed for a model system of known relative stereochemistry. The most obvious example was the dilactone system of avenaciolide (58) which must have the same approximate geometry.

Treatment of a sample of avenaciolide (58) with excess ethereal diazomethane in an identical manner to that described previously resulted in an oily solid, whose n.m.r. spectrum confirmed that pyrazoline formation had taken place but that two products had been formed in a 3/1 ratio. Due to decomposition these could not be separated by p.l.c., but the major component could be fractionally crystallised. A low field doublet in the n.m.r. at 4.57τ ($J=7.0\text{Hz}$) was ascribed to the resonance of the proton geminal to oxygen and α to carbonyl (H_A), while the other proton geminal to oxygen appeared as a 1H multiplet at 5.57τ . The 1H resonance at 6.83τ , a double doublet ($J_1 = J_2 = 7.0\text{Hz}$) was assigned to the proton H_B .

The relative stereochemistry shown for this compound (186) was postulated on the assumption that approach to the terminal methylene group from the α side would be hindered by the steric bulk of the *n*-octyl group. As the absolute stereochemistry of

Fig. 19

PYRAZOLINE A

+8.0 (221nm)

+15.6 (321)

200

250

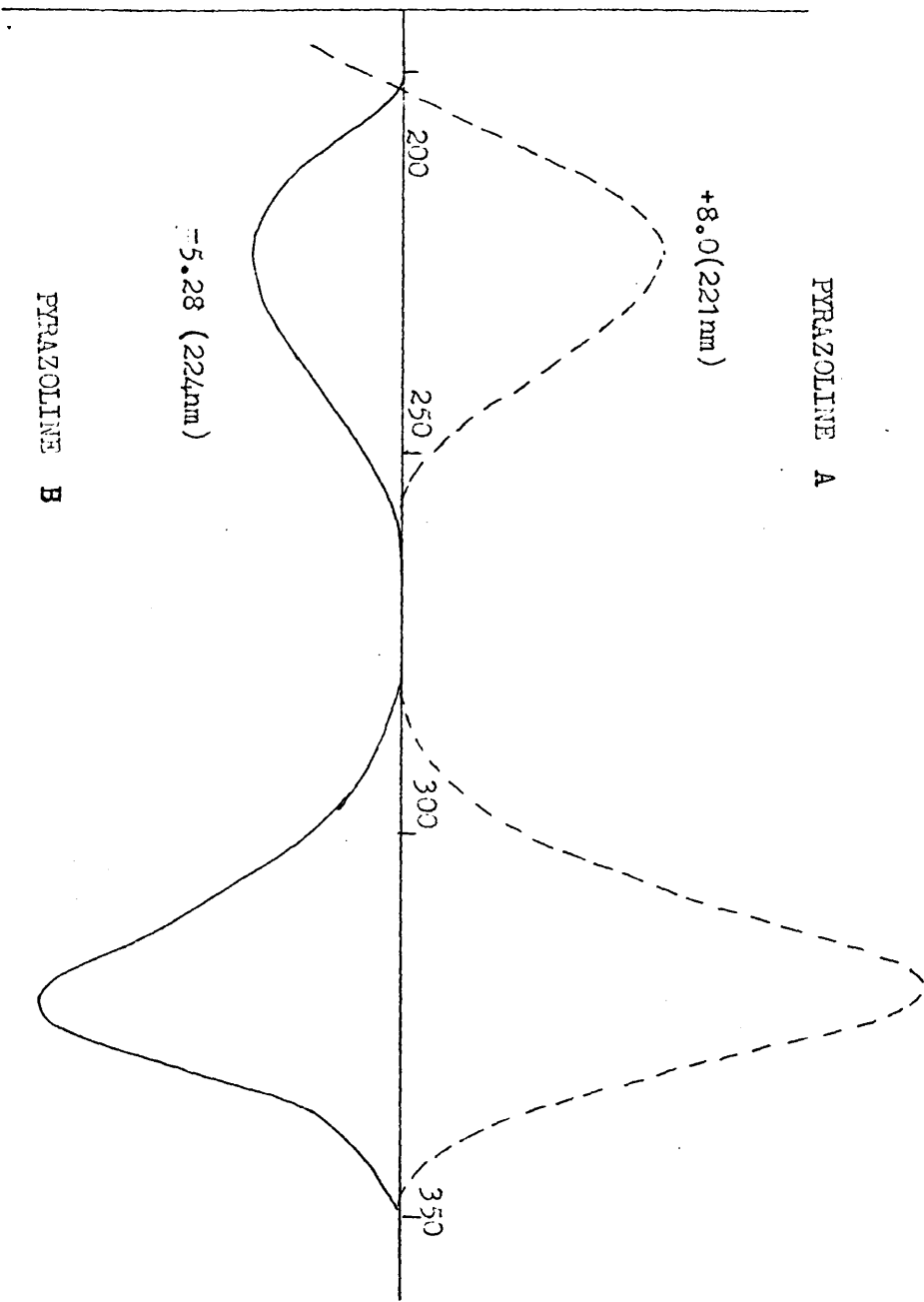
300

350

-5.28 (224nm)

PYRAZOLINE B

-12.25 (322nm)

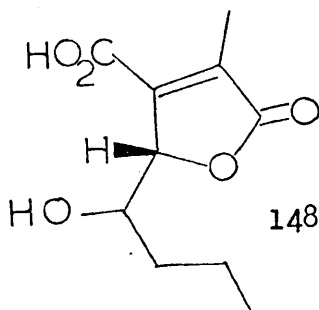
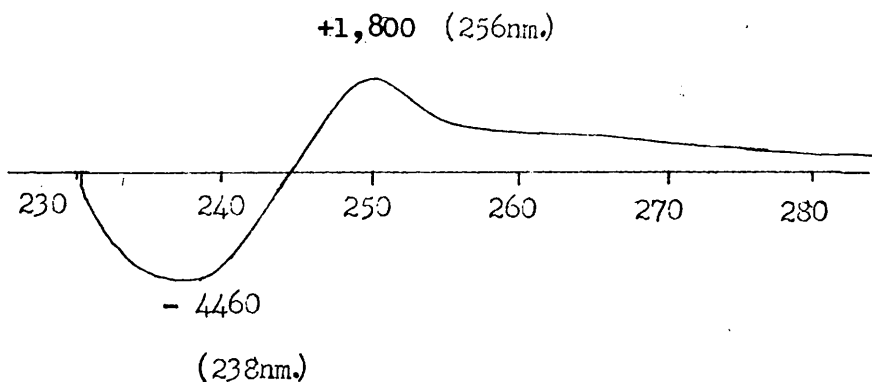


avenaciolide (56) had been determined by Turner⁵⁷ then the absolute stereochemistry at C-2 in the major pyrazoline compound (186) must also be that shown. The absolute stereochemistry of the new asymmetric centre can be determined from the c.d. curve of this pyrazoline compound as described earlier. This exhibited a negative Cotton effect at 324 nm. with $\Delta E = -6.9$, confirming it has the same absolute configuration as spiro-pyrazoline-camphor (184), as shown in (186). Thus the major pyrazoline compound has a trans orientation of H_B and the N=N group.

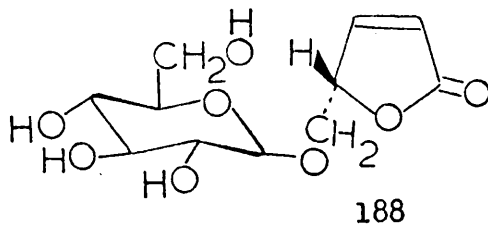
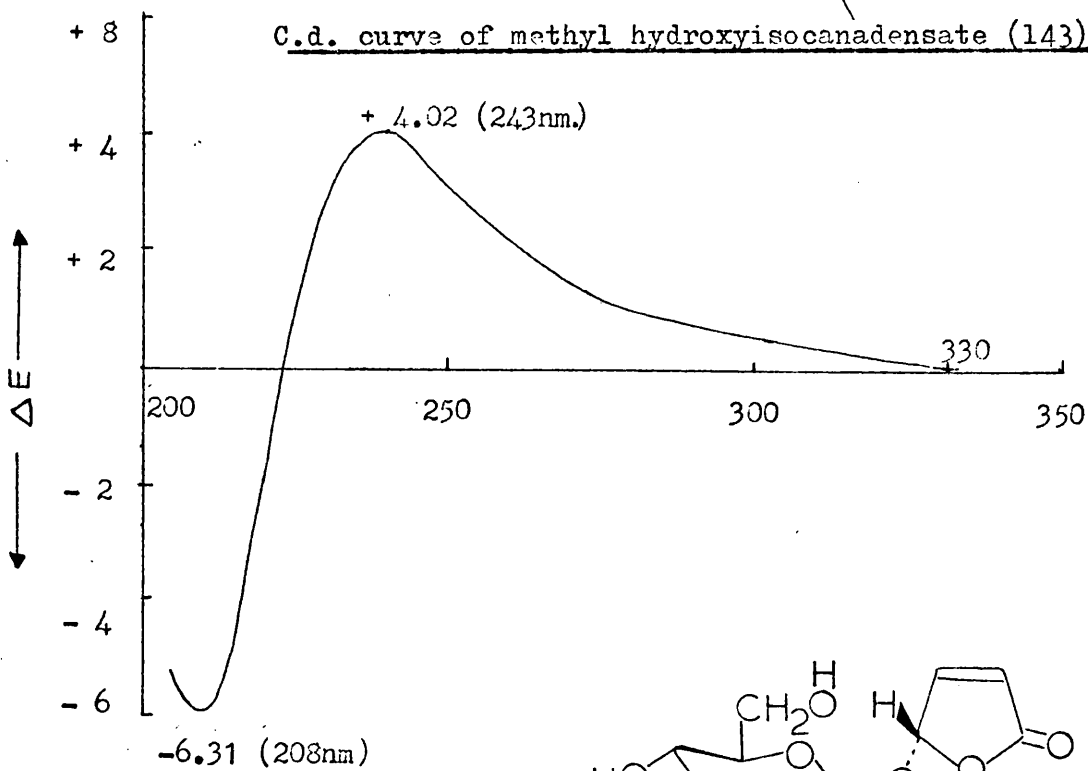
By comparing the n.m.r. spectrum of the major pyrazoline compound (186) with that of the 3/1 mixture it was possible to obtain the chemical shift values of the two protons H_A and H_B of the minor component (187) (Table 8). The proton (H_B) α to the carbon bearing the -N=N- group in the major trans pyrazoline compound (186) was, however, found to be only at slightly lower field (0.07 ppm) when in the minor isomer (187) which has a cis orientation of H_B and the N=N group. This difference in chemical shift value appears insignificant.

If, however, we compare the corresponding values for the other 'significant' proton H_A then in the case of the major (H_B /azo trans) pyrazoline compound (186) of avenaciolide

Fig. 20 O.r.d. curve of hydroxyisocanadensic acid (148).



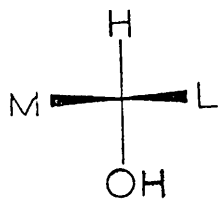
C.d. curve of methyl hydroxyisocanadensate (143).



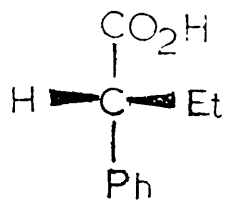
it occurs at lower field (4.57 τ) than in the case of the minor H_B /azo cis) isomer (4.83 τ). On this basis, pyrazoline B, having a value for H-3 lower by 0.26 p.p.m., would be assigned the H-2/azo trans configuration. Although this gives the correct absolute and relative stereochemistry for canadensolide (51) as established later it is unsatisfactory in that the origin of the shielding effects are uncertain. The mirror image c.d. curves of pyrazolines A and B are shown opposite (fig. 19). In fact, it required an interconversion of methyl hydroxyisocanadensate (143) to dihydrocanadensolide (54) to prove the absolute configuration at the bridgehead centres.

The absolute configuration at C-3 in hydroxyisocanadensic acid.

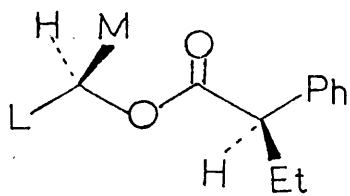
The 4S absolute configuration of the remaining metabolite, hydroxyisocanadensic acid (148), being a butenolide was readily determined from its associated Cotton effect¹²⁴. This was found to be positive with first extremum at 256 nm. in its o.r.d. curve (fig. 20), which must be due to the asymmetric absorption of the butenolide chromophore (λ_{\max} . 230 nm.) The absolute configuration at this centre in similar hydroxylated butenolides such as ranunculin (188) have been established in this way.¹²⁴ The analogous positive Cotton effect for methyl hydroxyisocanadensate (143) was readily detected in its c.d. curve, which showed a positive maximum (ΔE , + 4.02) at 243 nm. L-lichesterinic acid (183) has been reported¹²⁴ to exhibit a negative Cotton effect at 235 nm. (ΔE , - 3.5).



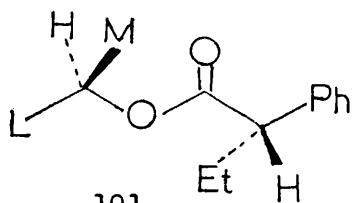
189



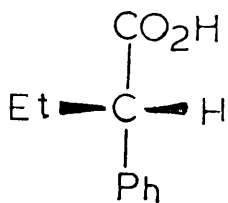
192



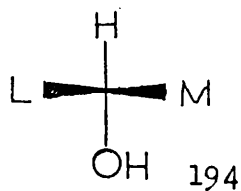
190



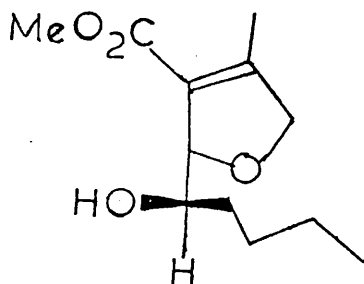
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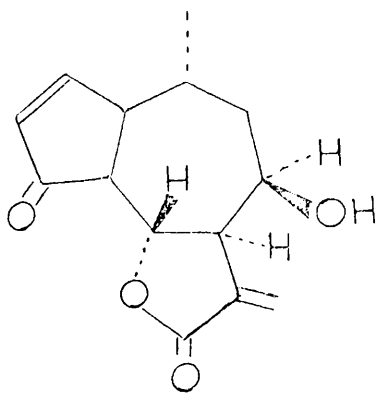


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Absolute Configuration at C-4 in Hydroxyisocanadensic acid.

An attempt was also made to determine the absolute configuration of the secondary alcohol group at C-4 in methyl hydroxyisocanadensate (143) using the Horeau method¹²⁸. This depends on the preferential reaction of an asymmetric alcohol (189) with one particular antipode of (+) α -phenylbutyric anhydride, and can be rationalised by considering the steric interactions occurring in the two possible diastereomeric esters (190) and (191). It can be seen that ester (191) would be formed more readily in this case, and hence the acid isolated after hydrolysis of the excess anhydride should be rich in (-) (R)- α -phenylbutyric acid (192).

When methyl hydroxyisocanadensate (143) was subjected to this technique, the acid obtained on hydrolysis was found to have a positive rotation, indicating an excess of (+) (S)- α -phenylbutyric acid (193) and hence that the starting alcohol had the absolute configuration (194). If the butenolide ring system of methyl hydroxyisocanadensate (143) is assigned the large group and the butyl group the medium one then this result means that this alcohol has the 4 R absolute configuration shown (195). This, however, is opposite to the known absolute configuration of this centre (4S) shown by the interconversion of methyl hydroxyisocanadensate (143) to dihydrocanadensolide (54) (see chapter 4). This contradiction might be explained by postulating that it is the butyl group and not the butenolide ring system which is the large group. Although this does



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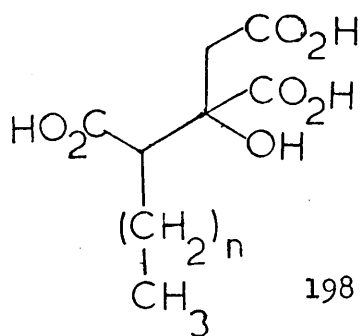
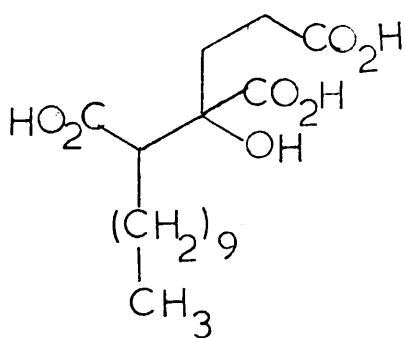
not seem likely on purely steric grounds, it is possible that in the "locked" conformer, postulated for this compound, the steric interactions of the butenolide group are minimal giving it less effective bulk than the n-butyl chain.

In agreement with this is the small (but significant) optical yield of ca. 11% obtained for the reaction, suggesting there is not a large effective steric bulk difference between these groups. In the case of cyclic sesquiterpene lactones, however, Horcau's method has been successfully applied, for example to 1-epiallohelanalin (196) which gave an optical yield of 15% (+)¹²⁹.

Alternatively this abnormal result might possibly be attributed to unknown, electronic factors introduced by the presence of the carbomethoxyl grouping.

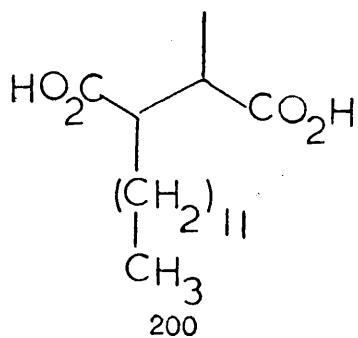
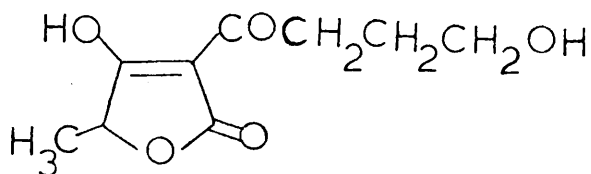
The limitations of this type of correlation has been stressed by Mislow¹³⁰ who said : "Thus in the assignment of configuration, a heavy burden of proof rests on the credibility of the transition state proposed in asymmetric syntheses, or on the critical analysis which underlies the weighting of conformational populations in mobile equilibria, whichever applies".

BIOSYNTHESIS OF CANADENSIC ACID.



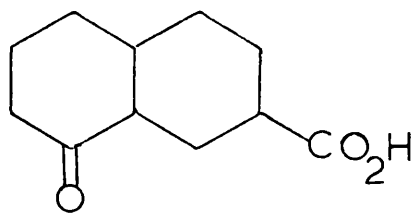
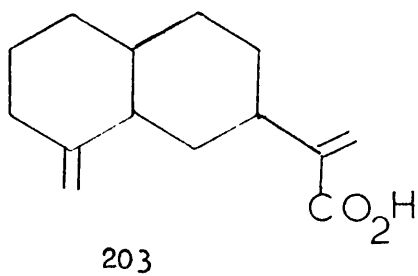
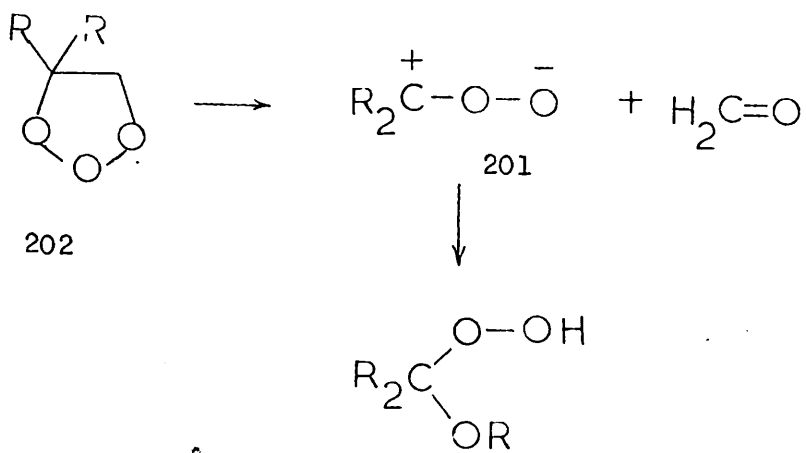
$n=13$ caperatic acid

$n=15$ agaricic acid



In fungi and lichens, as well as in plants, a smaller group of structurally related aliphatic acids or lactones have been found in which an alkyl chain is attached to shorter polycarboxylic moiety. The fungal metabolites include spiculisporic acid (197)¹³² (Penicillium spiculisporum), agaricic acid (198, n=15) (Fomes officinalis) the tetrionic acids exemplified by carolic acid (199)¹³⁴ (Penicillium charlesii). Related lichen metabolites¹³³ are rocellic acid (200), (Rocella tinctora), caperatic acid (198, n=13) (Parmelia caperata) and lichesterinic acid (183) (Cetraria islandica). It was obvious that the C₁₁ metabolites isolated from Penicillium canadense should be included in this group since their chemical structure indicated a similar biological pathway.

The structures of canadensolide (51), dihydrocanadensolide (54), canadensic acid (95) and hydroxyisocanadensic acid (148) can all be formally derived by canadensation of a fatty acid chain with a three carbon unit. Since at the time of commencement of this work biosynthetic studies¹³⁶ on the lichen acid protolichesterinic acid (214) had suggested that two different pathways might exist by which this type of compound might be formed in nature, it seemed desirable to establish the pathway by which the metabolites of



P. canadense were formed.

Prior to labelling experiments, however, it was necessary to select one particular metabolite for biosynthetic study, and establish a scheme for determining any distribution of label. As the antibiotic itself was difficult to purify, the then more readily available metabolite, canadensic acid (95), was chosen. This acid was also present in large amounts in the broth, providing ample material for dilution of radioactive material and for degradation purposes. Since this acid appeared to possess the functionality necessary for the establishment of a satisfactory degradation scheme numerous chemical reactions were directed towards achieving this.

Thus a Kuhn-Roth determination established that one C-methyl group was present in canadensic acid. The acetic acid isolated from this reaction enables C-7 and C-8 to be isolated in reasonable yield, making this reaction suitable for use with labelled material. A similar oxidation of the derived dihydrocanadensic acid* now gave the number of C-methyl groups as two. These reactions hence give an estimate of any label at C-10 and C-11.

Attempts were also directed at cleaving the terminal methylene group. It has been reported¹³⁷ that ozonolysis of tetrasubstituted and terminal methylene double bonds are best carried out in an alcoholic solution since the Criegee zwitterion (201) formed from the molozone (202) does not react with the co-formed formaldehyde but reacts

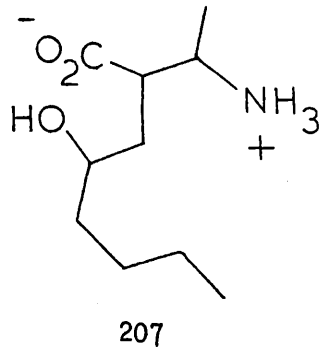
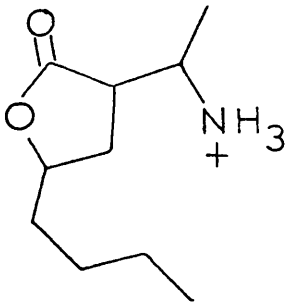
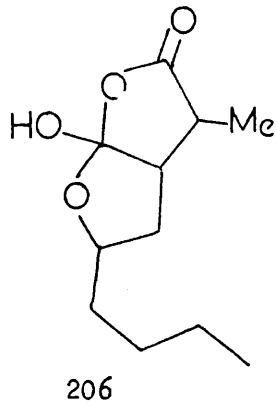
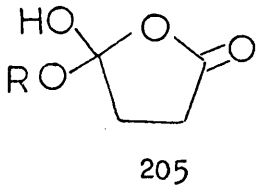
* Mixture of C-2, C-10 diastereoisomers

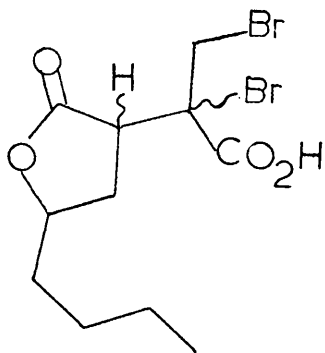
+ Mixture of C-2 epimers

preferentially with the alcohol of the reaction medium. The resulting α -hydroperoxyether can be readily hydrolysed to its corresponding ketone by mild treatment with water. In fact although ozonolysis of canadensic acid[†] by this procedure gave no recognisable ketonic fragment, a far higher yield of formaldehyde (isolated as its dimerone derivative) was obtained than in the previously reported reaction using acetic acid as solvent⁵⁴. It is of course known that double bonds conjugated to a carboxylic acid grouping can give rise to decarboxylated products on ozonolysis, as for example castic acid¹³⁸ (203) which gives the degraded acid (204). The "abnormal ozonolysis" has¹³⁹ been attributed to the formation of peracid.

Attention was then focussed on decarboxylating canadensic acid[†]. The first attempts utilising a Schmidt reaction gave only a poor yield of carbon dioxide (trapped as barium carbonate) and no isolable product. Although this could have been due to the presence of the conjugated double bond, a similar reaction on dihydrocanadensic acid^{*} was found to give only a slightly better yield of carbon dioxide. Moreover, in this latter case some dihydrocanadensic acid (20%) was recovered but in spite of exhaustive extraction procedures no other characterisable product was detected. Succinic acid derivatives have, however, been reported to give poor yields of carbon dioxide when subjected to Schmidt reaction conditions.¹⁴⁰ This has been postulated to

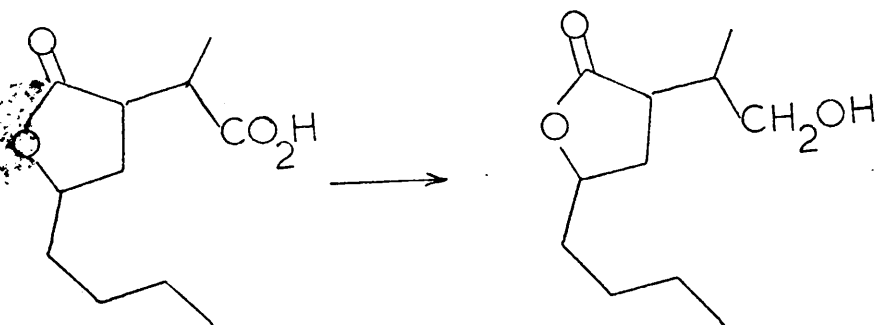
[†] Mixture of C-2 epimers * Quaternary mixture of C-2, C-10 diastereoisomers.



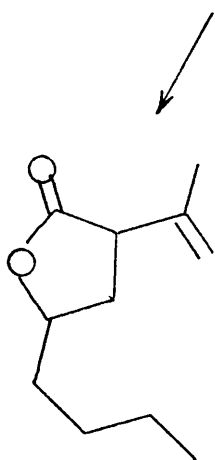


208

Diagram 6



210



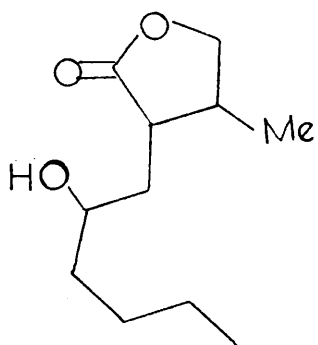
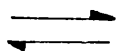
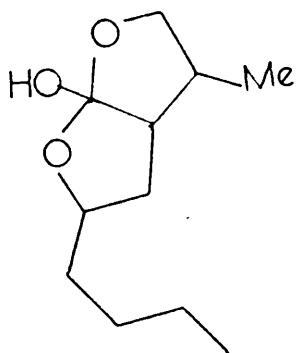
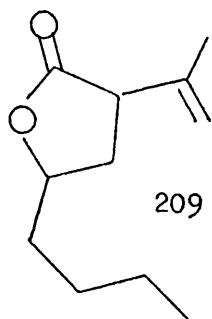
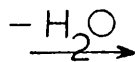
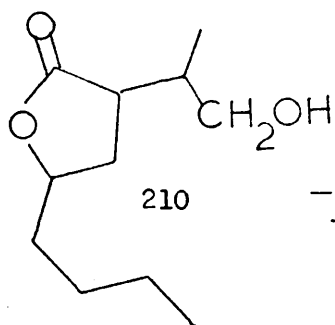
209

be due to the formation of the hemiacetal like species (205) in the acidic medium. The formation of the analogous compound from dihydrocanadensic acid* 156 could explain the relatively small yield of carbon dioxide and the reluctance of starting material to react. Formation of the zwitterionic compound (201) on work-up would result in any product formed being extremely water soluble and difficult to isolate.

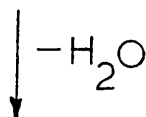
The next approach utilised the double bond of canadensic acid to introduce a bromine atom β to the carboxyl group. It was thought that a decarboxylation-elimination reaction would then liberate carbon dioxide with formation of an olefin, which might be suitable for further degradation. Bromination of canadensic acid⁺ was achieved by refluxing with carbon tetrachloride and an excess of bromine to give an oil, whose i.r. spectrum did not show any olefinic absorption. Peaks at 1775 cm.^{-1} and 1710 cm.^{-1} were assigned to γ -lactone and carboxylic acid groups respectively. The dibromo acid* structure (208) was also supported by its n.m.r. spectrum. Decarboxylation of dibromocanadensic acid (208) was achieved in reasonable yield by refluxing in ethyl acetate with an equivalent of triethylamine. However, attempts to isolate any other product from this reaction were unsuccessful due to decomposition both on preparative t.l.c. and g.l.c. Although this represented a feasible decarboxylation procedure, a more satisfactory method seemed desirable.

+ Mixture of C-2 epimers.

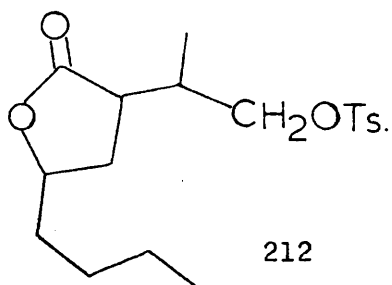
* Quaternary Mixture of C-2, C-10 diastereoisomers.



211



several products



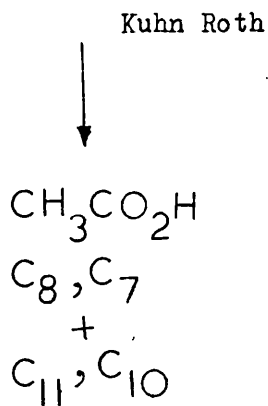
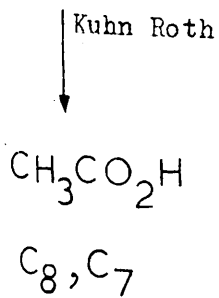
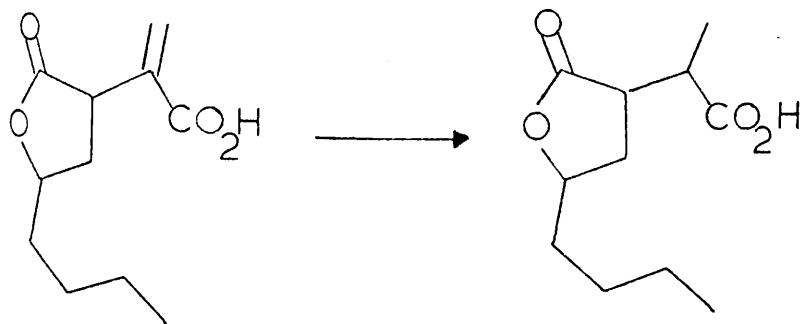
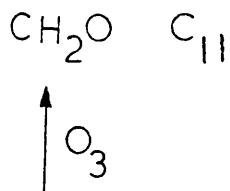
Thus if dihydrocanadensic acid* was converted by the sequence shown (diag.6) into the olefin (209) it seemed likely that ozonolysis of this latter compound would enable a step-wise degradation to be achieved. Hence dihydrocanadensic acid* was converted by refluxing with oxalyl chloride in benzene into the corresponding acid chloride which was reduced by sodium borohydride to dihydrocanadensol* (210). In agreement with this structure was a strong hydroxyl absorption at 3550 cm.^{-1} in the i.r. spectrum which also showed only a single peak at 1760 cm.^{-1} (γ -lactone) in the carbonyl region. The primary alcohol grouping was also indicated by the appearance of a 2H multiplet at 6.35τ ($\text{CH}_2\text{-OH}$) in the n.m.r. spectrum.

Dehydration of the mixture of alcohols (210) to the desired olefin (209)⁺ was not thought likely to readily occur by acid catalysis since primary alcohols cannot easily support any positive charge on the primary carbon atom. These alcohols are in fact believed to dehydrate via bimolecular attack of anions or nucleophilic solvent molecules on conjugate acids of the alcohols. As the β hydrogen atom in dihydrocanadensol* (210) was attached to a tertiary carbon atom it was, however, unlikely to be acidic. In spite of this, it was found that even a brief reflux in benzene with a trace of p-toluenesulphonic acid transformed dihydrocanadensol* into several less polar products. While the multiplicity of products might be due partly to double bond isomerisations, further

* Mixture of C-2, C-10 diastereoisomers.

+ Mixture of C-2 epimers.

Diagram 7 Degradation of canadensis acid⁺



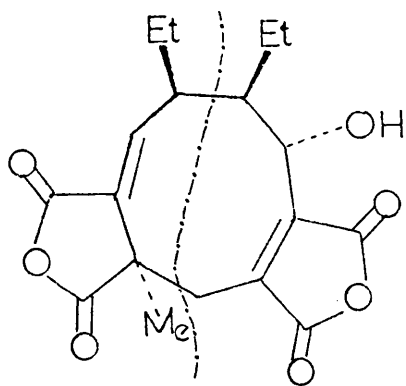
isomers could arise by dehydration of the isomeric lactone (211) via the ortho ester type of compound. Alternative methods of dehydration were therefore sought.

Dihydrocanadensol* (210) unfortunately resisted mild dehydration using refluxing benzene and iodine or base catalysed elimination on active alumina. Thus in order that a pyrolytic elimination reaction could be attempted, the corresponding tosylate* (212) was prepared by treatment of dihydrocanadensol* with tosyl chloride in the usual way. Its structure was supported by the lack of any hydroxyl absorption in the i.r. spectrum, and by the characteristic resonances of the aromatic ring (two doublets at 2.17 and 2.64 τ) and aromatic methyl group protons (singlet at 7.58 τ) in the n.m.r. spectrum. Likewise there was a downfield esterification shift of 0.45 ppm. of the resonance previously assigned to the hydroxymethylene protons (2H-10).

Preliminary attempts to eliminate this tosylate (212) by pyrolysis and to effect Hoffman eliminations after quaternising triethylamine proved unpromising, and were not investigated further when it was found that canadensic acid could be efficiently degraded as indicated earlier (cf. chapter 3) by means of a carboxy-inversion reaction. A preliminary investigation of the biosynthesis of canadensic acid⁺ had, however, been initiated using the degradation scheme outlined in diagram 7. This sequence of reactions enabled a fairly good approximation of the activities at the indicated positions to be obtained, although a more detailed study using the carboxy-inversion reaction can be envisaged.

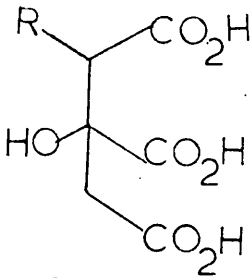
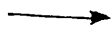
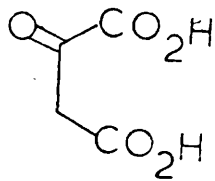
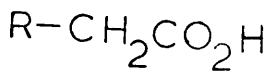
+ Mixture of C-2 epimers

* Quaternary mixture of C-2, C-10 diastereoisomers.

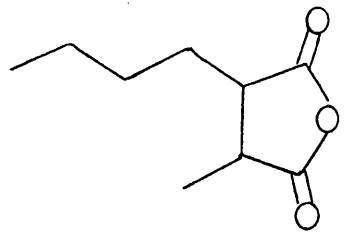


213

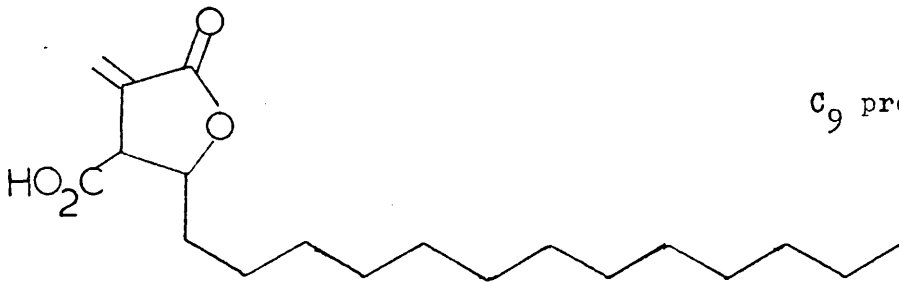
Fig. 21.



215



C₉ precursor of gluconic acid



214

Biosynthetic studies on various fungal metabolites which appear to be similarly derived have been carried out. Thus the nonadrides exemplified by glauconic acid (213) was postulated¹⁴¹ to arise from the coupling of two C_9 units of identical carbon skeleton as indicated. That the C_9 precursor was derived by condensation of a hexanoyl derivative with oxaloacetic acid to give the substituted citric acid (fig. 21) was indicated¹⁴² by a high incorporation of ^{14}C labelled acetate into the fatty acid portion, while only (1, 4- ^{14}C) succinate was stereospecifically incorporated into the 3 carbon unit. On the other hand, a similar biosynthesis might be predicted for the lichen metabolites, exemplified by (+) - protolichesterinic acid (214), but derivation from a C_{16} fatty acid chain and oxaloacetic acid seemed to be contradicted by studies which suggested that the C_3 unit was derived from acetate rather than oxaloacetate¹³⁶. (Subsequent contradictory experiments with protolichesterinic acid were reported later, when a good incorporation of succinate was obtained, and seasonal variation in secondary metabolite production by lichens was postulated¹⁴³).

The feasibility of this mechanism occurring in fungal secondary metabolism was demonstrated by Gatenbeck who isolated¹⁴⁴ (+)-decylcitric acid and (-)-decylcitric acid (215, R=n. C_{10}) from a strain of Penicillium spiculisporum, and demonstrated their biosynthesis by condensation of lauryl co-enzyme A and oxalacetate enzymically¹⁴⁵. The purified enzyme system obtained was also capable of condensing fatty acids of

Table 2.

Incorporations of labelled substrates into canadensis acid.

Compound	Total activity fed (d.p.m.)	Diluted by inactive acid (r.f.)	Activity isolated d.p.m. / mg.	Incorporation %
1 - ^{14}C - acetate	1.37×10^7	133	3.79×10^5	2.8
2 - ^{14}C - acetate	7.40×10^6	150	8.63×10^4	1.5
2,3 - ^3H - succinate	1.66×10^{10}	54	3.77×10^4	0.01
2 - ^{14}C - DL - mevalonic acid lactone	5.55×10^7	131	1.48×10^3	0.35
3 - ^{14}C - pyruvate	5.55×10^7	140	2.83×10^3	0.71
1 - ^{14}C - glyoxalate	5.55×10^7	148	7.06×10^2	0.19

smaller chain length than the natural one. Thus the hexyl citric acid ($215, R=nC_6$) obtained from a similar condensation of octanoyl co-enzyme A with oxalacetate, could by decarboxylative elimination of water and lactonisation produce canadensic acid (95).

Growth-time studies with Penicillium canadense indicated that canadensic acid was first produced in detectable amount by t.l.c. after six days. Good incorporations were obtained by feeding labelled precursors to the fungus on the 2nd day and harvesting on the 6th day, and hence all the labelling experiments were carried out on this time basis. The experiments were carried out on a small scale using 5 roux bottles (as opposed to 100 for a normal fermentation), and the charcoal-acetone extract of the broth diluted with inactive carrier material. The labelled canadensic acid isolated as a mixture of C-2 epimers was separated from the non-acidic metabolites by bicarbonate extraction, and was then crystallised to constant activity.

The incorporations achieved for several labelled substrates are given in (table 9). The high incorporation of sodium ^{14}C -acetate was in agreement with the postulated biosynthesis of canadensic acid, and degradation of the resulting labelled acid⁺ was carried out as indicated earlier to determine the distribution of activity. The acetic acid (C-3 and C-7) obtained by Kuhn Roth oxidation of 2- ^{14}C acetate labelled canadensic acid⁺ (table 10) was found to possess ca. 33% of the

+ Mixture of C-2 epimers.

Diag. 8.

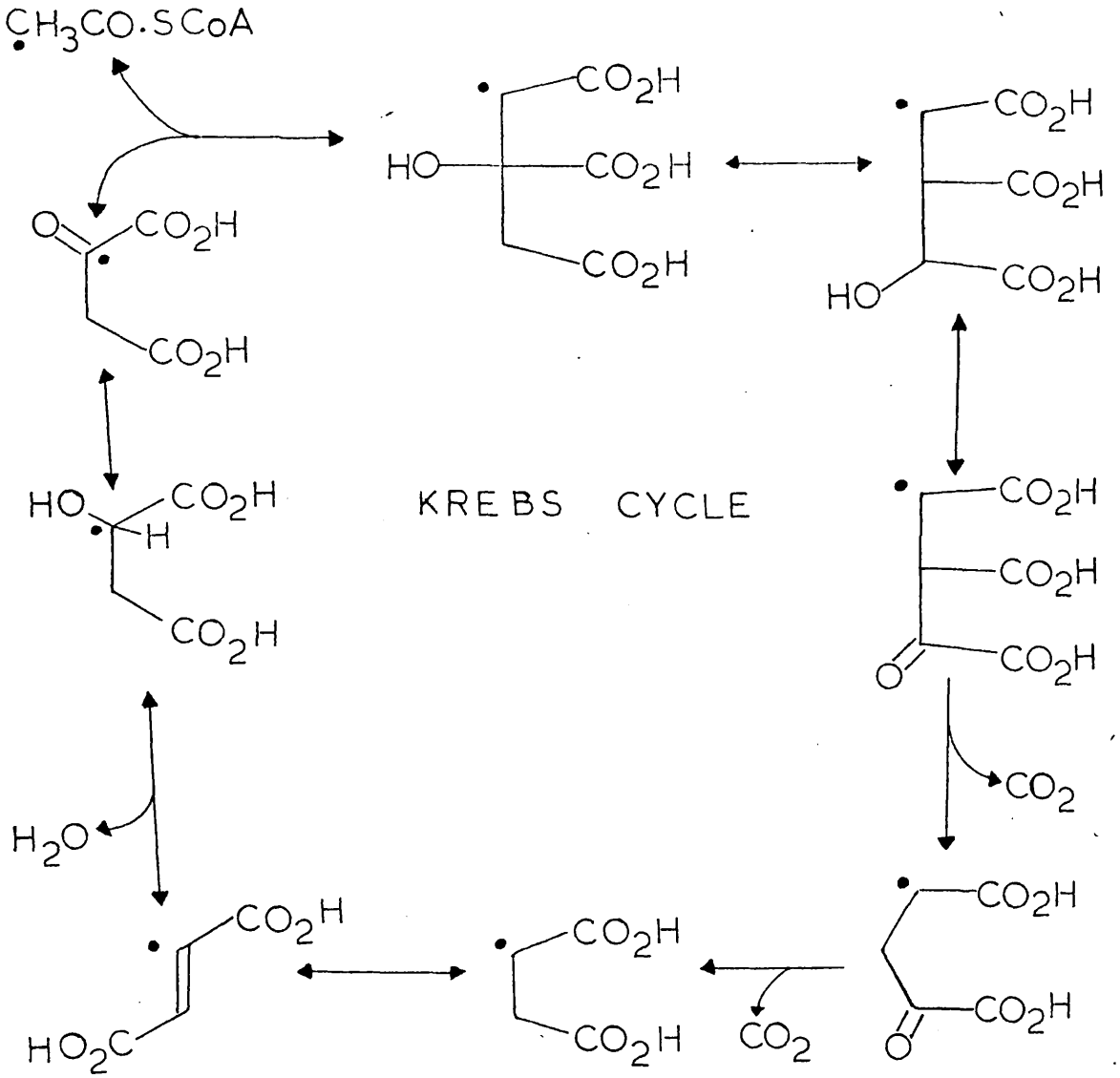


Table 11.

Degradation of labelled acid from 1 - ^{14}C - acetate feed.

Compound	Relative molar activity (d.p.m./m.mole)	Carbon atoms	Total activity. (%)
Canadensic Acid epimers	1.540×10^5	All	100
Acetic acid (from unsaturated acid)	5.475×10^4	C-8, C-7	35.6
Acetic acid (from dihydro acid)	2.780×10^4	C-8, C-7, C-11, C-10	38.2†
Formaldehyde dimedone	0	C-11	0
		C-10	2.6†

† Since only 86% of the theoretical amount of acetic acid was obtained in Kuhn Roth oxidation, results were calculated on the assumption that the acetic acid had been formed in equal amounts from the two C-methyl groups.

oxidation of labelled acid from 2 - ¹⁴C - acetate

Compound	Relative molar activity (d.p.m./m.mole)	Carbon atoms	Total activity. (%)
Canadensic Acid epimers	11,760	ALL	100
Acetic acid (from unsaturated acid)	3,905	C-8, C-7	33.2
Acetic acid (from dihydro acid)	2,185	C-11, C,10,C-8, C-7.	37.2 [‡]
Formaldehyde dimerone	290	C-11. C-10.	2.5 1.5 [‡]

‡ Since only 86% of the theoretical amount of acetic acid was obtained in the Kuhn Roth oxidation, results were calculated on the assumption that the acetic acid had been formed in equal amounts from the two C-methyl groups.

total activity of the molecule. This is greater than the 25% expected at these positions for uniform labelling of an acetate derived octanoate chain. This result can, however, be explained by a higher incorporation of acetate into the starter unit. The acetic acid obtained by oxidation of the derived dihydrocanadensic acid* was found in both feeds to possess total activity only slightly greater than that of the acetic acid obtained from canadensic acid⁺ confirming that there was not a significant amount of (¹⁴C-acetate derived) label at C-10 or C-11. This conclusion was supported (at least as far as C-11 is concerned) by ozonolysis of the labelled canadensic acid⁺ to give formaldehyde dimedone which provided an accurate determination of the activity at C-11. This showed that although no detectable labelling of this position occurred in the acid⁺ derived from 1-¹⁴C-acetate, a small amount of activity (2.5%) was present in the acid⁺ derived from 2-¹⁴C-acetate. This result is consistent with the precursor for the C₃ unit being succinic acid (or oxaloacetic acid) since conversion of 2-¹⁴C-acetate into 2,3-¹⁴C-succinate could take place via the tricarboxylic acid cycle, while 1-¹⁴C acetate would not be expected to label these positions (see diag. 8).

Confirmation of the postulated fatty acid derivation of carbon atoms C-1 to C-8 in canadensic acid was also attempted using experiments with sodium D₃-acetate. The good incorporation of ¹⁴C-acetate into the

+ Mixture of C-2 epimers.

* Quaternary mixture of C-2, C-10 diastereoisomers.

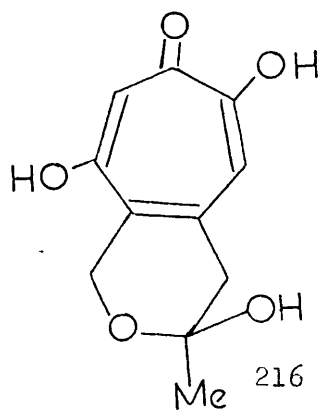


Table 12.

Degradation of labelled acid from 2,3-³H - succinate feed.

Compound	Relative molar activity (d.p.m./M.mole)	Carbon atoms	Total activity %
Canadensis acid epimers	1.865×10^6	All	100
Formaldehyde dimedone	2.851×10^5	C-11	15.3
Acetic acid (from unsaturated acid)	1.384×10^5	C-8, C-7	7.4
Acetic acid (from dihydro acid)	2.460×10^5	C-8, C-7, C-11, C-10	23.4

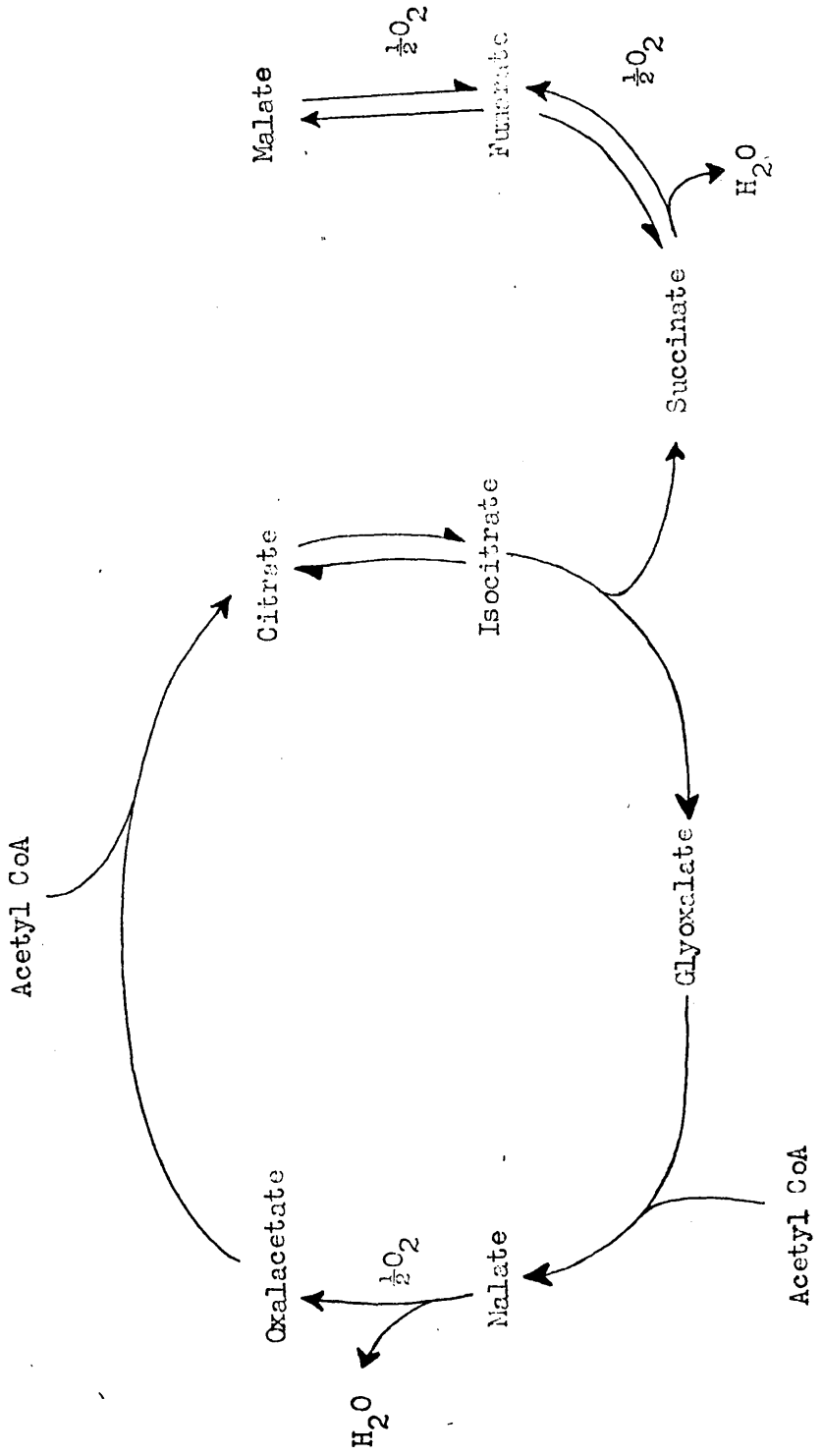
molecule suggested that a substantial deuterium enrichment might be obtained if a large amount of substrate was fed to the fungus. This type of technique, combined with mass spectrometry, has been used to establish the biosynthesis of patulin (29), ¹⁴⁶, while similar feedings of ¹³C-labelled precursors established the biosynthesis of the tropolone metabolite sepedonin (216). ¹⁴⁷ In our case, however, no deuterium enrichment of either canadensic acid⁺ or dihydrocanadensolide was detected from exploratory experiments using mass spectrometry.

Several feeding experiments with probable precursors of the C₃ unit of canadensic acid were also carried out. Since succinic acid is converted to oxaloacetate via the Krebs cycle, it was expected to be more specifically incorporated into this unit than had acetate. Although the incorporation of 2,3-³H-succinate was found to be small (0.01%) a large amount of the activity (15%) was in fact present at C-11 (Table 12). Some scrambling of the label was apparent, and presumably occurred by partial degradation of the succinate to acetate. As has been shown above, however, acetate was not readily incorporated into the C₃ unit and hence the activity at C-11 was significant, and this result thus suggested that succinate was indeed closer to the immediate biosynthetic precursor of this unit than acetate.

Although time prevented degradation of the labelled canadensic acid⁺ from the remaining precursor experiments, certain conclusions can be drawn from the observed incorporations (Table 9). Both 3-¹⁴C-pyruvate

+ Mixture of C-2 epimers

Diag. 9 Glyoxalate cycle.



(0.71%) and 2-¹⁴C-mevalonate (0.35%) were well incorporated, but it is possible that these compounds are simply providing a source of acetate. Pyruvate, of course, would be readily converted biologically in the presence of thiamine pyrophosphate and lipoic acid¹⁴⁸ into acetyl co-enzyme A, while mevalonate although theoretically non-convertible to acetate (from which it is derived) has in practice been incorporated into acetate derived molecules possibly by degradation of terpene intermediates^{149,150}. Besides, the intact incorporation of mevalonate into the carbon skeleton of canadensic acid would presumably require prenylation of a fatty acid, an unexpected process biogenetically. The remaining labelled precursor, sodium 2-¹⁴C-glyoxalate (incorporation 0.19%), however, is unlikely to be converted to acetate by fungi. Apart from oxidation to carbon dioxide, the most probable biochemical pathway involves condensation with acetate via the Glyoxalate cycle (diag. 9) with the eventual formation of oxaloacetate. An indication that this pathway could be important in the biosynthesis of the metabolites of P.canadense was provided by the isolation on one occasion of small amounts of fumaric and succinic acids. As these are possible end products of the glyoxalate cycle, their presence in isolable quantities suggests that this cycle is more active than normally encountered in primary metabolism.

Although these preliminary results appear to be in agreement with the postulated biological formation of canadensic acid by condensation of an octanoate chain with oxaloacetic acid, further

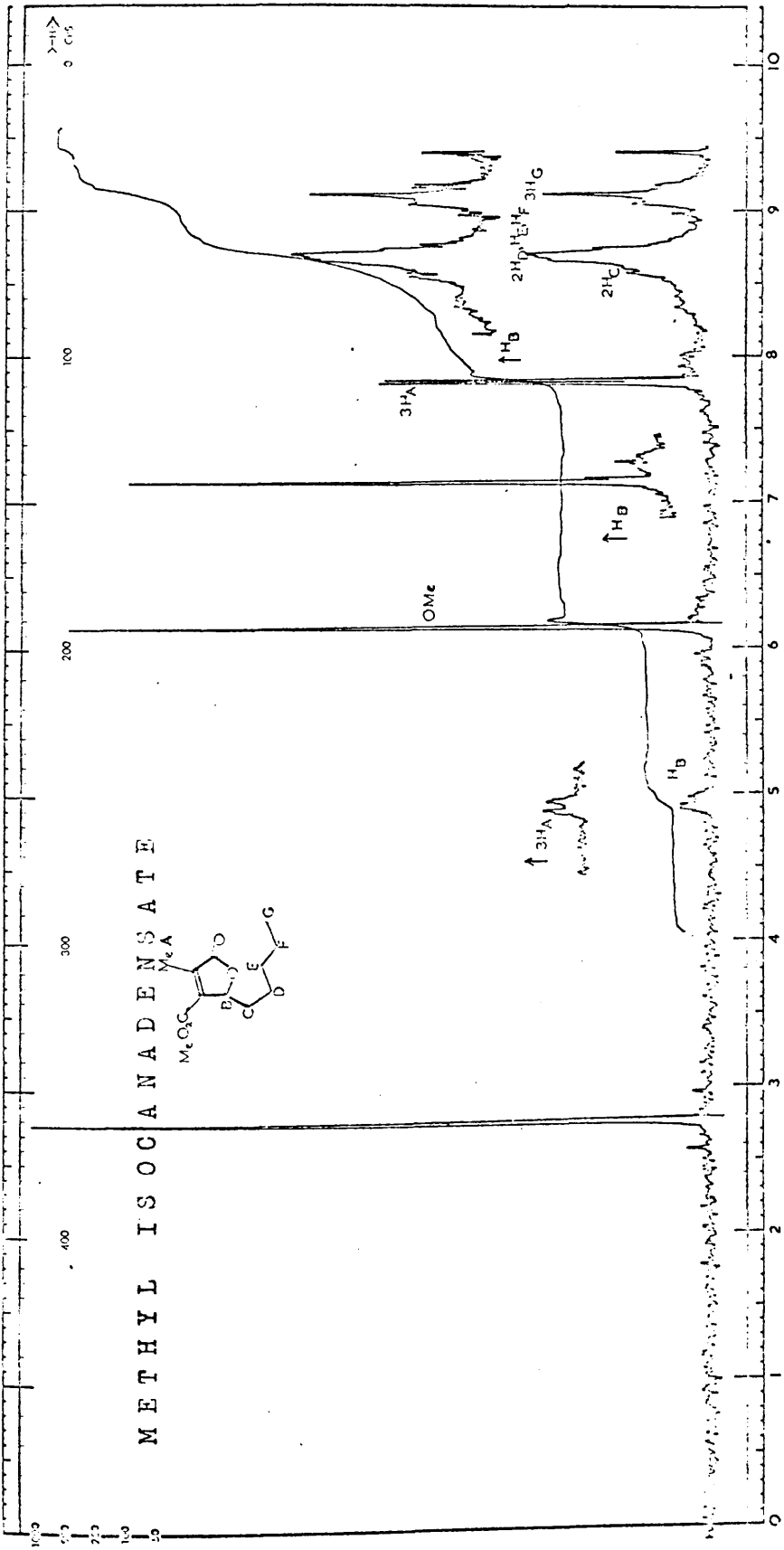
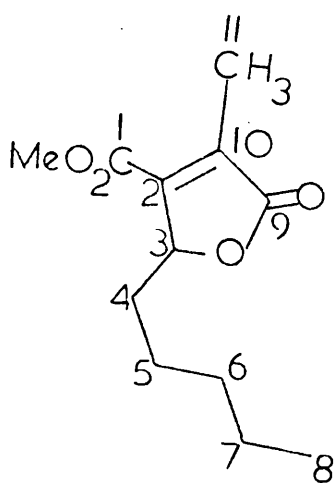


Fig. 22.

experiments with labelled precursors and using the more extensive degradation scheme are required. However, in order to clarify the biogenetic relationships of the various metabolites of P. canadense, culture filtrates were examined after six days growth for the presence of possible biosynthetic intermediates. The gum obtained from the organic extract of the broth, after chromatography on a silver nitrate impregnated silicic acid column afforded substantial quantities of canadensolide, dihydrocanadensolide and canadensic acid as well as fractions containing smaller amounts of two unknown acids. Separation of these acids was achieved by esterifying the fractions using diazomethane and p.l.c.

The less polar acid (217), an oil, analysed as $C_{12}H_{18}O_4$ and showed strong absorption at 1770 and 1728 cm^{-1} in its i.r. spectrum due to the presence of a δ -lactone system and carbomethoxyl group respectively. Although no peak attributable to a double bond appeared to be present, the u.v. spectrum, by strong absorption at 229nm. (ϵ 13,500), suggested this ester possessed a similar chromophoric system to that of methyl hydroxyisocanadensate (143).

Likewise the n.m.r. spectrum (fig. 22) showed a 3H doublet ($J = 2.0$ Hz., $3H_A$) typical of a vinyl methyl group at 7.83 τ . Double irradiation showed $3H_A$ was coupled to a 1H multiplet (H_B) at 4.94 τ , assigned to an allylic proton geminal to oxygen. Confirmation of the presence of the carbomethoxyl group was also provided by the 3H



217

singlet at 6.14 τ . At higher field, the 2H multiplet at 8.35 τ was assigned to methylene protons on a carbon atom β to oxygen, while the remaining resonances centred at 8.6 τ (6H) and 9.12 τ (3H) had the pattern expected for the n-butyl part of a n-pentyl chain. This evidence strongly suggested that the ester had structure (217) and was accordingly named methyl isocanadensate. This was supported by the mass spectrum whose base peak occurred at m/e 156 ($M^+ - 70$) due to the expected loss of pentene from the molecular ion.

An important feature of the n.m.r. spectrum of this ester, methyl isocanadensate (217), was the apparent non-equivalence of the methylene protons (2H-4). This was apparent when irradiation at the 3H doublet at 7.83 (3H-11) resulted in the allylic proton (H-3) collapsing to a double doublet ($J_1 = 8.0$, $J_2 = 1.5$ Hz.) rather than a triplet. This non-equivalence is typical of the protons of a methylene group adjacent to an asymmetric centre⁷⁸ and can be explained by the different environment of the two methylene protons in the three conformers about the (C-3)-(C-4) bond. It was also noted that one of the methylene protons had the same small coupling constant to that ($J_{3,4} = 1.5$ Hz.) found for the corresponding protons of methyl hydroxyisocanadensate (143).

The more abundant ester (218) in the esterified fraction was again obtained as an oil, $C_{12}H_{20}O_4$. Strong absorptions at 1790 cm^{-1} and 1746 cm^{-1} in the i.r. spectrum (CCl_4) again suggested the presence of a

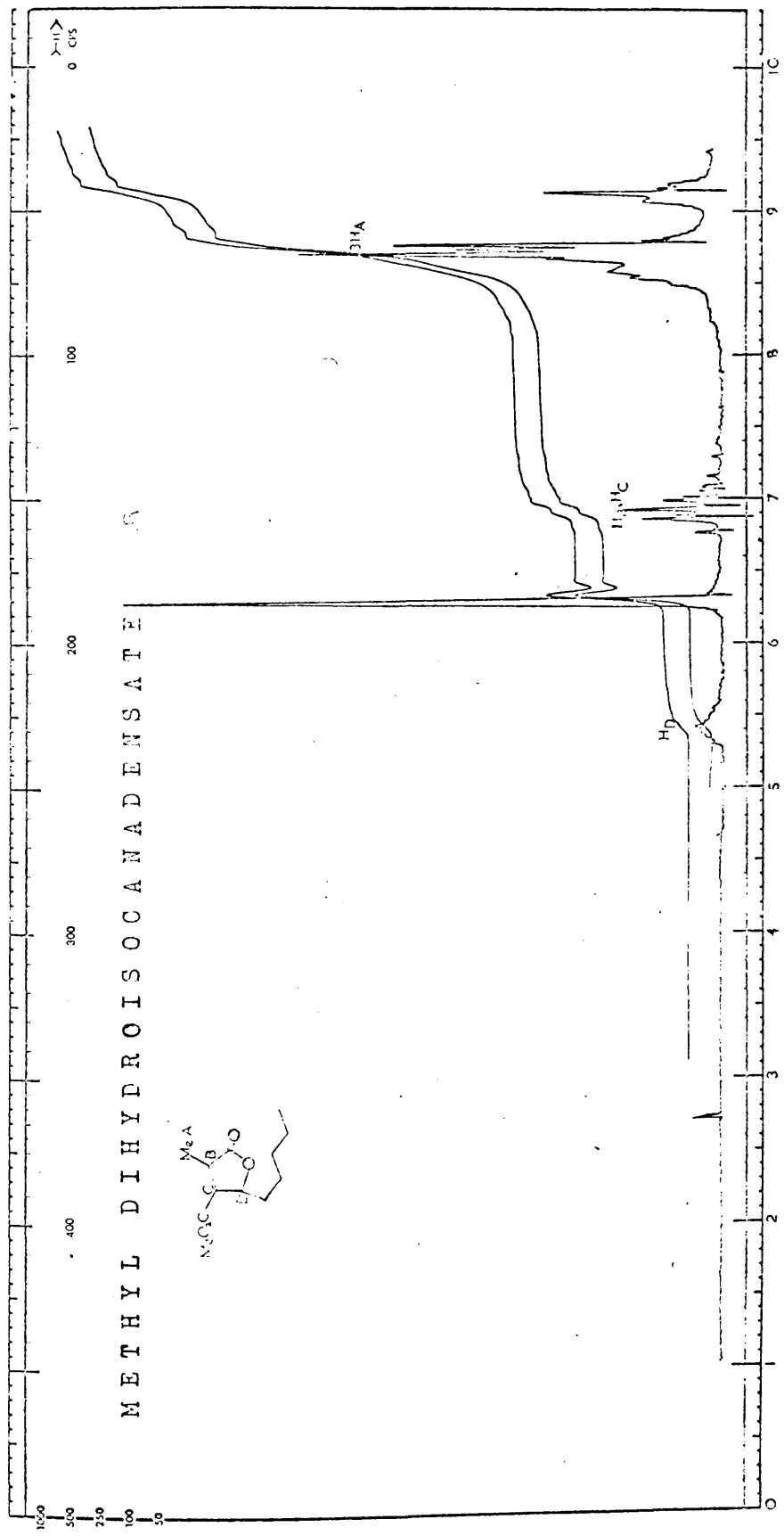
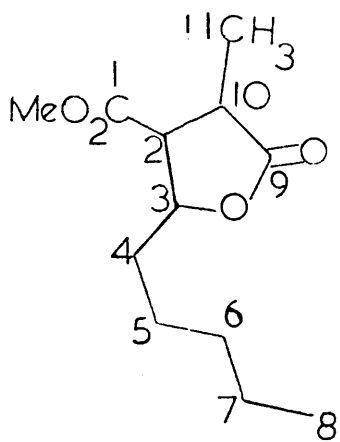
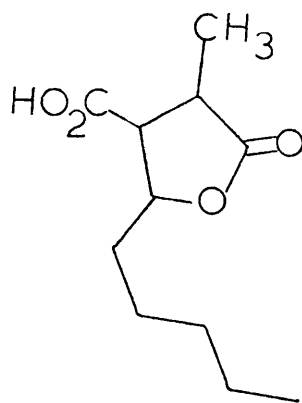


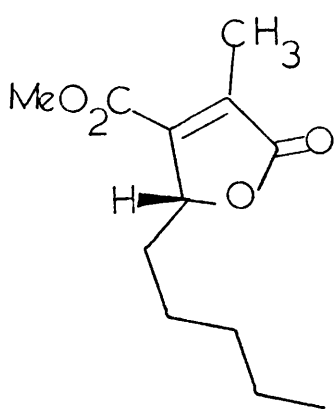
Fig. 23.



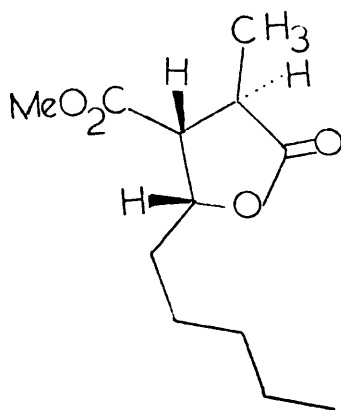
218



219



217



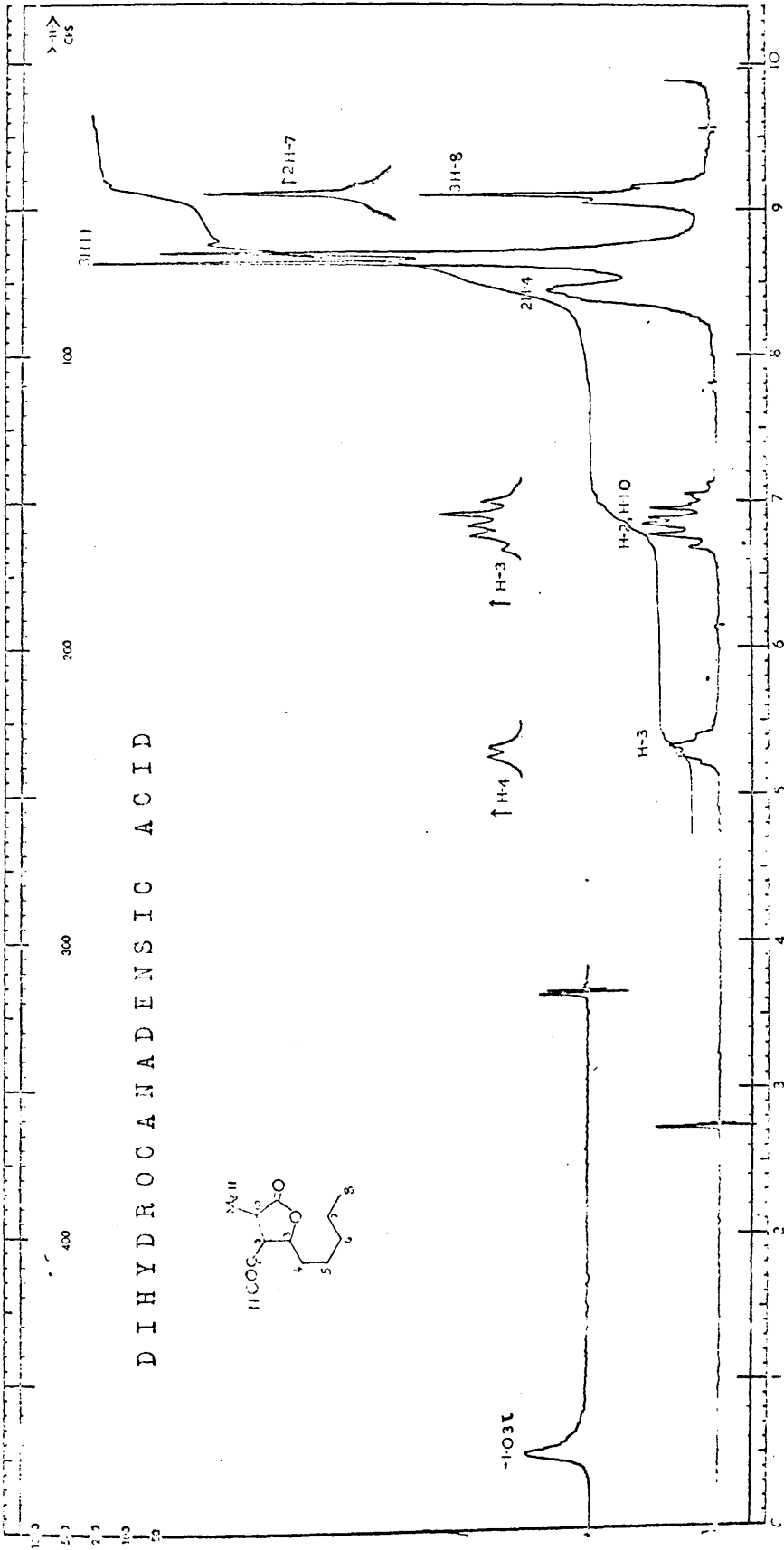
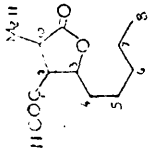
220

γ -lactone system and a carbomethoxy group respectively. The absence of any conjugation in this molecule, however, was shown by its u.v. spectrum which had no absorption maximum above 200 nm.

Similarly, the n.m.r. spectrum (fig.23) in place of a vinyl methyl resonance had a 3H doublet ($3H_A$) ($J = 6.0$ Hz.) at 8.73τ , assigned to a secondary methyl group β to carbonyl. These protons were shown by double irradiation to be coupled to H_B , forming with H_C a 2H multiplet centred at 6.90τ , both presumably being on carbon atoms α to carbonyl. The 1H broad multiplet at 5.42τ (H_D) due to the proton geminal to oxygen of the γ -lactone was also shown by double irradiation to be vicinal to the 2H multiplet centred at 8.2τ and again assigned to the methylene protons α to carbon bearing oxygen. Resonances centred at 8.6τ (6H) and 9.12τ (3H) had the same pattern observed previously for methyl isocanadensate (127) and typical of the *n*-butyl moiety. Thus structure (218) was postulated for this compound, methyl dihydroisocanadensate.

Dihydroisocanadensic acid (219) itself was later isolated in minor quantities from a normal 10 day culture, its i.r. spectrum (KBr) showing absorption at $3,200-2,800\text{ cm.}^{-1}$ and 1742 cm.^{-1} due to the presence of the carboxyl group. The n.m.r. spectrum, apart from lacking the 3H singlet of the methoxyl group (6.28τ), showed similar resonances to those found in the corresponding methyl ester. In this case, however, double irradiation enabled the chemical shift

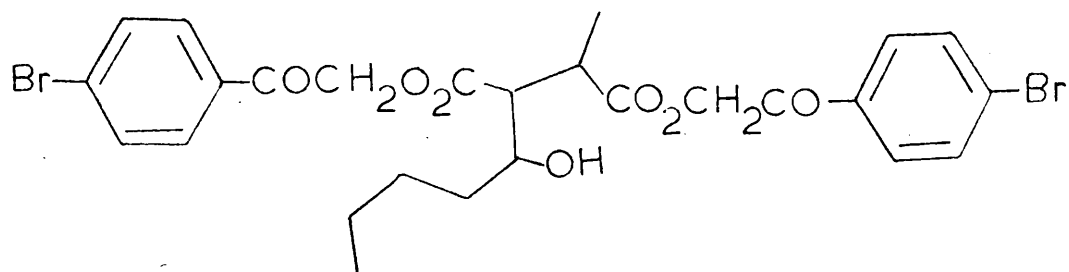
DIHYDROCANADENSIC ACID



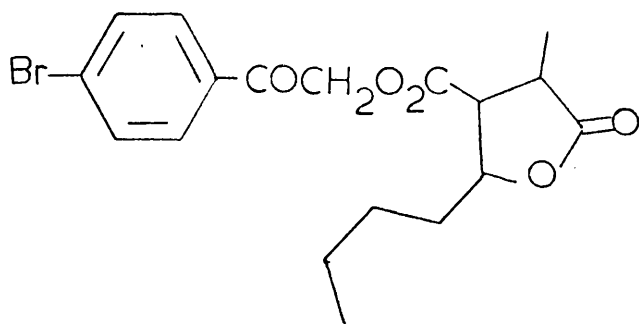
values of H-2 (6.82 τ) and H-10 (6.96 τ), and the corresponding coupling constants ($J_{2,10} = 9.0$, $J_{10,11} = 8.0$, $J_{2,3} = 7.0$ Hz.) to be determined. As in the case of canadensic acid (95), however, it was not possible to determine the relative stereochemistry by application of the Karplus equation due to the flexibility of the ring system. Evidence for a n-pentyl side chain was provided by an ion at m/e 143 ($M^+ - 71$ mass units), the base peak in the mass spectrum, due to the favoured cleavage at the γ -carbon atom¹³¹ of the saturated lactone ring.

The 3R absolute configuration of methyl isocanadensate (217) was readily determined from its o.r.d. and c.d. curves which exhibited positive Cotton effects, showing it to have the same absolute configuration at C-3 to that postulated for hydroxyisocanadensic acid (148). Although a similar determination could not be made in the more complex case of methyl dihydrocanadensate (218), if this molecule is assumed to possess the same relative stereochemistry as dihydrocanadensolide (54), it may be represented as in (220).

Confirmation of the above assignment for dihydroisocanadensic acid (219) could possibly have been achieved by interconversion of the two esters. As specific hydrogenation of the butenolide system of methyl isocanadensate (217) was unlikely to be any more successful than in the case of its hydroxyanalogue (143), desaturation of methyl dihydroisocanadensate (218) using 2,3-dichloro-5,6-dicyano-p-benzoquinone (D.D.Q.) was considered more feasible. Although this reagent usually



221

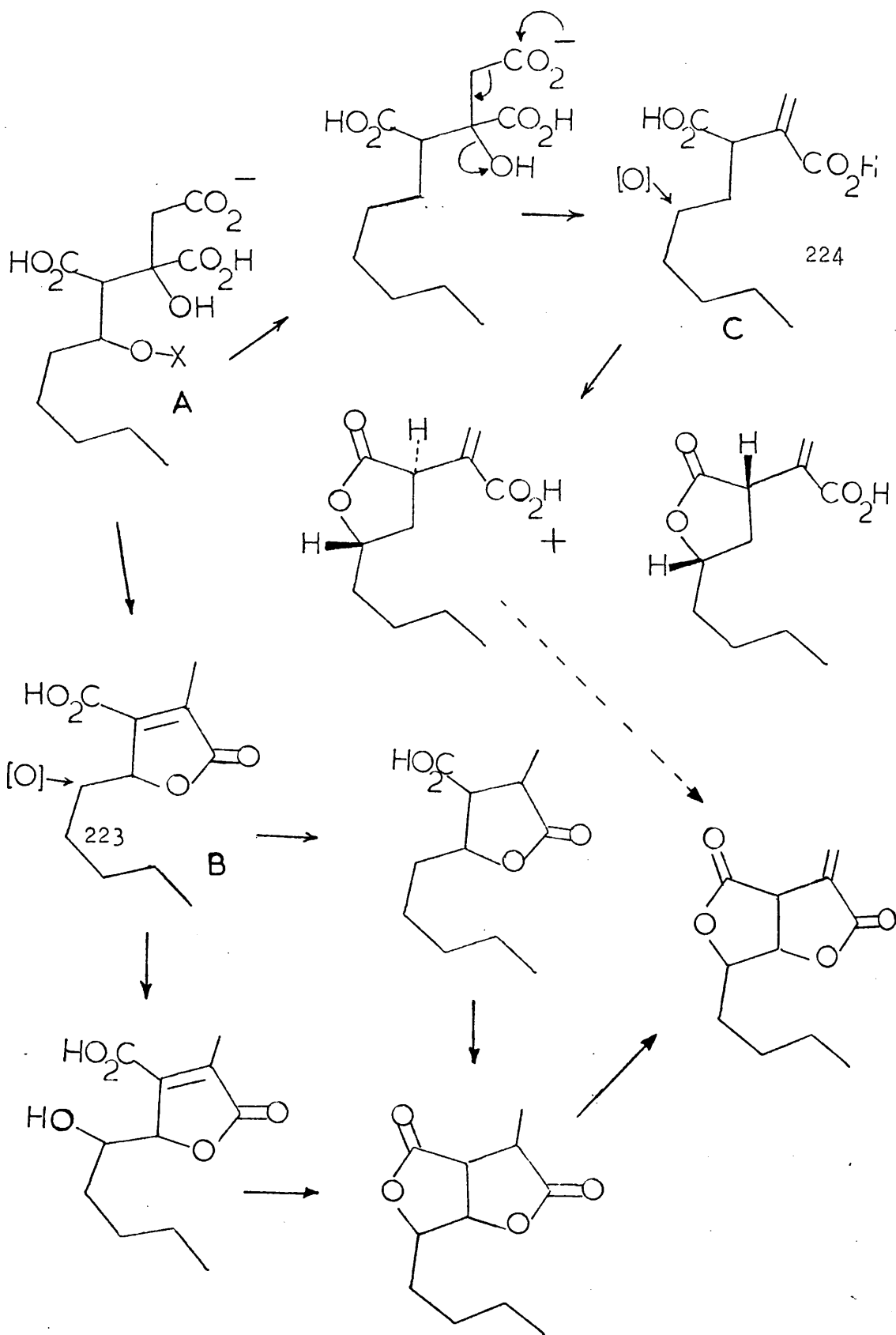


222

dehydrogenates δ -lactones to the corresponding α,β unsaturated lactones in fair yield⁹², it was found not to effect the required transformation on the dihydroester (218). A further attempt was made to prepare the required butenolide system by making the hydroxyl group of the γ -lactone system in dihydroisocanadensic acid (219) available for oxidation, via the bis phenacyl ester derivative (221)¹⁵¹. However, treatment of the di sodium salt of dihydroisocanadensic acid (219) with 2 equivalents of *p*.bromophenacyl bromide gave only the mono-phenacyl ester (222), presumably due to the ready cyclisation of the corresponding diester. The reported formation of *p*.bromophenacyl-santonate¹⁵¹ in high yield from sodium santonate is possibly not a good analogy for the case in question, since the santonate ester formed could adopt a skew-boat conformation making recyclisation unlikely.

It was realised that saturation of the double bond of methyl isocanadensate (217) would probably have been achieved using zinc dust and acetic acid as described for its hydroxy analogue. This, however, would have almost certainly been non-specific resulting in a mixture of epimers at C-2 and C-10 and was not carried out on the small quantity of unsaturated ester available. These new metabolites isolated from the culture filtrates of the 6 day culture could thus represent intermediates occurring in the early stages of the biosynthesis of some of the metabolites of Penicillium canadense. Isocanadensic acid (223) could possibly result from concerted decarboxylation - elimination of

Scheme 2. Postulated biosynthesis of C_{11} metabolites of *P. canadense*.

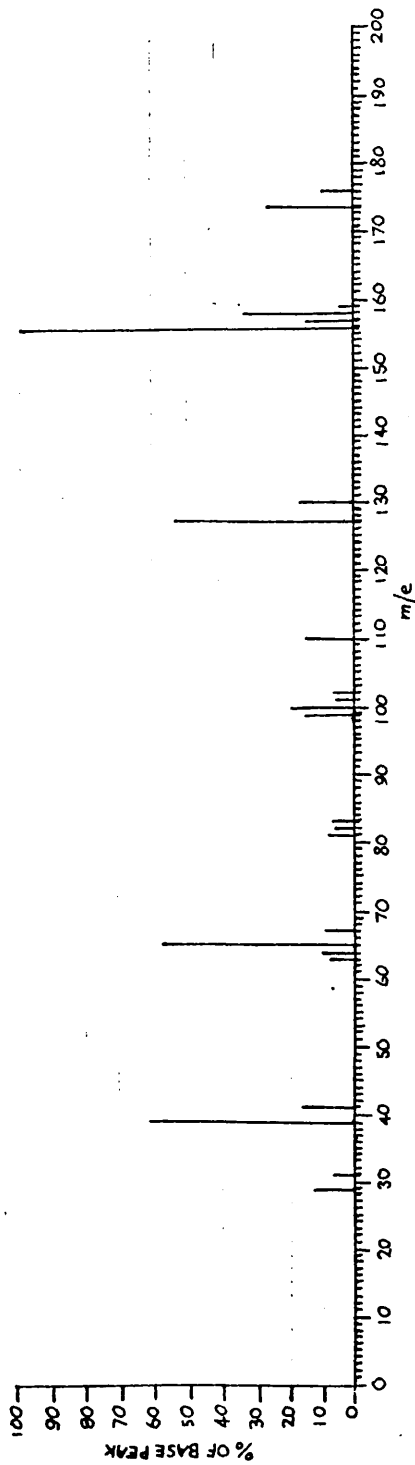


water followed by double bond isomerisation of an octylcitric acid intermediate. By simple stages of oxidation and reduction this butenolide acid (223) could be converted into the metabolites shown (A→B Scheme 2). (Incidentally, attempts to effect in vitro desaturation of 10-epidihydrocanadensolide (61) to the antibiotic using D.D.Q. were, however, unsuccessful).

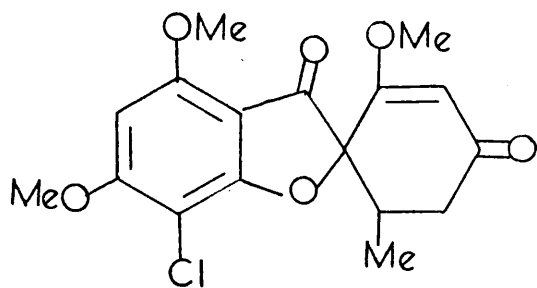
The formation of canadensic acid (95) would appear to involve the alternative decarboxylative-elimination of water to the unsaturated diacid (224) with its subsequent lactonisation (A →C). If both (+) and (-) nexylcitric acids are produced by this fungus with analogy to the decylcitric acids¹⁴⁴, then the corresponding C-2 epicanadensic acid (125) could be a natural metabolite, which could cyclise to the dilactone system of canadensolide.

CHLORINE CONTAINING METABOLITE.

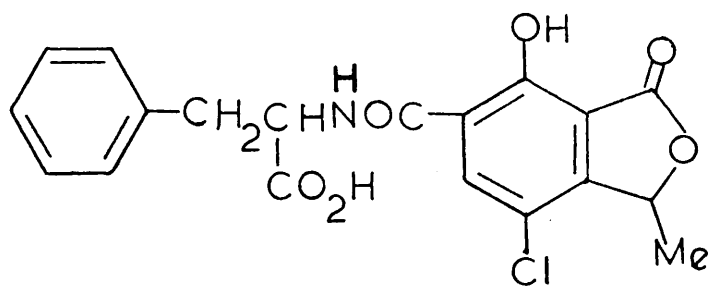
Disc. 10. Mass spectra data of chlorine containing metabolite



<u>Transition</u>	<u>m/e</u>	<u>Metastable ion</u>
174 ⁺ → 156 ⁺	18	139.8
156 ⁺ → 128 ⁺	28	105.1
128 ⁺ → 100 ⁺	28	78.2
100 ⁺ → 65 ⁺	35	-



225



226

A metabolite, structurally unrelated to those previously described, was isolated in minor quantities from the culture fluids of Penicillium canadense on one occasion. This compound was obtained from column fractions immediately following those containing canadensic acid (95), and crystallised as needles (m.p. 144°) from ethyl acetate - light petroleum. It appeared to be phenolic (from its purple reaction with methanol ferric chloride on t.l.c) and was shown to contain chlorine by a Beilstein test. Mass spectrometry gave a molecular ion at 174.00 75 ($C_7H_7^{35}ClO_3$ requires 174.00 84) with the characteristic $M+2$ peak of approximately one-third the intensity of the parent due to the presence of molecular ions containing the ^{37}Cl isotope. This type of characteristic doublet occurred at various parts of the mass spectrum corresponding to fragment ions containing chlorine as shown (diag. 10). The interesting biological properties shown by various naturally occurring chlorophenols e.g. griseofulvin (225)¹⁵², ochratoxin A (226)¹⁵³, etc. suggested that this compound would merit further examination.

The i.r. spectrum of this metabolite showed no carbonyl absorption but exhibited broad absorption at 3,425 and 3,045 $cm.^{-1}$ due to hydroxylic functions, and absorption at 1597 $cm.^{-1}$ together with intense peaks at 700 - 900 $cm.^{-1}$ attributable to an aromatic ring.

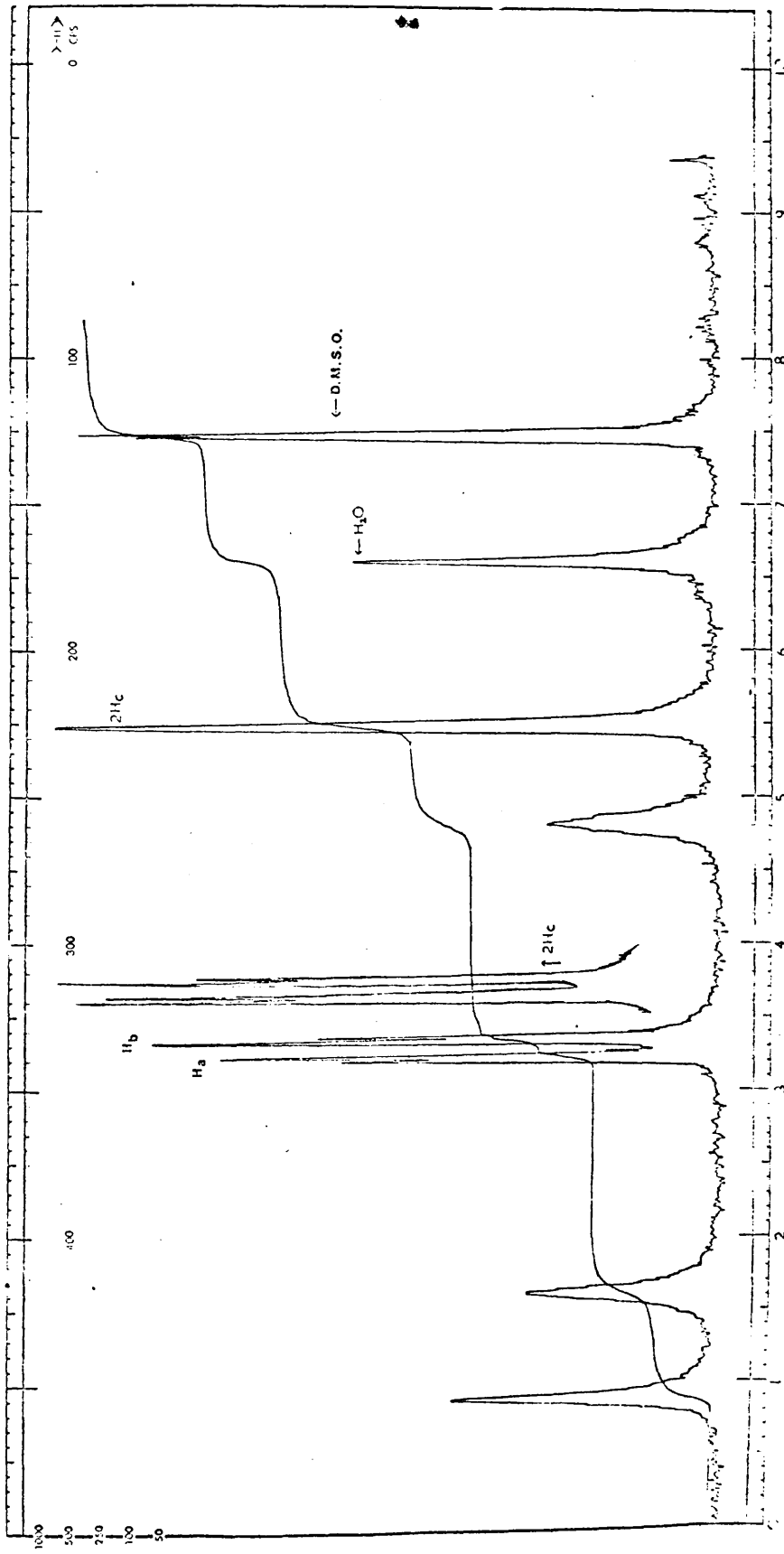
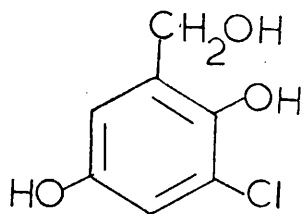


Fig. 24. Chlorine containing metabolite

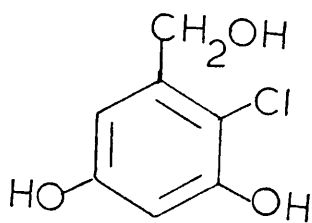
It's u.v. spectrum showed a strong band at 295 nm. (ϵ 3,910) which underwent a complex base shift (vide infra.).

The n.m.r. spectrum (fig. 24) in D_6 dimethyl sulphoxide showed broad multiplets at 0.88 τ (1H) and 1.61 τ (1H) assigned to phenolic protons and a 1H multiplet at 4.92 τ due to a hydroxyl group (all three resonances disappeared on adding D_2O). Two 1H doublets at 3.21 τ ($J = 3.0$ Hz.) and 3.35 τ ($J = 3.0$ Hz.) were assigned to aromatic protons, while the size of their coupling constant suggested they were situated meta to one another. The remaining 2H singlet at 5.48 τ was assigned to an aryl hydroxymethyl group. Irradiation at this 2H singlet resulted in an increase in intensity of the low field doublet (3.21 τ), presumably due to the removal of long range coupling. This result suggested that the low field aromatic proton was ortho to the hydroxymethyl group.

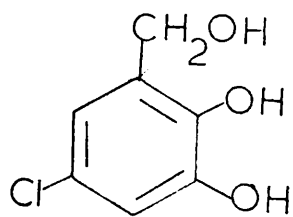
From the above data the metabolite appeared to be a chlorodihydroxybenzyl alcohol and this was confirmed by acetylation to give a triacetate, which although shown to be homogeneous by t.l.c., was an oil. It's i.r. spectrum (thin film) in place of hydroxyl absorption, showed a strong band at 1770 cm^{-1} assigned to two phenolic acetate groups while a peak at 1745 cm^{-1} indicated the presence of a benzylic acetate group. The u.v. spectrum showed only



I



II



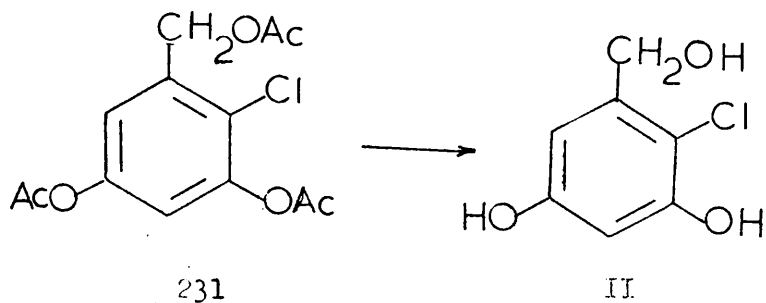
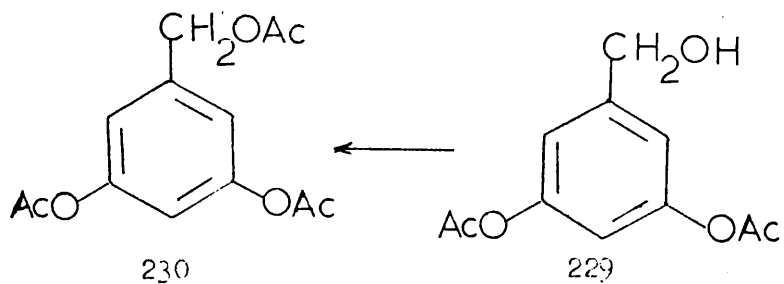
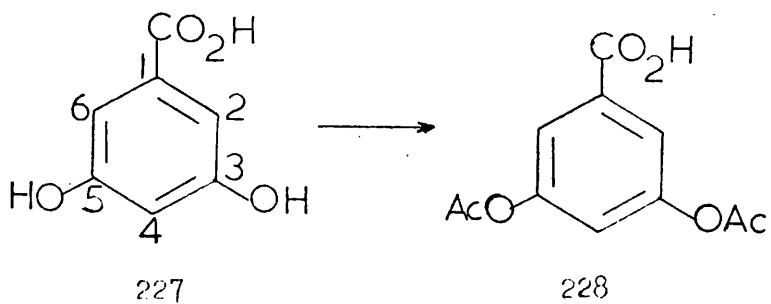
III

end absorption¹⁵⁴ confirming the absence of any phenolic group. The expected acetylation shifts were found for the meta protons (to 2.78 and 2.39 τ) and for the benzylic methylene protons (to 4.98 τ) in the n.m.r. spectrum.

Hydrolysis of one of the acetate groups of the triacetate derivative readily occurred on p.l.c. The diacetate, again an oil, gave a green colouration with methanolic ferric chloride on t.l.c. and a base shift in its u.v. spectrum, suggesting the presence of a phenolic group. Its i.r. spectrum (thin film) showed broad absorption at 3,500 cm.^{-1} (-OH) and 1750 cm.^{-1} ($\text{CH}_3\text{CO.O-}$). Two 3H singlets at 7.68 and 7.84 τ in the n.m.r. spectrum indicated the presence of only two acetate groupings. Although the resonance of the benzylic methylene protons still appeared at 5.02 τ , the two 1H doublets (at 3.15 and 3.24 τ) of the aromatic protons occurred at higher field than in the triacetate derivative, showing that a phenolic acetate group ortho or para to two protons had been hydrolysed.

The 1,2,3,5 substitution of this chlorodihydroxybenzyl alcohol suggested by the n.m.r. spectrum was confirmed by the characteristic pattern of weak overtone and combination tone bands between 1740 and 1950 cm.^{-1} ¹⁵⁵. Three structures having a proton ortho to the hydroxymethyl group can be considered, namely the hydroquinone (I) the resorcinol (II) and the catechol (III). Hydroquinones are

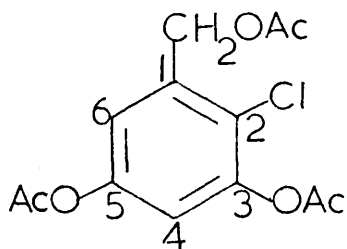
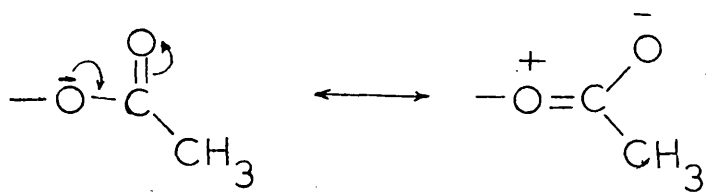
Scheme 3.



generally differentiated¹⁵⁵ from the other two types by their u.v. spectra which exhibit a hypsochromic shift on basification. However, the u.v. spectrum of the original metabolite upon addition of base gave peaks at longer wavelength, a result which was interpreted as a bathochromic shift, hence, making the hydroquinone structure (I) seem unlikely. Since the metabolite gave a negative vanillin - sulphuric acid test for a catechol¹⁵⁷ the resorcinol structure (II) seemed at this stage more feasible, and confirmation was sought by synthesis.

The first synthetic approach to this system used 3,4-dinitro-2-chlorobenzoic acid¹⁵⁸. Since attempts to convert the nitro groups into the desired hydroxyl functions were unsuccessful, chlorination of a suitable resorcinol was considered. The readily available secondary metabolite α -resorcylic acid (227), which has the desired substitution was hence selected (scheme 3). The phenolic groups were protected by acetylation to the known diacetoxy compound (228)¹⁵⁹, ν_{\max} . 3,200 - 2,800 cm.^{-1} (CO_2H), 1770 ($\text{CH}_3\text{CO.O-}$), and 1695 (COOH). The presence of meta oriented aromatic protons was shown by doublets ($J = 2.5 \text{ Hz.}$) at 2.67 τ (2H) and 3.13 τ (1H) in the n.m.r. spectrum. The two acetate groups gave rise to a 6H singlet at 7.80 τ . Selective reduction of the carboxylic function of the diacetoxyacid (228) was carried out in good yield by using exactly one equivalent of diborane¹⁶⁰ (attack at the acetate group

Diag. 11.



231

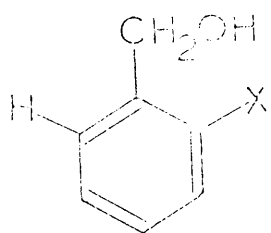
occurred with an excess of the reagent). The diacetoxy benzyl alcohol (229), an oil $C_{11}H_{12}O_5$, in place of the band at 1695 cm.^{-1} showed absorption at $3,520\text{ cm.}^{-1}$ ($-\text{OH}$) and retained a strong band at 1760 cm.^{-1} ($\text{CH}_3\text{CO.O}$). Formation of the hydroxymethyl group was indicated by the 2H singlet at 5.19τ and by the upfield shift of the aryl protons to 3.06τ (2H, $J = 2.0\text{ Hz.}$) and 3.22τ (1H, $J = 2.0\text{ Hz.}$).

The triacetate derivative (230), $C_{13}H_{14}O_4$ was prepared prior to chlorination of the aromatic nucleus, and was obtained as an oil which lacked hydroxyl absorption in the i.r. but showed peaks at 1770 cm.^{-1} (phenolic acetate) and 1740 cm.^{-1} (benzylic acetate) in the carbonyl region and also singlets in the n.m.r. at 7.71τ (6H) and 7.90τ (3H) due to the two types of acetate groupings. It had been considered that activation of the aromatic ring would be provided mainly by the weak inductive (+I) or hyperconjugative effect of the acetoxymethyl group. The lone pairs of the phenolic oxygen atoms should not contribute greatly to the electron density of the aromatic ring due to delocalisation involving the acetate carbonyl group as shown (diag.11) (cf. u.v. spectra of acetylated phenols which resemble those of the parent hydrocarbon¹⁵⁴). Electrophilic attack of chlorine at the desired position ortho to the acetoxymethyl group rather than para hence seemed likely not only on the basis that an increased electron density at the former positions would result if an inductive effect was operative, but also because there was a

statistically greater chance of ortho substitution (2 possible positions) rather than para (only 1 position). Consideration of steric crowding would not appear to favour one position rather than another.

In fact chlorination of the triacetoxy compound (230) occurred slowly in acetic acid to give apparently only one chloro compound, obtained as an oil, $C_{13}H_{13}ClO_6$, showing carbonyl absorptions at 1770 and 1738 $cm.^{-1}$. The n.m.r. spectrum showed that the predicted 2-chloro-3,5 - diacetoxybenzyl acetate (231) had been formed. Two 1H doublets ($J = 2.5$ Hz.) at 2.83 and 2.98 τ were assigned to the meta protons H-6 and H-4, while in addition to the 2H singlet of the benzylic methylene protons at 4.73 τ , three 3H singlets at 7.70, 7.77 and 7.83 τ indicated the presence of three non-equivalent acetate groups. Comparison of these spectra with the i.r. and n.m.r. spectra of the triacetate derivative of the natural product showed that although strikingly similar they were not identical.

Hydrolysis of this chloro compound (231) was achieved by refluxing in methanol with hydrochloric acid to give the chlororesorcinol (II) as needles m.p. 147°. Its i.r. spectrum (KBr) was similar but not identical to that of the chlorine containing metabolite. The n.m.r. spectrum (in D_6 acetone) showed 1H doublets ($J = 2.0$ Hz.) at 3.30 and 3.58 τ assigned to H-6 and H-4 respectively and the 2H singlet of the benzylic methylene protons at 5.27 τ . The mass spectrum



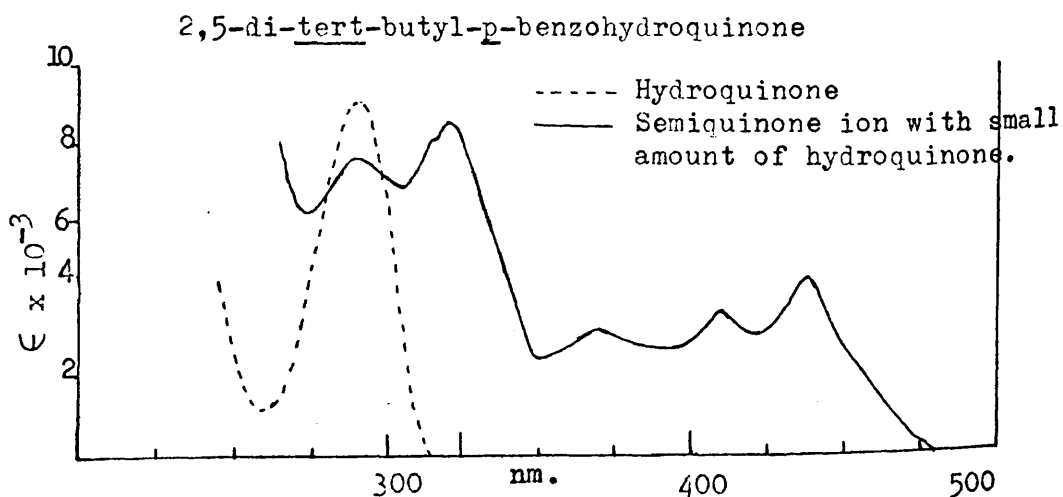
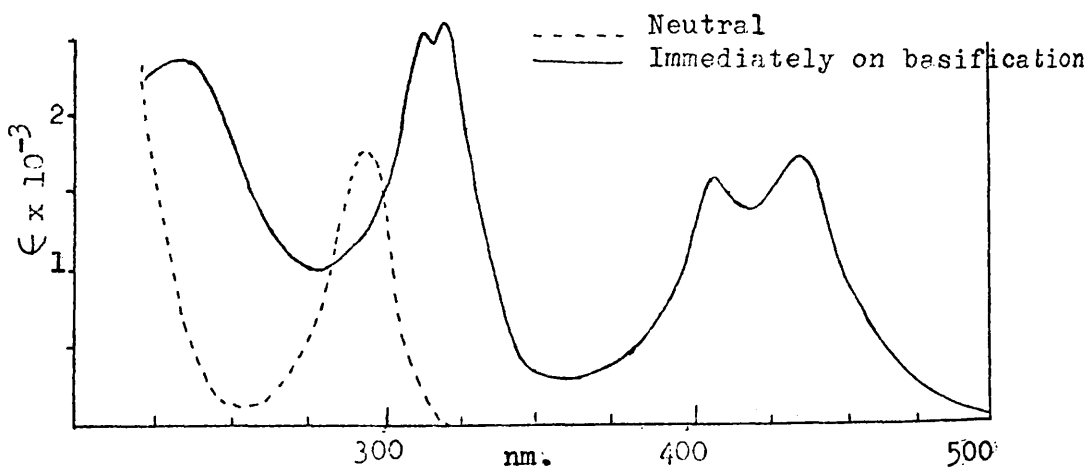
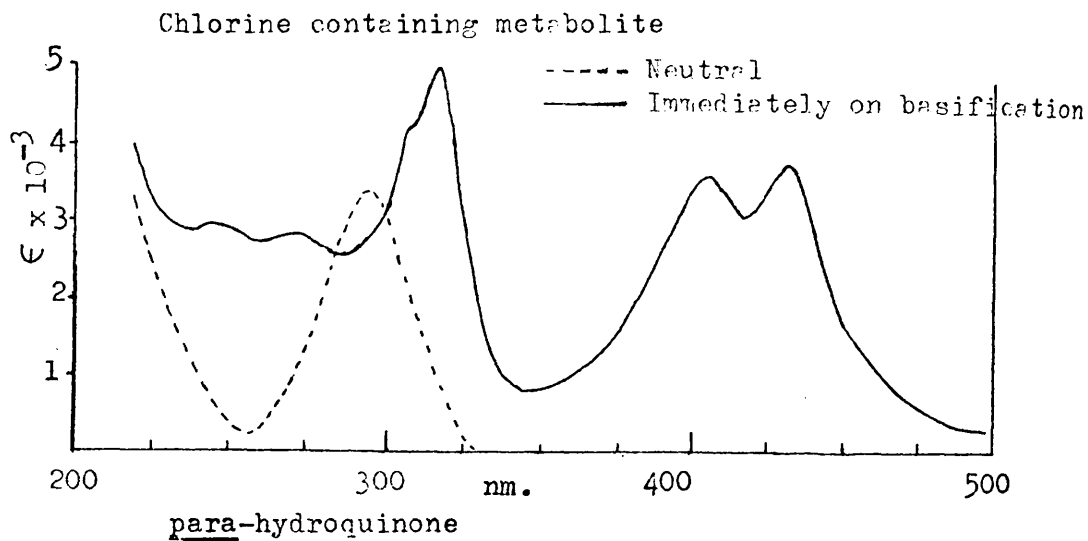
233

showed a molecular ion at m/e 174 (^{35}Cl), the base peak, from which the loss of water was not particularly favoured (cf. the mass spectrum of the chloro metabolite where $M^+ - \text{H}_2\text{O}$ was the base peak). This is in accord with the recorded spectra of meta - hydroxybenzyl alcohols¹⁶⁶ which exhibit strong molecular ions, and since no mechanism can be written for the loss of water only weak $M^+ - \text{H}_2\text{O}$ peaks occur. It was, therefore, now evident that the mass spectrum of the metabolite provided evidence, previously overlooked, against the resorcinol structure II. Likewise the u.v. spectrum showed λ_{max} at 233 nm. (ϵ 2,365) which underwent a normal bathochromic shift to 303 nm. (ϵ 3,840) on basification.

Although this synthesis did not provide direct evidence about the structure of the metabolite, certain conclusions could be drawn from the data obtained for the chloro resorcinol (II). Slight broadening of the doublet of the proton H-6, ortho to the aromatic methylene group relative to that of the para proton H-4 was observed in the n.m.r. spectrum, providing confirmatory evidence for the similar partial structure (233) previously postulated for the metabolite. Also the close similarity in polarity (oh t.l.c.) of the chlororesorcinol (II) to the metabolite suggested that of the two alternate structures that of the hydroquinone (I) was more likely since the catechol (II) would be expected to be much less polar than the other two isomers.

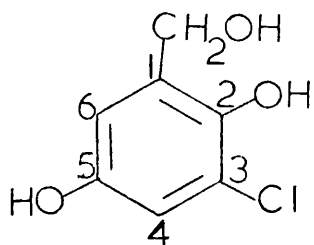
As stated previously, however, the u.v. spectrum of the metabolite

Fig. 25. U.V. spectra of Hydroquinones.

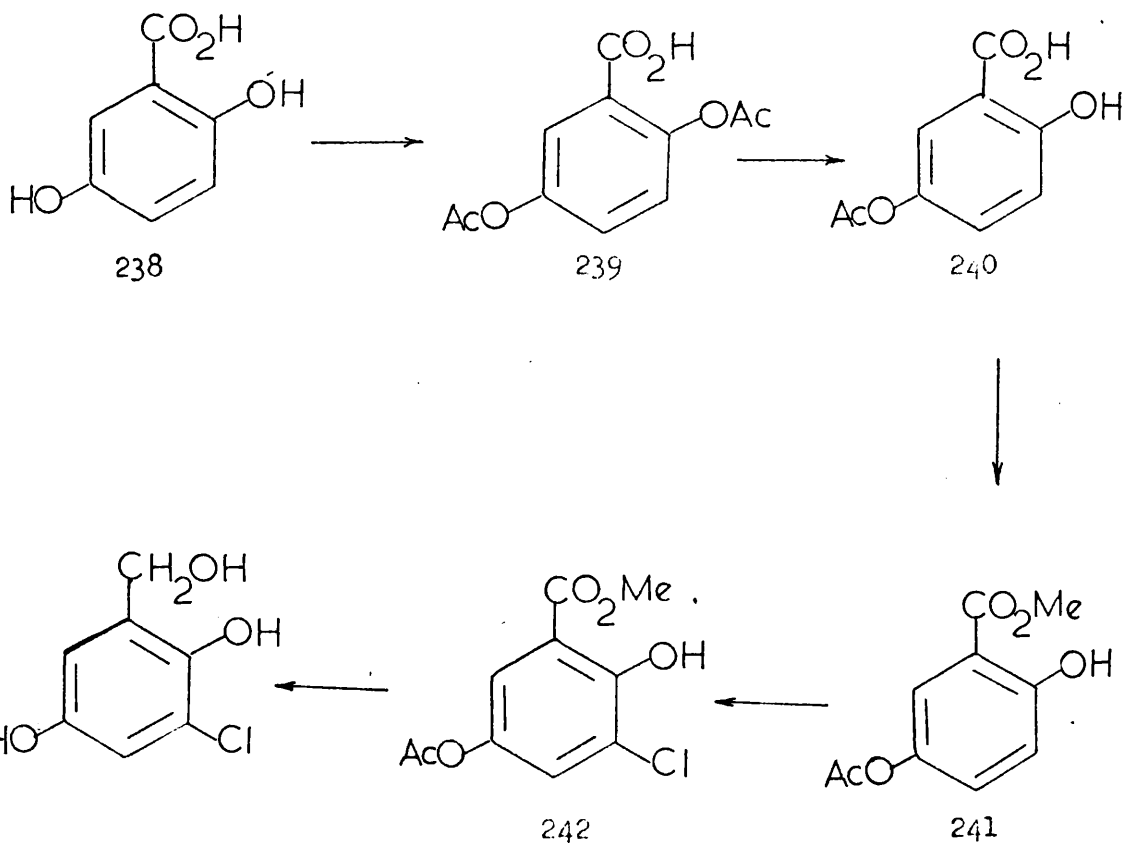


did not show a hypsochromic shift on basification of its ethanolic solution. Instead a series of intense peaks immediately appeared at 314, 320, 408 and 433 nm. (fig. 25), and were found to decrease with time, the final spectrum showing only a broad band at 267 nm. which remained unchanged on acidification. Contrary to the fact that hydroquinone itself, is known¹⁵⁶ to give a hypsochromic shift, its u.v. spectrum, recorded under identical conditions to those used in the above measurements, was found to give a similar pattern of absorption peaks on basification (fig. 25), strong bands occurring at 312, 318, 410 and 430 nm., collapsing rapidly to a broad band at 311nm. Apart from one paper¹⁶⁴ which records peaks at 405 nm. (log. ϵ 2.0) and 430 nm. (log. ϵ 2.0) in addition to the normal "hypsochromic" peak at 235 nm. (log. ϵ 2.7), these anomalous peaks recorded for a basified solution of hydroquinone do not appear to have been reported in the literature, nor are any special precautions specifically used in measurement of these structural types stipulated¹⁶⁵.

It was suspected that the unusual absorption spectra recorded were due to the presence of semiquinone ions by autoxidation of hydroquinone in the basic solution, with these reactive ions undergoing further reactions such as oxidation, coupling, etc. This deduction appeared to be substantiated by the "normal" hypsochromic shifts obtained for both hydroquinones when their u.v. spectra were recorded under nitrogen using deoxygenated aqueous sodium hydroxide as base.



Scheme 4.



In fact a study of the absorption spectra of semiquinone ions, generated by aerial oxidation of the corresponding hydroquinone with basic catalysis, has been made¹⁶⁶. Although the unsubstituted *p*-benzoquinone ion itself was too labile to be examined, the stable 2,5 di-*tert*-butyl-*p*-benzoquinone ion was reported to give weak absorption bands at 436, 411 and 374 nm. and a strong band at 323 nm. (fig. 25). The similarity of this spectrum to those reported above provided further evidence for the involvement of species of this type.

Synthesis of the 3-chlorogentisyl alcohol (I) now postulated as the structure of the metabolite was achieved as indicated in (scheme 4), from gentisic acid (238), which can be readily obtained from *p*-hydroquinone¹⁶⁷. The first step in the synthesis required preparation of the diacetate (239), a crystalline compound $C_{11}H_{10}O_6$. The presence of the carboxylic acid grouping was shown by broad absorption from 3,250 - 2,800 $cm.^{-1}$ and a strong peak at 1705 $cm.^{-1}$ in the i.r. spectrum. The acetate groups gave rise to strong bands at 1759 and 1727 $cm.^{-1}$ due to phenolic and hydrogen bonded phenolic acetate groupings, and a 6H singlet at 7.67 τ in the n.m.r. spectrum.

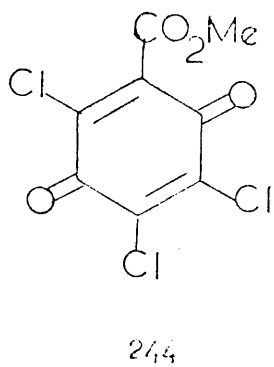
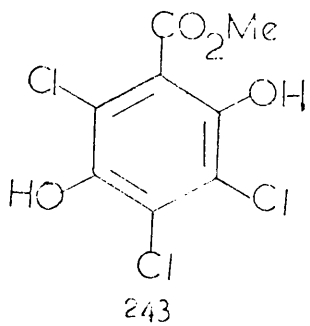
Selective hydrolysis of the 2-acetate grouping was obtained under reflux in dioxan - water at pH 6.0 due to neighbouring group participation¹⁶⁸ involving the carboxylate ion. The facile hydrolysis of acetylsalicylic acid is of course, well known¹⁶⁹. Evidence for the

phenolic grouping in the 5-acetoxysalicylic acid (240) was its blue colouration with methanolic ferric chloride on t.l.c., and its u.v. spectrum which showed strong absorption at 308 nm. (ϵ 3,850) and a base shift. Only a single absorption attributable to an acetate group was likewise evident (1768 cm.^{-1}) in the i.r. spectrum.

Of particular importance in the n.m.r. spectrum were the resonances of the aromatic protons which established the substitution. A low field doublet ($J = 3.0 \text{ Hz.}$) at 2.36τ was assigned to the proton H-6 which was coupled to the meta proton H-4, a double doublet ($J = 3.0 \text{ Hz.}$, 9.0 Hz.) at 2.73τ . The large ortho coupling (9.0 Hz.) was evident in the resonance of proton H-3, a doublet at 3.10τ .

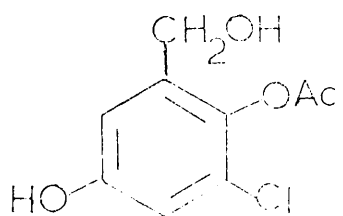
Brief treatment of 5-acetoxysalicylic acid (240) with diazomethane produced the corresponding methyl ester (241), a crystalline solid (m.p. 87°). ($\nu_{\text{max.}}$ at 1670 cm.^{-1}) showing a 3H singlet at 6.06τ in the n.m.r. spectrum. The only unsubstituted position activated by the phenolic group was that at C-3, and hence the desired monochlorination at this position was achieved using chlorine water, or chlorine in acetic acid when needle crystals (m.p. 124°) were obtained.

Although the presence of the expected functional groups could be inferred from the i.r. spectrum $\nu_{\text{max.}}$ 3,450 (-OH), 1761 (acetate) and 1682 (carbomethoxyl group) and the u.v. spectrum, conclusive proof



of the structure was provided by the n.m.r. spectrum, which displayed only two aromatic proton resonances. Doublets ($J = 3.0$ Hz.) at 2.47τ (1H) and 2.67τ (1H) were consistent only with resonances of the meta protons H-6 and H-4 respectively, while the remaining resonances were also consistent with structure (242).

Treatment of the above methyl ester (241) with a large excess of chlorine in methanol gave, on the other hand, a moderate yield of a compound of similar polarity as yellow needles (m.p. 119°). Perchlorination was evident from the low hydrogen content given by elemental analysis, and the single resonance at 6.03τ (due to the methoxyl group) shown in its n.m.r. spectrum. It was thought that hydrolysis of the acetate group with subsequent chlorination had occurred to give the trichlorohydroquinone (243). However, an alternative possibility was the corresponding quinone (244) which could result from oxidation of the former compound by, for example, the hypochlorous acid present in the solution. The quinone structure was suggested by the apparent lack of any absorption due to phenolic groups above $3,000 \text{ cm.}^{-1}$ in the i.r. spectrum, although this might be explained by the strong hydrogen bonding likely to be present in the hydroquinone structure (243). The high ester frequency at 1735 cm.^{-1} and the strong bands at 1690 and 1680 cm.^{-1} ¹⁷⁰, however, seemed more consistent with a quinone. Similarly the u.v. spectrum showed long wave absorption at 272 nm. which did not exhibit a base shift although the molecular absorption coefficient ($\epsilon_{3,540}$) appeared low for a quinone.

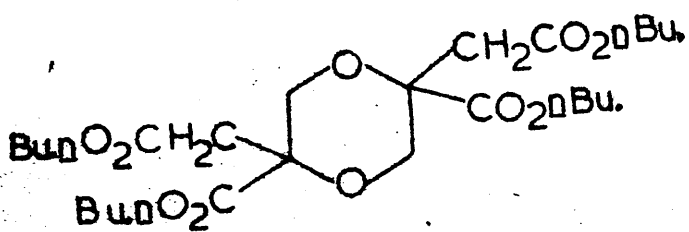


945

Confirmatory evidence was provided by the mass spectrum, however, which showed the molecular ion (m/e 269) expected of the chloroquinone structure (244) with ions at $M+2$, $M+4$ and $M+6$ of the correct intensity for a compound containing three chlorine atoms¹⁷², and a breakdown pattern consistent with this structure.

The final step in the synthetic sequence required reduction of the carboxylic acid group of the chloro ester (242) to the alcohol and hydrolysis of the acetate. Since the formation of metal-oxide bonds¹⁷³ might have reduced the effectiveness of lithium aluminium hydride for this reaction, attempts were made to achieve the required transformation using complex borohydrides (174) such as sodium borohydride, lithium borohydrides, etc. in methanol. Limited success was obtained possibly due to the formation of boron compounds of the final product¹⁷⁴. By using a modified work-up and a large molar excess of lithium aluminium hydride, a moderate yield of material of similar R_f and staining to the chlorine containing metabolite was obtained. Crystallisation from ethyl acetate - light petroleum gave needles (m.p. 144°), $C_{11}H_{11}ClO_3$, which were identical in i.r. and u.v. spectra and mixed m.p. to a sample of the natural product. The metabolite is hence 3-chloro-2,5-dihydroxybenzyl alcohol, and the diacetate derivative must have structure (245).

CANDIPOLIN.



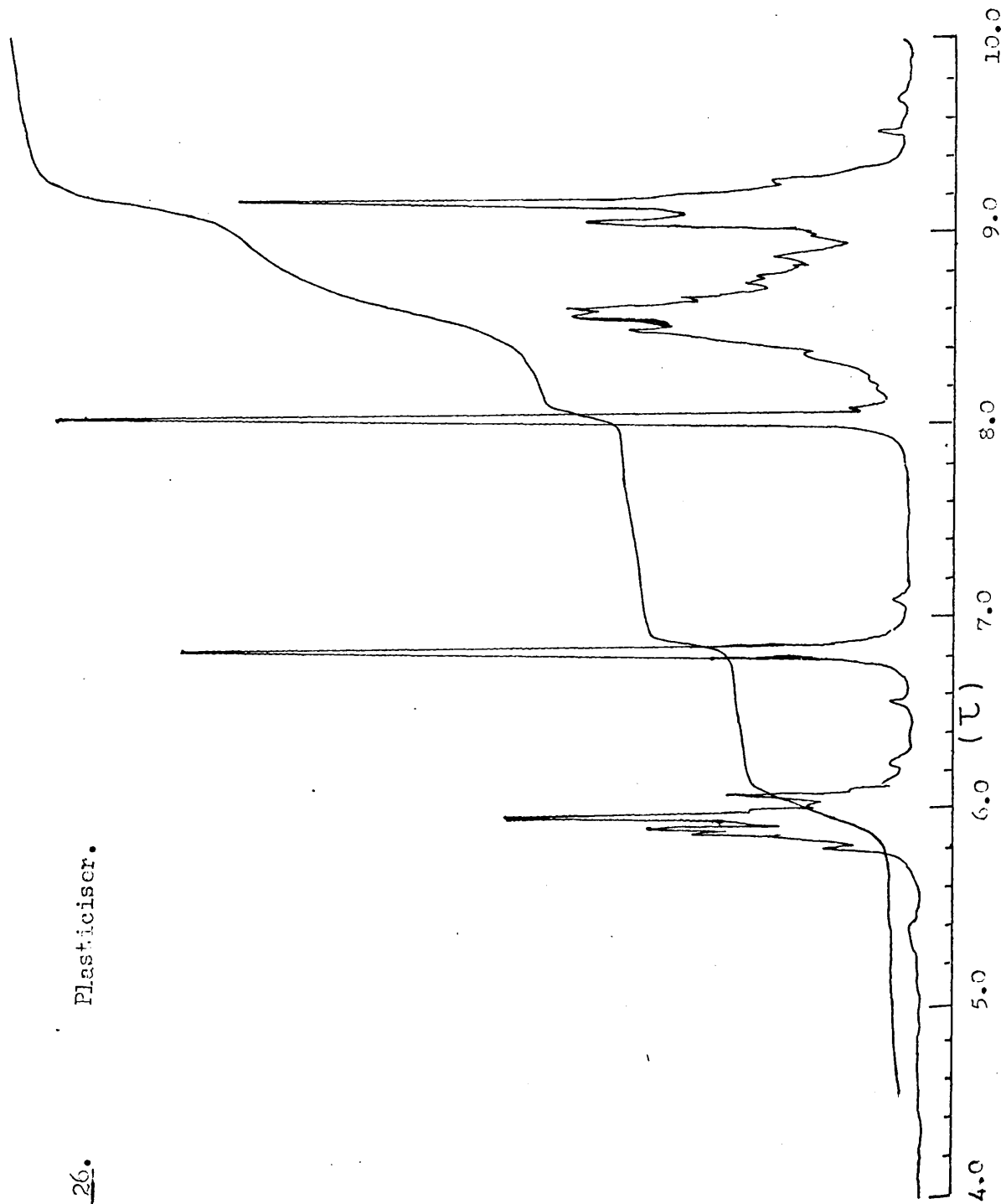
246

Since a small amount of antifungal activity was known to be present in the chloroform extract of the mycelium of Penicillium canadense, an investigation of the metabolites present in this extract was carried out by R.L. Baxter 175. Although no antifungal antibiotic was characterised, several inactive metabolites were isolated. In addition to trace amounts of canadensic acid, a neutral oil (thought to be an artefact) and a crystalline compound (of postulated amino acid origin) were present in reasonable quantities. Although tentative structures had been assigned to both these compounds, further evidence seemed necessary.

(a) Isolation of a Plasticiser.

The colourless, neutral oil was at first assigned the dioxan structure (246) on the basis of its spectral data. Strong absorption at 1745 cm.^{-1} in its i.r. spectrum suggested the presence of the ester groupings, which were confirmed by the series of overlapping triplets (8H) at ca. 5.9τ in its n.m.r. spectrum (fig. 26), assigned to methylene protons geminal to oxygen of carboxyl. The AB doublet (4H) centred at 6.9 and 7.0τ could be assigned to the methylene protons of the dioxan ring, the coupling constant (15 Hz.) appearing to rule out the corresponding epoxide structure. The singlet at 7.94τ , although at the position usually associated with an acetate grouping, appeared to integrate better as 4 protons rather than 3. These protons were, therefore,

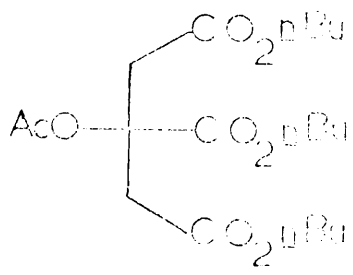
Fig. 26. Plasticiser.



attributed tentatively to 4 protons α to carbonyl. Evidence for the propyl residue of the *n*-butyl ester groups was provided by the resonances at 8.3 - 8.8 τ (16H) and a triplet (12H) at 9.10 τ ($J = 7.0$ Hz.)

Since *n*-butyl esters appear to be unknown in fungal metabolites, whereas, this grouping is a common feature of many known plasticisers¹⁷⁶, this compound was suspected of being an artefact. Moreover, it had also been isolated in large quantities¹⁷⁷ from the broth of another fungus, Penicillium brevicompactum, grown in our laboratories. Credence was lent to the dioxan structure (246) by the fact that it might represent a cyclic dimer of an epoxidised methylenesuccinic ester which are known plasticisers¹⁷⁸. It seemed possible that the dioxan product could have been extracted from P.V.C. or rubber tubing during work-up procedure or have been a fungal modified plasticiser (by oxidative cleavage of *n*-butyl pthalate for example).

However, although microanalysis was consistent with the dioxan structure (246), $C_{24}H_{44}O_{10}$, the mass spectrum did not show the expected molecular ion (m/e 516), the highest peak occurring at m/e 403 (which might have been interpreted as an $M+1$ ion). Losses of butanol and butene evident in the spectrum did, however, further confirm the butyl ester groupings. Further, molecular



2,7

weight determinations by osmotic pressure measurements, furnished a molecular weight of 403 ± 5 , and saponification, assuming the presence of 4 ester groupings gave a similar value for the molecular weight. This clearly was inconsistent with the dioxan structure (246).

That the signal at 7.98τ in fact represented the three protons of an acetate grouping (although the integration had suggested the presence of 4 protons) was suggested by a loss of 60 mass units in the mass spectrum, and was established by a selective hydrolysis by heating to 200° in the presence of 3,5-dinitrobenzoic acid to give the corresponding alcohol (ν_{\max} 3550 cm^{-1} , absence of 7.98τ resonance in the n.m.r. spectrum). One spectroscopic feature of interest was the sizable acetylation shift of 0.5 ppm shown by the methylene protons vicinal to the hydroxyl group, a feature which has appeared to have escaped notice until recently.¹⁷⁹

The compound was deduced to be acetyl tributyl citrate (247), $\text{C}_{20}\text{H}_{34}\text{O}_8$ and this was confirmed by comparison of the derived alcohol with tributylcitrate.

This compound was shown to be present in P.V.C. tubing by brief treatment with ether when a substantial quantity of essentially pure plasticiser was obtained. A recent paper¹⁸⁰ also described this compound as a contaminant of various solvents

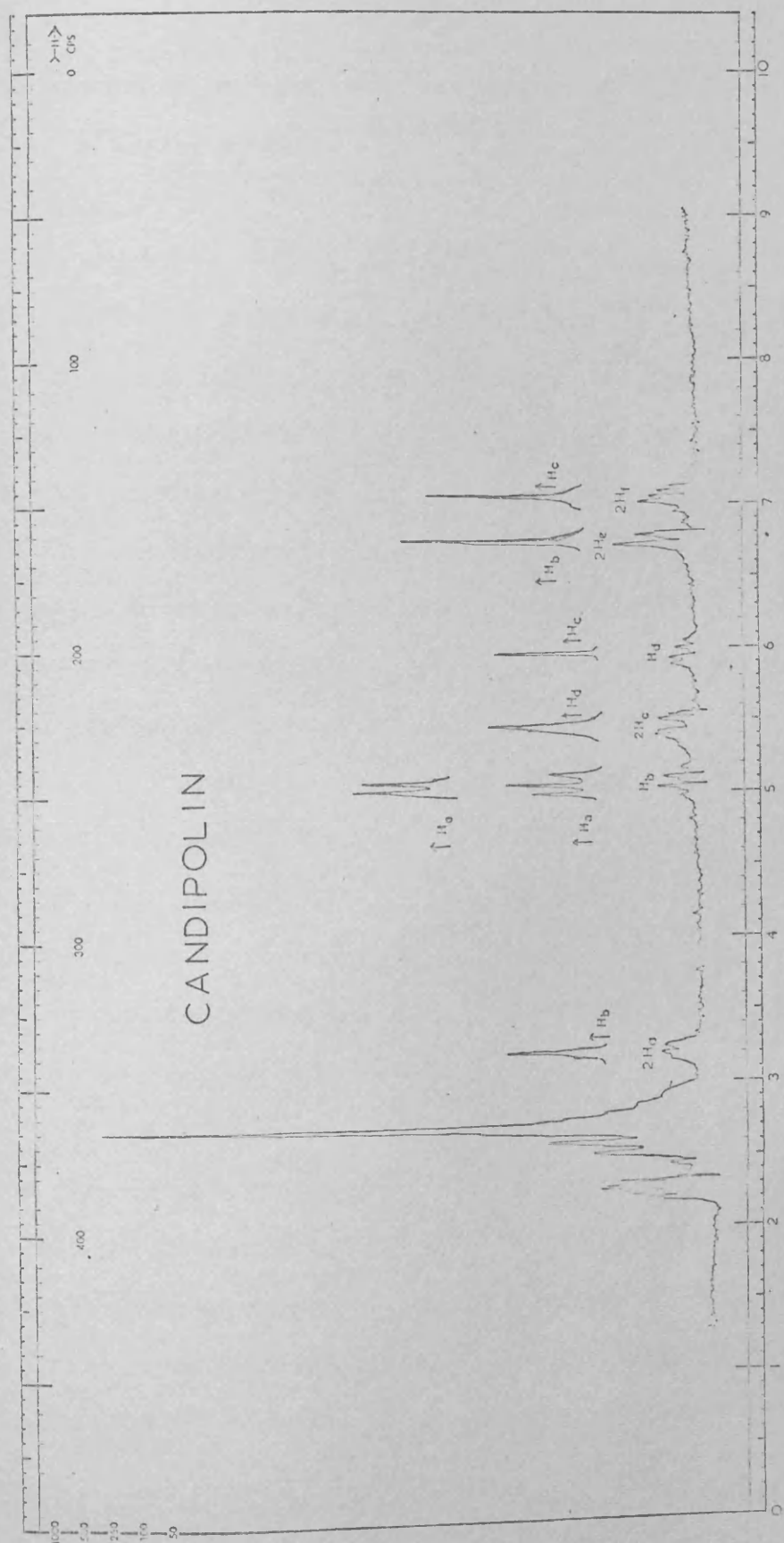
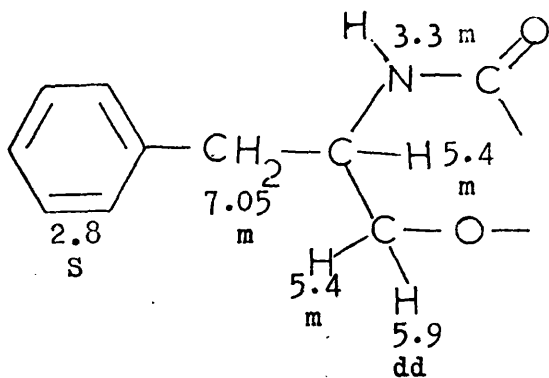
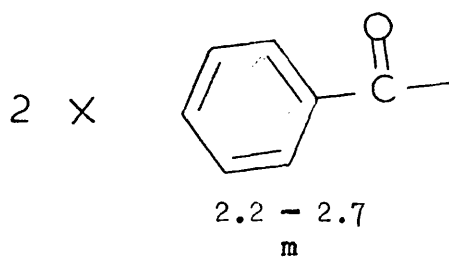
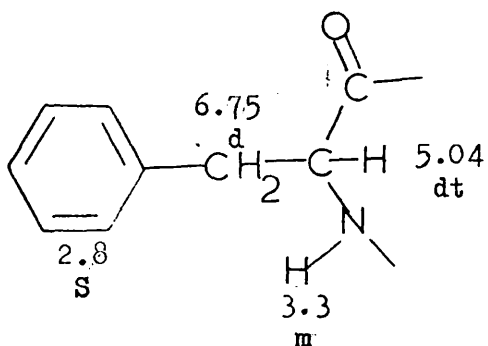


Fig. 27

Diag. 12 Structural features present in the n.m.r. spectrum of candipolin.



in addition to its occurrence in P.V.C. tubing.

(b) Candipolin.

The crystalline metabolite (m.p. 210°) isolated from the chloroform extract of the mycelium was assigned the molecular formula $C_{32}H_{30}N_2O_5$ on the basis of elemental analysis and mass spectrometry. Infrared absorption bands at $3,435\text{ cm.}^{-1}$ ($\epsilon 530$), 1660 cm.^{-1} ($\epsilon 900$) and 1510 cm.^{-1} in chloroform solution suggested the presence of at least one secondary amide group. Absorption at 1742 cm.^{-1} ($\epsilon 400$) was assigned to an ester grouping, while bands at 1600 , 1579 cm.^{-1} indicated the presence of aromatic groups, which appeared to be monosubstituted benzene rings (from bands at 694 cm.^{-1} and 794 cm.^{-1}). In agreement with this were the abundant peaks at m/e 91 and m/e 105 in the mass spectrum which suggested the presence of benzyl and benzoyl groupings.

The n.m.r. spectrum (fig. 27) of candipolin showed the presence of the structural features represented in (diag. 12). These partial structures have the appropriate n.m.r. signal indicated, and sequences of vicinal protons were demonstrated by double irradiation experiments. Exchange of the amide protons ($2H$, m, 3.3τ) did not occur with D_2O but was rapidly achieved using CF_3CO_2D .

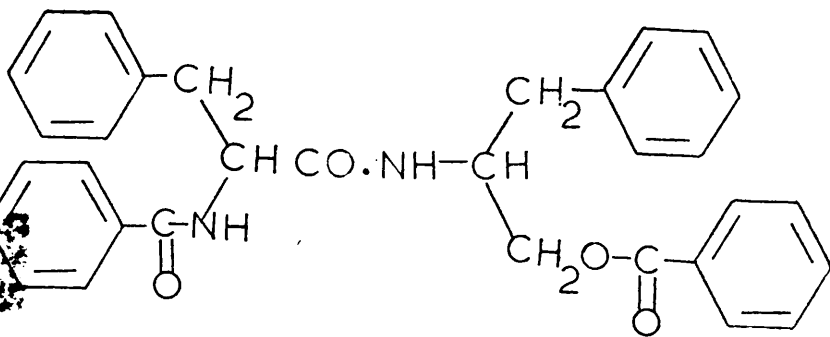
With the knowledge that the molecule possesses two benzoyl groups, and an ester function the most obvious way of combining these partial structures gives the 'peptide' structure (248). The mass spectrum (Table 12) seemed to be in accord with this structure. Loss

Table 12.

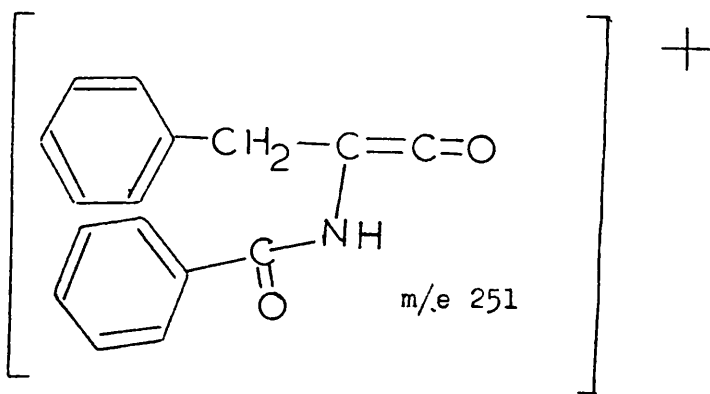
Mass Spectral Data for Candipolin.

Proposed Mass Spectral Breakdown.

<u>m/e</u>	<u>Relative Abundance (%)</u>	
506	0.05	M^+ (m/e 506)
415	0.13	\swarrow $\text{Ph}\cdot\text{CH}_2\text{-C}=\text{C}=\text{O}^+$ (m/e 251) $\text{NH}\cdot\text{CO}\cdot\text{Ph}$ \searrow m/e 415
294	0.20	
269	1.5	
251	3.3	$\text{Ph}\cdot\text{CH}_2\text{-C}=\text{C}=\text{O}^+$ (m/e 251)
223	2.2	$\text{Ph}\cdot\text{CH}_2\cdot\text{CH}=\text{N}^+\text{-COPh}$ (m/e 223)
148	33	$\text{Ph}\cdot\text{CH}_2\cdot\text{CH}=\text{N}^+\text{-COPh}$ (m/e 223) $\downarrow - 28$ m * 95.5 $\downarrow - 77$
147	17	
146	91	$\text{Ph}\cdot\text{CH}_2\cdot\text{CH}^+\text{-N}=\text{C}=\text{O}$ (m/e 148)
118	34	$\text{Ph}\cdot\text{CH}_2\cdot\text{CH}^+\text{-N}=\text{C}=\text{O}$ (m/e 148) $\downarrow - 28$ m * 95.5
91	100	$\text{Ph}\cdot\text{CH}_2\cdot\text{CH}=\text{N}^+$ (m/e 118)
77	72	
64	17	
51	77	



248

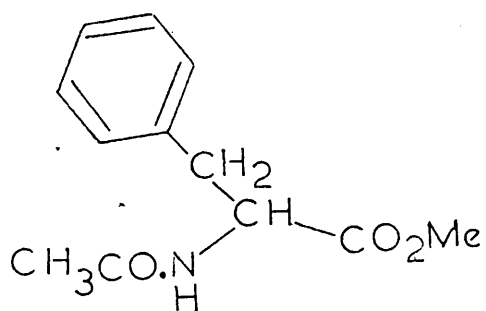


249

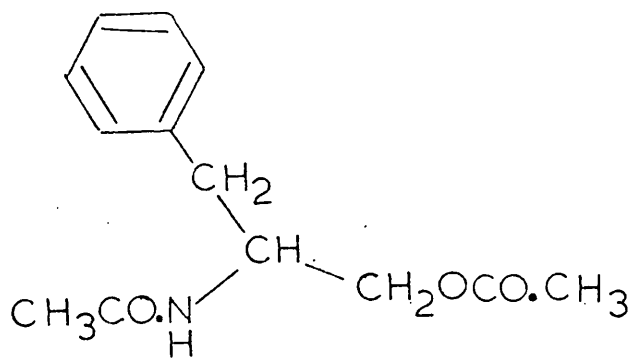
of a benzyl radical from the molecular ion (m/e 506) could account for the ion at m/e 415, while the strong peak at m/e 251 indicated the ion (245) which could arise by cleavage at the peptide bond. Loss of CO from this ion would give the ion at m/e 223.

Hydrolysis of candipolin was achieved by heating with 5N hydrochloric acid in a sealed glass tube¹⁸¹ at 130°. Analytical t.l.c. (using silica gel G) showed the presence of two polar compounds active to ninhydrin, these being identical in R_f and staining to samples of phenylalanine (orange/red on standing) and phenylalaninol (purple/red only on heating). This was further substantiated by methylation followed by trifluoroacetylation¹⁸² of part of the hydrolysate, when two peaks of equal intensity, corresponding to those recorded for authentic samples of bistrifluoroacetyl-phenylalaninol, and trifluoroacetyl-phenylalanine methyl ester, were obtained on g.l.c. (using a 1% N.G.S. column at 140°).

An ether extract of the above hydrolysate gave a crystalline acid, which was purified by dissolving in aqueous sodium bicarbonate and precipitation with hydrochloric acid to give benzoic acid (m.p. 122°) identical in its i.r. spectrum and mixed m.p. to an authentic sample. The remaining amine hydrochlorides, however, could not be separated without prior formation of suitable derivatives. Thus, esterification (using methanol and hydrogen chloride) followed by acetylation gave an oil, which although apparently homogeneous by analytical t.l.c., separated



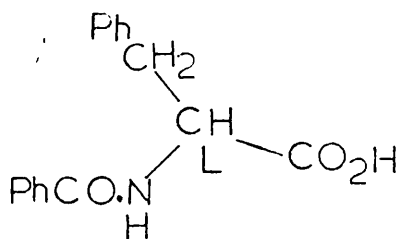
250



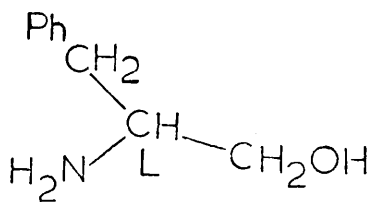
251

into two bands on multiple elution preparative layer chromatography, enabling two crystalline compounds to be isolated. The less polar material, $C_{12}H_{15}NO_3$ (m.p. 90°) was found to be N-acetylphenylalanine methyl ester (250)¹⁸², its i.r. spectrum (KBr) showing a band at 1748 cm.^{-1} due to a carbomethoxyl group, and absorption at 3,300, 1645 and 1527 cm.^{-1} indicative of a secondary amide grouping. The presence of these groups was further confirmed by 3H singlets at 6.20 τ (OMe) and 7.90 τ ($-\text{NH}\cdot\text{CO}\cdot\text{CH}_3$) in the n.m.r. spectrum. The methine proton appeared at 5.05 τ as a 1H double triplet ($J = 7.0, 8.0\text{ Hz.}$) due to coupling with the benzylic methylene protons (6.80 τ) and the amide proton (3.93 τ). This compound was found to be dextro-rotatory, $[\alpha]_D^{25} + 25.0^\circ$ (CHCl_3) and hence the L optical isomer (literature value $[\alpha]_D^{25} + 19.5^\circ$ (MeOH)¹⁸³).

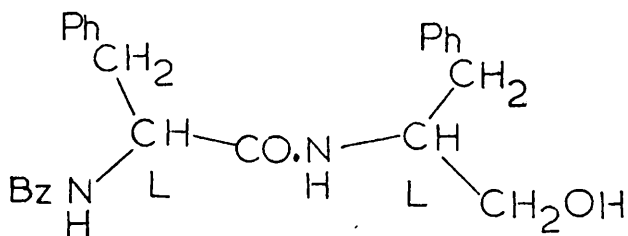
The other compound, $C_{13}H_{17}NO_3$ (m.p. 128°) was found to be N,O diacetyl phenylalaninol (251). Its i.r. spectrum was characterised by a lower ester frequency (1725 cm.^{-1} , $\text{CH}_3\text{CO}\cdot\text{O}$) than that found for the previous compound, the postulated structure being supported by 3H singlets at 7.90 τ ($\text{CH}_3\text{CO}\cdot\text{NH}-$) and 8.07 τ ($\text{CH}_3\text{CO}\cdot\text{O}-$) in its n.m.r. spectrum. Other important features were the 2H doublet ($J = 5.0\text{ Hz.}$) at 5.97 τ assigned to the methylene protons geminal to acetate, and the increase in chemical shift (0.55 p.p.m.) of the methine proton (5.60 τ).



252



253



254

relative to that of acetylphenylalanine methyl ester (250). This compound was found to laevorotatory, and comparison with a synthetic sample of *N,O*-diacetyl-L-phenylalaninol (vide infra), $[\alpha]_D - 55.5^\circ$ (CHCl_3), proved it also to be the L optical isomer.

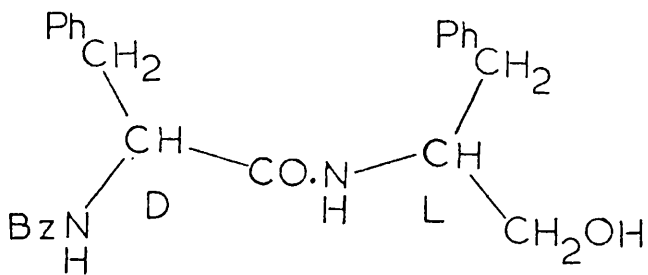
Hence, candipolin appeared to be derived from one molecule each of L-phenylalanine and L-phenylalaninol and two molecules of benzoic acid. Since peptide synthesis is well documented, the total synthesis of this molecule seemed a comparatively easy task, and the general approach using coupling of L-benzoylphenylalanine (252) with phenylalaninol (253) to give the peptide alcohol (254) was employed. In fact the use of a benzoyl protecting group introduced difficulties, although a successful route was eventually achieved.

Minor problems were also encountered in the apparently straightforward synthesis of the starting material. Thus the protected amino acid (252) could not be prepared by treating phenylalanine with benzoyl chloride in pyridine possible due to mixed or benzoic anhydride formation. However, by acid hydrolysis of the easily prepared benzoylphenylalanine ethyl ester (m.p. 103°) $\nu_{\text{max.}} 1752 \text{ cm.}^{-1}$ (CO_2Et), 1645 and 1525 cm.^{-1} (amide), needle crystals (m.p. 141°) of benzoylphenylalanine (252) were obtained. Its structure was supported by strong i.r. absorption at 1705 cm.^{-1} due to the presence of the carboxylic acid, and apart from the absence of ester group resonances the similarity of the n.m.r. spectrum to that of the original

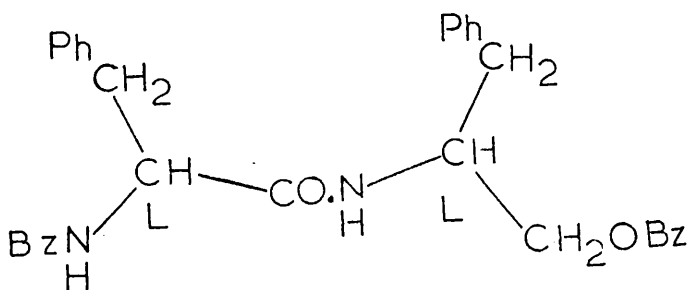
ester.

Phenylalaninol (253), the other compound essential for the synthetic scheme, is reported¹⁸⁴ to be formed in high yield by reduction of phenylalanine ethyl ester by sodium borohydride in ethanol via participation of the amino group. In our hands, however, under varied conditions, only indifferent yields of the desired alcohol were obtained with hydrolysis of the ester group the predominant reaction. It was more convenient to effect reduction using lithium aluminium hydride which gave a high yield of optically pure phenylalaninol (253). (It seems probably that the racemisation previously reported for this reaction¹⁸⁵ resulted from the alkaline conditions used in the work-up). This material lacked any carbonyl absorption, but strong i.r. peaks at 3,325 and 3,295 cm.^{-1} together with broader absorption at 3,200 cm.^{-1} was in accord with the presence of both amine and alcohol functions. Confirmation of its structure was obtained by preparation of the diacetyl derivative, which had identical physical properties to those reported above for one of the products of the hydrolysis of candipolin.

Preliminary attempts at coupling benzoylphenylalanine and phenylalaninol using the acid chloride¹⁸⁶ or mixed anhydride techniques¹⁸⁷ were totally unsuccessful. The former method, however, has been shown to be unreliable¹⁸⁸, and the latter, depending on the competitive action of two different acyl radicals, has also been criticised¹⁸⁹. A partially successful route was achieved using the active ester method¹⁹⁰



255



256

whereby the *p*-nitrophenyl ester of benzoylphenylalanine (252) was allowed to react with phenylalaninol in an inert solvent. Although exceptionally clean this reaction produced two products of similar R_f which were eventually shown to be isomeric alcohols of the general structure (254), but epimeric at the asymmetric centre present in L-benzoylphenylalanine (252). Since the *p*-nitrophenyl ester was optically inactive it appeared that racemisation had occurred on treating benzoylphenylalanine with *p*-nitrophenol in the presence of dicyclohexylcarbodiimide (D.C.C.). Although this reagent has been reported to cause racemisation¹⁹¹, the above result at first seemed unusual since the corresponding active esters of carbobenzoxy protected amino acids have been prepared in high optical purity¹⁹⁰. It was, however, suspected that the benzoyl protecting group used in our case was not as efficient as the more usual carbobenzoxy, presumably because the former is more electronegative, and it has been reported¹⁹² that benzyloxycarbonyl - L -leucine retains some of its optical activity even after several days in acetic anhydride with sodium hydroxide, whereas benzoyl-L-leucine is completely racemised after a few hours.

The above difficulty was circumvented by applying the azide coupling technique¹⁹³ which is generally exceptional in avoiding racemisation. Thus the hydrazide of L-benzoylphenylalanine was prepared from the corresponding ethyl ester by treatment with hydrazine hydrate, and converted into the required azide using nitrous

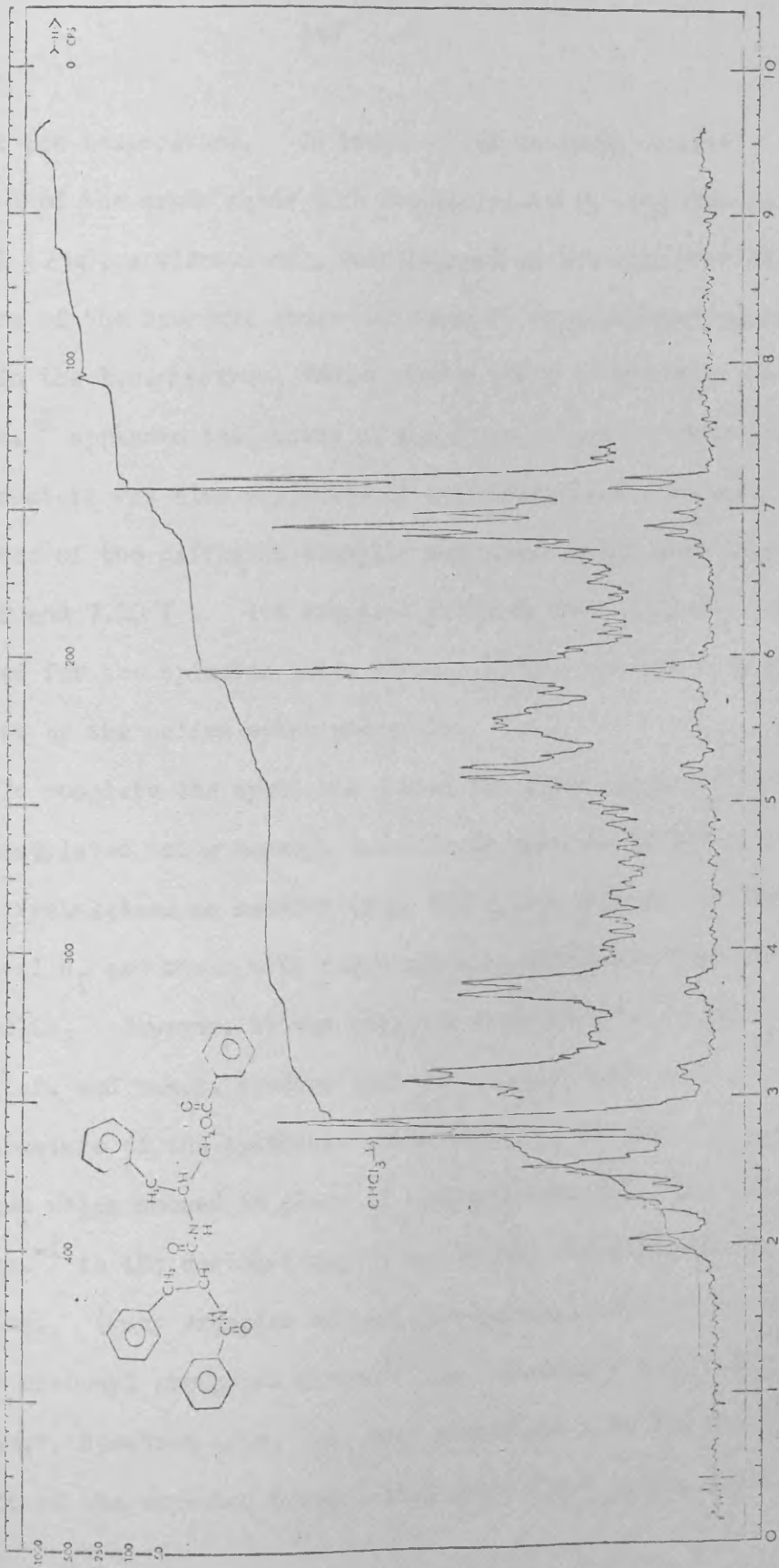
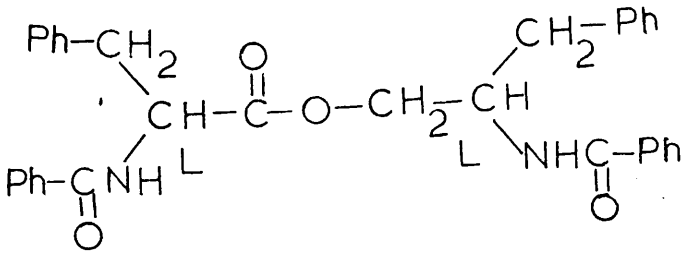
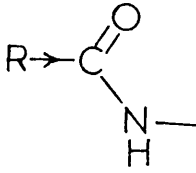
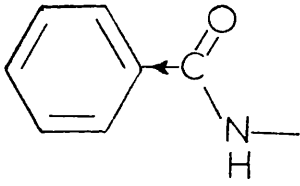


Fig. 28

acid at ice temperature. On treatment of an ethyl acetate solution of the crude azide with L-phenylalaninol (253) the amide alcohol (254), a viscous oil, was obtained as the sole product. The presence of the hydroxyl group was shown by broad absorption centred at $3,450\text{ cm.}^{-1}$ in the i.r. spectrum, while strong, sharp absorptions at 1655 and 1630 cm.^{-1} appeared indicative of two types of amide functions. Its structure was also supported by its n.m.r. spectrum where the resonance of two different benzylic methylene groups were apparent at 6.7δ and 7.20τ . Its spectral features were similar to those recorded for the epimeric amide alcohol (255) which had been previously prepared by the active ester procedure.

To complete the synthetic scheme the amide alcohol (254) was benzoylated using benzoyl chloride in pyridine to give a compound which crystallised as needles (m.p. 208°) from ethanol, and which had an identical R_f and stain with ceric ammonium nitrate to that observed for candipolin. However, it was only too apparent from a comparison of their i.r. and n.m.r. spectra that the two compounds were not identical. The structure of the synthetic amide (256) was validated by its i.r. spectrum which showed in place of hydroxyl absorption new peaks at 1722 and 1710 cm.^{-1} in the carbonyl region due to the formation of the benzoate grouping. (Many examples of multiple carbonyl absorptions due to a single carbonyl group are known¹⁹⁴). Similarly the proton resonances in its n.m.r. spectrum (fig. 28) were consistent with its structure and showed the expected benzoylation shift (0.6 p.p.m. downfield) for



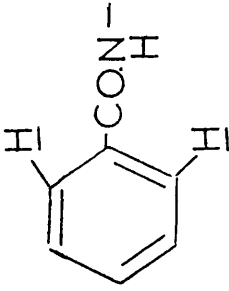
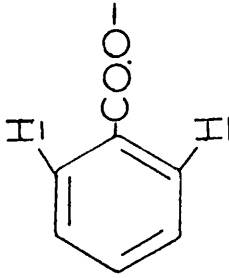
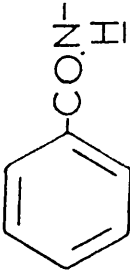
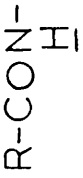
the methylene protons which were geminal to hydroxyl and now appeared at 5.88 τ .

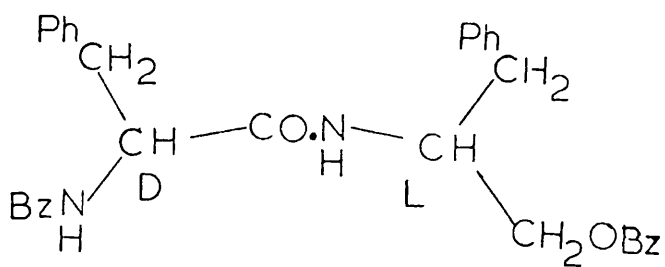
Critical resonances in the above n.m.r. are, however, those assigned to the aromatic and amide protons. The synthetic benzoylated amide (256) showed two 2H narrow multiplets centred at 2.00 and 2.28 τ , which were assigned to the ortho protons of the benzoate and benzamide groups respectively, these protons being deshielded by lying in the plane of the carbonyl group. In the n.m.r. of candipolin, however, only a single 4H narrow multiplet at 2.27 τ was apparent (cf. fig. 27), suggesting that the two sets of ortho protons were attached to similar (benzamide) groups. Also the synthetic amide (256) exhibited two 1H multiplets at 3.16 and 3.75 τ assigned to the protons of aromatic and 'aliphatic' amide groups respectively. This effect was attributed to the greater acidity of the aromatic and amide proton because of the weak - I affect expected of a benzene ring compared to the + I affect of the alkyl group in an aliphatic amide (diag. 13). Candipolin, however, again showed only a narrow 2H multiplet at 3.3 τ indicating that its two amide protons were of the same type and probably benzamide protons.

The above evidence suggested that candipolin had the benzoylated ester structure (257). In agreement with this was the low benzoate frequency (ca. 1720 cm.^{-1}) shown in the i.r. spectrum of the synthetic peptide which indicated that the 1742 cm.^{-1} band in the i.r. spectrum of

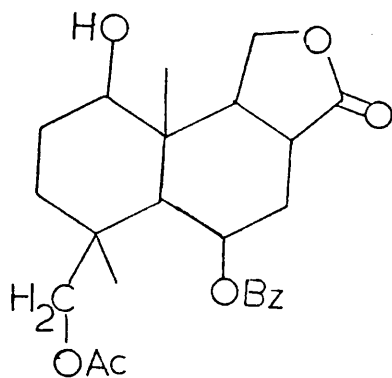
Table 13

τ value (Integration) of Proton(s) indicated.

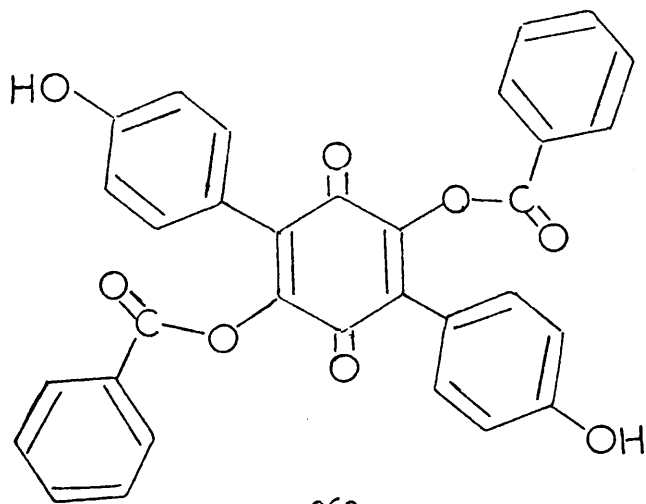
COMPOUND				
Candipolin (257)	2.27 (4H)	-	3.30 (2H)	-
Benzoylated amide (256)	2.28 (2H)	2.00 (2H)	3.16 (1H)	3.75 (1H)
Isomeric benzoylated amide (258)	2.35 (2H)	2.00 (2H)	3.26 (1H)	3.68 (1H)
N,O-Dibenzoyl phenylalaninol	2.30 (2H)	1.98 (2H)	3.43 (1H)	-
N-Benzoyl phenylalaninol	2.30 (2H)	-	3.20 (1H)	-
N-Benzoyl phenylalanine	2.27 (2H)	-	3.20 (1H)	-
ethyl ester				
Acetyl phenylalanine	-	-	-	3.93 (1H)
methyl ester (250)				
Diacetyl phenylalaninol (251)	-	-	-	4.30 (1H)



258



259



260

candipolin (which had been assigned to this type of grouping) was in fact more in accord with a saturated ester. To substantiate the above assignments it was necessary to examine the relevant physical features of other *N*- and *O*-benzoylated derivatives. Thus the carbonyl absorption of the benzoate group in the i.r. spectra of the epimeric synthetic amide (258) and dibenzoylphenylalaninol again occurred ca. 1720 cm.^{-1} providing corroborating evidence for this group frequency. Moreover, more concrete structural evidence was obtained from a comparison of the chemical shift values of the critical protons (Table 13). This confirmed that the typical value for the ortho protons of a benzoate group was ca. $2.0(\pm 0.1)\tau$ while the corresponding protons of a benzamide group occurred at ca. $2.3(\pm 0.1)\tau$. Similarly it was apparent that the amide proton of a benzamide group had a characteristic chemical shift value of ca. 3.3τ while that of a peptide bond was ca. 3.7τ with slightly higher values for 'aliphatic' secondary amide protons. This data thus completely supported the 'ester' structure of candipolin.

This metabolite is hence interesting because of the unexpected ester linkage between the molecules of phenylalanine and phenylalaninol and the presence of benzoyl residues. Thus, although benzoyl esterases¹⁹⁵ are known in fungal systems, they are relatively rare. Secondary metabolites known to possess this feature are pebrolide (259), a sesquiterpene benzoate from Penicillium brevicompactum¹⁹⁶ and

aurantiacin (260)¹⁹⁷. L-phenylalaninol itself has been also reported to be a constituent of another fungal metabolite, a biologically active polypeptide, anti amoebin, from Emericellopsis poonensis.¹⁹⁸

Attempts to synthesise candipolin (257) from benzoyl-L-phenylalanine (252) and N-benzoyl-L-phenylalaninol using normal peptide coupling techniques were unsuccessful. The formation of an ester bond, however, requires much stronger activation than for peptide bonds and this problem has been encountered in the synthesis of the cyclododecadeptide valinomycin, which contains two amino acids (L-valine and D-valine) and two hydroxyacids (D- α -hydroxyisovaleric acid and L-lactic acid) arranged in a 36 membered ring regularly alternating between amino and hydroxy acids¹⁹⁹. In this case the ester linkages were eventually formed in good yield by using N,N-carbonyldiimidazole (CDI)²⁰⁰ as coupling reagent. Preliminary experiments using this reagent for the coupling of benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol have proved promising, and the synthesis of the ester (257) is at present in progress.

EXPERIMENTAL

7

Melting points were determined by a Fisher melting apparatus and are uncorrected, boiling points are uncorrected. Elemental analyses were obtained on a Micro 2000 automatic microanalyzer. Infrared and spectra were recorded with a Perkin-Elmer 521 instrument and a Perkin-Elmer 200 spectrophotometer. Mass spectra were recorded with a Perkin-Elmer 215 instrument.

EXPERIMENTAL.

Elemental analyses listed in Table I were obtained on a Perkin-Elmer 2000 instrument. Infrared spectra were obtained with a Perkin-Elmer 521 instrument. Mass spectra were obtained with a Perkin-Elmer 215 instrument. Boiling points were obtained on a Fisher boiling point apparatus. Melting points were obtained on a Fisher melting point apparatus. All reagents were of the highest quality available and used without further purification unless otherwise stated. Solvents were dried and distilled under reduced pressure immediately before use.

Preparation of 2,4-Dinitrophenol

2,4-Dinitrophenol was prepared from phenol by the method of H. G. W. Clarke, J. Chem. Soc. 1925, 1031. Phenol (100 g, 0.93 mol) was dissolved in 200 ml of concentrated sulfuric acid and cooled to 0°C. Nitric acid (100 g, 0.93 mol) was added and the mixture stirred for 24 hours. The mixture was poured into water and the solid product washed with water. The solid was dried and purified by recrystallization from water. Yield 85%.

EXPERIMENTAL.INSTRUMENTATION

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected, boiling points are uncorrected. Ultraviolet spectra were obtained on a Unicam S.P. 800 recording spectrophotometer. Infra red spectra were measured with a Unicam S.P. 200 instrument and a Perkin Elmer 237 spectrometer. Nuclear magnetic resonance spectra were recorded with a Perkin-Elmer R 10 60 Mc/s spectrometer and with a Varian HA-100 Mc/s spectrometer. Unless otherwise stated all values quoted are recorded in chloroform with tetramethylsilane as internal standard. Mass spectra were obtained with an A.E.I. M.S. 12 mass spectrometer. Analytical gas-liquid chromatography was performed on a Pye Argon chromatograph, while combined mass spectrometry - gas chromatographic determinations were obtained from an LKB-9000 instrument. Optical rotations, in methanol (unless otherwise stated) were measured on a Hilger and Watts microptic photoelectric polarimeter.

Thin Layer Chromatography.

R_f values were determined from elution on 0.25 mm. layers of Kieselgel G, the compounds being located by spraying with ceric ammonium nitrate/sulphuric acid (1% in 10%) and heating. The colour

observed for a particular compound is given in brackets immediately after its R_f value. The dyes sudan yellow and p-aminoazobenzene were used to standardise all R_f values. The R_f values of these dyes in the solvents employed are given below :

<u>Solvent</u>	<u>p-aminoazobenzene</u>	<u>sudan yellow</u>
100% CHCl_3	0.51	0.62
1% MeOH in CHCl_3	0.90	-

GENERAL.

Diazomethane was prepared by the method of Moore and Reed²⁰¹ from bis (N-methyl-N-nitroso)-terephthalimide. All organic extracts were dried over anhydrous magnesium sulphate and solvents were removed using a rotary film evaporator. 'Light petroleum', unless otherwise stated, refers to light petroleum b.p. 60-80°.

Concentrations for optical measurements are given in g./l.

The following abbreviations are used in reporting spectral data: S, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br., broad; and infl., point of inflexion.

In the following typical description of n.m.r. data

5.6 (1H.m, irr 2.7 \rightarrow d ($J=2$), irr 4.3 \rightarrow t ($J_1=2$, $J_2=3$), H-4), irradiation at 2.7 τ has resulted in the collapse of the 1H multiplet at 5.6 τ to a doublet $J=2\text{Hz.}$, etc.

Sat. $-\text{CH}_2-$ refers to protons of the type indicated in $\text{X}-\text{CH}_2-\underline{\text{CH}_2}-\text{CH}_2-\text{Y}$

where X and Y can be either hydrogen or carbon atoms.

REFERENCES

1. J. H. Goldstein, *Journal of Polymer Science*, **10**, 1 (1953).

The data used for describing polymer systems are as follows: the first number refers to the number of units of $\frac{1}{2}$, the second number refers to the number of units of $\frac{1}{2}$, the third number refers to the number of units of $\frac{1}{2}$, the fourth number refers to the number of units of $\frac{1}{2}$, the fifth number refers to the number of units of $\frac{1}{2}$, the sixth number refers to the number of units of $\frac{1}{2}$, the seventh number refers to the number of units of $\frac{1}{2}$, the eighth number refers to the number of units of $\frac{1}{2}$, the ninth number refers to the number of units of $\frac{1}{2}$, the tenth number refers to the number of units of $\frac{1}{2}$.

2. J. H. Goldstein, *Journal of Polymer Science*, **10**, 1 (1953).

The data used for describing polymer systems are as follows: the first number refers to the number of units of $\frac{1}{2}$, the second number refers to the number of units of $\frac{1}{2}$, the third number refers to the number of units of $\frac{1}{2}$, the fourth number refers to the number of units of $\frac{1}{2}$, the fifth number refers to the number of units of $\frac{1}{2}$, the sixth number refers to the number of units of $\frac{1}{2}$, the seventh number refers to the number of units of $\frac{1}{2}$, the eighth number refers to the number of units of $\frac{1}{2}$, the ninth number refers to the number of units of $\frac{1}{2}$, the tenth number refers to the number of units of $\frac{1}{2}$.

Received

March 1954

EXPERIMENTAL.CHAPTER 1.Nomenclature used in Activity Assay Results.

The code used for describing activity assay results is as follows : the first number refers to the dilution i.e. 1 is a dilution of $\frac{1}{2}$, 2= $\frac{1}{4}$, 3= $\frac{1}{8}$, etc. A fungistatic effect is indicated by the presence of a first figure which is the lowest dilution at which no germination or germination less than 1% is observed. The letter S indicates stunting and the following number the lowest dilution at which this occurs. Thus 35S indicates no germination at the lowest dilution of $\frac{1}{8}$, and that stunting occurs at a dilution of $\frac{1}{32}$.

Growth and Extraction of the Mold²⁰²

Penicillium canadense (Commonwealth Mycological Institute No. 95, 493) was subcultured onto 2% malt agar slants and thence to agar seed bottles (15 x 9 cm.). A spare suspension, prepared from 12 such bottles and distilled water (2l.), was used to inoculate 100 Roux surface culture bottles which had previously been sterilised (0.5 hours with steam at 242°F and 12 p.s.i.) and each contained 200 ml. of the following medium :

Glucose	50g.
Ammonium tartate	2.8g.

Dipotassium hydrogen phosphate	5.0g.
Magnesium sulphate	1.0g.
Sodium chloride	1.0g.
Difco yeast extract	0.5g.
FeSO ₄ ·7H ₂ O (0.1g), CuSO ₄ ·5H ₂ O (0.015g), ZnSO ₄ ·7H ₂ O (0.05g), MnSO ₄ (0.01g), 1 ml Na ₂ MoO ₄ (0.01g), 100 ml distilled water.	

Distilled water was added to make 1 litre solution.

Cultures were allowed to grow undisturbed at 25° and 70% relative humidity, artificial illumination being provided by Mazda fluorescent tubes for approximately 8 hours per day. After the prescribed period of growth had elapsed, the mycelial mats were separated from the broth, dried at 40°, powdered in a mortar and subjected to soxhlet extraction with chloroform for 24 hours. The pH of the broth was adjusted to 7 (recorded on a pH meter) and in one procedure extracted with activated charcoal (10g. per litre of broth) at room temperature for 24 hours. Thereafter, the charcoal was filtered off and extracted in a soxhlet for 24 hours with acetone. In the later procedure the broth was subjected to a constant liquid-liquid extraction with ethyl acetate for 24 hours.

Activity Assays

Initial assays for antifungal activity using the serial dilution

Table 14. Initial Activity Assays on the Culture filtrate of P. canadense.

Fungus	Days of Growth		
	6	12	18
P. canadense	559	457	154

Table 15. Activity assays of a typical growth period of P. canadense (Large Scale Production).

	Days of Growth		
	3	6	10
	359	5510	5511

spore germination test on the conidia of Botrytus alii were performed on replicate cultures of the fungus. After intervals of 6, 12 and 18 days growth, the broth of each medium was filtered through sterile No.1 Whatman paper and the crude filtrates were used for the assays (Table 14). From this data it appeared that the optimum time for antibiotic production was 10 days and hence cultures were harvested after this time.

Large scale production on groups of cultures (100 Roux bottles) were then carried out. Assays for antibiotic activity at intervals throughout the growth period of 10 days gave the results shown in (Table 15).

Similar assays performed on the mycelial extracts had shown only very weak activity.

Chromatographic Separation of the P. Canadense Metabolites .

The dry broth extracts (5.4 g. from 1 l. broth) were dissolved in chloroform, silicic acid 10g. added and the solvent removed on a rotary film evaporator. The silicic acid with the adsorbed organic material was allowed to dry thoroughly at room temperature, finely powdered and introduced onto a column of silicic acid (200g). Separation was achieved by elution of the column with petrol containing increasing percentages of chloroform (10,20,30, etc. % of chloroform). Fractions were collected at 15 minute intervals (50ml.). Table 16 exhibits a typical fractionation distribution, these quantities of metabolites are only approximate, since there is always a variation in the relative proportions

TABLE 16

Summary of Chromatographic Separation of the P. canadense Metabolites.

<u>Fractions:</u>	<u>Eluting Solvent:</u>	<u>Weight:</u>	<u>Constituents:</u>
1 - 10	100% Light Petroleum ---	0.05g	non polar oils
	30% Light Petroleum/20% Chloroform		
11 - 18	80% Chloroform/20% Light Petroleum	0.6g	canadensolide, dihydrocanadensolide
19	85% Chloroform/15% Light Petroleum	0.05g	canadensolide, dihydrocanaden- solide, canadensic acid.
20 - 24	85% Chloroform/15% Light Petroleum	1.4g	canadensic acid.
25 - 29	90% Chloroform/10% Light Petroleum	0.2g	canadensic acid, hydroxyisocanadensic acid.
30 - 60	100% Chloroform-100% ethylacetate	ca.0.1g	minor metabolites.

produced by the fungus.

Fractions 11-18 were combined and the mixture separated by preparative layer chromatography on a Kieselgel HF₂₅₄ plate (1m x 20 cm x 0.75 cm) using multiple elution with 80% chloroform/20% petroleum ether. The less polar band containing dihydrocanadensolide was detected using an iodine spray reagent. Elution of this band with ethyl acetate gave pure dihydrocanadensolide (200 mgs), which crystallised as needles (m.pt. 94.5°) from benzene/light petroleum. The band corresponding to canadensolide could be detected by its pale blue fluorescence under u.v. light (254 nm.) and its staining with iodine. Canadensolide was obtained as an oil (250 mg.) on eluting this band with ethyl acetate. It crystallised as needles from ether/light petroleum (m.pt. 50°) on leaving at ice temperature.

The more polar fractions (20-24) were crystallised from benzene/light petroleum mixtures to give canadensic acid. This was purified by dissolving in ice-cold freshly prepared aqueous sodium bicarbonate solution and then immediately reprecipitated with ice cold dilute 5N hydrochloric acid. The filtered solid was washed with water and recrystallised (after drying).

The later polar fractions (25-29) contained varying concentrations of canadensic acid and hydroxyisocanadensic acid which resulted in jelly formation on attempted crystallisation. Pure samples of this were obtained by very careful partial crystallisation from ether/light petroleum of fractions which contained essentially pure hydroxyisocanaden-

sic acid. It could also be obtained by subjecting a chloroform solution (200 ml.) of crude extract (20g.) to one extraction of a saturated freshly prepared solution (20ml.) of sodium bicarbonate. Acidification of this aqueous solution at ice temperature with 5N hydrochloric acid gave a precipitate of hydroxyisocanadensic acid (0.8g.), which was crystallised as needles from ether/light petroleum.

Continued elution of the column with chloroform containing increasing concentrations of ethyl acetate (0,10,20 % etc.) gave reddish oils, which consisted of several polar materials. (Fr30 -). Minor quantities of pure metabolites were obtained from these.

Canadensolide (51)

The antibiotic was isolated as previously described as an oil which crystallised from ether-light petroleum as colourless needles m.p. 50°.

$$[\alpha]_D - 141^\circ \text{ (C 0.86).}$$

O.R.D. in methanol (C 0.57): $[\Phi]_{400} - 90$; $[\Phi]_{250} - 2,860$;

$[\Phi]_{280} - 21,700$; $[\Phi]_{239} - 35,400$.

T.l.c. 0.50, CHCl_3 (yellow-brown).

i.r. ν_{max} (KBr.): 1764 (γ -lactone), 1666 (C=C), 1185, 1060, 959, 918, 908 cm^{-1} .

ν_{max} ((1.46 mM CHCl_3): 1775 cm^{-1} (ϵ 1320, $\Delta \nu_{\frac{1}{2}}$ 23 cm^{-1})
1663 cm^{-1} (ϵ 73).

u.v. $\lambda_{\text{max.}}$ (EtOH): 213 nm. (ϵ 11,700).

n.m.r. 100 Mc/s (CDCl₃) τ : 3.58 (1H, d, J = 2.5 Hz., H-11c),
3.97 (1H, d, J = 3.0 Hz., H-11t), 4.88 (1H, dd, J = 6.5,
4.5 Hz., H-3), 5.38 (1H, m, J = 6.2, 4.5 Hz., H-4), 6.05
(1H, dd, J=6.5, 2.0 Hz., H-2), 8.08 (2H, m, 2H-5),
8.5 (4H, m, sat. -CH₂-), 9.05 (3H, t, J = 6.0 Hz., CH₃-CH₂).

m.s. m/e (Rel abundance): M⁺ 210 (0.5), 182 (1.5), 166 (3),
153 (2), 151 (3), 137 (7), 123 (18), 109 (19), 110 (40),
98 (67), 96 (100), 85 (22), 69 (31), 68 (54), 67 (21),
57 (16), 55 (23).

Analysis Found : C, 62.84; H, 6.64%; M⁺ at m/e 210.

C₁₁H₁₆O₄ requires C, 62.85; H, 6.71%; M.W. 210.

Ozonolysis of Canadensolide (51).

A stream of ozonised oxygen was passed through a solution of canadensolide (150 mgs.) in glacial acetic acid (15 ml.) for 1½ hours. 2 N hydrochloric acid (6 ml.) was added and the solution was left at R.T. for 2 hours. After the addition of more acetic acid (6mls.), the solution was stirred overnight.

The solution was steam distilled into an ethanolic solution of dimedone (40 mgs. in 10 ml.). After heating for a few minutes on a hot plate, the distillate was allowed to cool. The white solid obtained was crystallised from ethanol-water as needles m.p. 191° (30 mg., 14%). The melting point remained unchanged on admixture with

an authentic sample of formaldehyde dimedone.

Cleavage of Canadensolide (51) with Sodium Metaperiodate.

(a) Canadensolide (25mg.) in methanol (1ml.), and 3 mls. 0.1N sodium hydroxide, was refluxed for $\frac{1}{2}$ hour. The cooled solution was diluted to 15 ml. with water and adjusted to pH 8.5. Sodium metaperiodate (35mg) was added. A chloroform solution containing 2,4-dinitrophenylhydrazine (25mg. in 10ml.) was stirred vigorously with the aqueous solution overnight.

The chloroform layer was separated, washed with water (2x10 ml.), dried and evaporated. P.l.c. of the resulting oil gave a solid, which crystallised as needles from ethanol-water, m.p. 106° (4mg., 13%). The melting point was undepressed on admixture with an authentic sample of n-valeraldehyde dinitrophenylhydrazone.

(b) ⁵⁴ Canadensolide (21mg.) was treated with potassium hydroxide (13.5 mg.) in water (1ml.) and gently warmed for a few minutes. Sodium metaperiodate (22 mg.) in the minimum volume of water was added dropwise with stirring. A white precipitate formed rapidly and stirring was continued for 4 hours. The aqueous solution was then filtered and the filtrate carefully steam distilled. The distillate was examined by g.l.c. using an Aerograph 200 instrument and two separate columns. Column (1): 20% Carbowax 1500 on 60/80 Cromosorb W.,

TABLE 17

Column	Degraded	Synthetic	Combined	Product	n-Valer-	n-Pentanol
	n-Valer- aldehyde	n-Valer- aldehyde	Injection	Reduced	aldehyde	
d.n.p. 60°	R _t 8.8 min.	R _t 8.9 min.	R _t 8.9 min.	R _t 23.1 min.	R _t 22.8 min.	R _t 23.0 min.
					Reduced	
Carbowax 60°	R _t 2.0 min.	R _t 2.0 min.	R _t 2.0 min.	R _t 13.8 min.	R _t 13.8 min.	R _t 14.0 min.

5' x $\frac{1}{8}$ " . Column temperature 60°, detector temperature 170°, injection temperature 120°: Flow rate 20 ml./min. N₂ 58 p.s.i. and H₂ 18 p.s.i. Column (2): 15% D.N.P. on 60/80 Chromosorb W., 6' x $\frac{1}{8}$ " (Column temperature 60°, detector temperature 120°: Flow rate 18 ml./min., N₂ 58 p.s.i., and H₂ 18 p.s.i.)

On both columns the n-valeraldehyde present in the distillate had the same retention time as an authentic sample (Table 17).

The walls of a 1ml. syringe were coated with a 50% aqueous solution of sodium borohydride. Vapour samples (0.2ml.) from the distillate were introduced into the syringe and allowed to react with the borohydride for 10 minutes. Subsequent g.l.c. examination of this vapour on the two columns showed one peak corresponding in retention time to n-pentanol (Table 17). The vapour from an aqueous solution of an authentic sample of n-valeraldehyde was similarly treated with borohydride, g.l.c. examination showing complete reduction to pentanol (Table 17).

Dihydrocanadensolide (54)

This compound was isolated as described earlier and crystallised from benzene-light petroleum as colourless needles m.p. 94.5 - 95°.

$[\alpha]_D - 31^\circ$ (C 0.53).

O.R.D. in methanol (C 0.52): $[\Phi]_{400}$; - 540; $[\Phi]_{274}$, - 890;

$[\Phi]_{236}$, -3,860; $[\Phi]_{215}$, +6,110.

T.l.c. 0.52, CHCl₃ (purple-brown).

i.r. ν max. (KBr.): 1766 (lactone), 1292, 1185, 1068, 958, 916, 908 cm.^{-1} .

ν max. (0.78 mM CHCl_3): 1783 cm.^{-1} (ϵ 925), 1789 cm.^{-1} (shoulder) (ϵ 850).

u.v. λ max. (EtOH): no absorption > 200 n.m.

n.m.r. 100 Mc/s (CDCl_3) τ : 4.85 (1H, dd, $J=6.0$, 4.0Hz., H-3), 5.44 (1H, m, $J=7.0$, 4.0Hz., H-4), 6.83 (1H, dd, $J=6.0$, 1.0Hz., H-2), 7.06 (1H, dq, $J=8.0$, 1.0Hz., H-10), 8.2 (2H, m, 2H-5), 8.5 (4H, m, sat. CH_2), 8.53 (3H, d, $J=8.0$ Hz., 3H - 11), 9.06 (3H, t, $J=6.0$ Hz., $\text{CH}_3\text{-CH}_2$).

m.s. m/e (Rel. abundance): M^+ 212 (5), 166 (4), 155 (3), 153 (4), 139 (4), 125 (10), 112 (12), 111 (7), 99 (21), 98(100), 70 (34), 69 (71), 57 (11), 56 (11), 55 (16), 41 (42).

Analysis Found : C, 62.49; H, 7.31%; M^+ at m/e 212.

$\text{C}_{11}\text{H}_{16}\text{O}_4$ requires C, 62.25; H, 7.60%; M.W. 212.

Catalytic Reduction of Canadensolide (51) (Dihydrocanadensolide) ⁵⁴.

Canadensolide (21mg.) in ethanol (3ml.) was shaken overnight with 5% palladium-on-charcoal (10mg.) in an atmosphere of hydrogen. The catalyst was separated on glass paper and the solvent removed in vacuo to yield an oil (23mg.) T.l.c. employing chloroform as eluant showed two spots more polar than the starting material.

ν max. (thin film): 3,600 - 3,200, 1780 - 1720 cm.^{-1} .

τ values (CDCl_3): 3.95 (OH, b), 4.9 (1H, m), 5.4 (1H, m)

6-7 (2H, m).

Methyl doublets ($J = 6 \text{ Hz.}$) of approximately equal relative intensity at 2.55, 8.65 and 8.68 τ . This mixture was treated with Jones reagent (0.2ml.) for 20 minutes, diluted with water (2ml.) and extracted with ether. The organic extract was washed with bicarbonate, water, and dried, to give an oil which was separated by preparative t.l.c. on one Kieselguhr HF₂₅₄ plate (20 cm. x 20 cm. x 0.7 mm.) and elution with chloroform. The major band (R_f 0.45) was extracted from the silica by elution with chloroform, removal of the solvent affording a colourless solid which crystallised from benzene - light petroleum as colourless needles m.p. 89-93 $^{\circ}$, (4mg., 20%). These needles had identical i.r., m.s., and t.l.c. properties as naturally occurring dihydrocanadensolide (54).

Canadensic Acid: Mixture of C-2 Epimers (55).

This substance was isolated as described earlier and crystallised from benzene-light petroleum as colourless needles m.p. 130 $^{\circ}$.

$[\alpha]_D$ in methanol, +37.3 $^{\circ}$ (C 1.06).

O.R.D. in methanol (C 1.2): $[\Phi]_{400}$, + 26.5; $[\Phi]_{250}$, + 835; $[\Phi]_{227}$, + 1410.

T.l.c. 0.60, benzene (35)-dioxan (5)-acetic acid (1) (brown).

i.r. ν_{max} (KCl): 3,500 - 2,500 (COOH), 1761 (δ -lactone), 1692 (C=C), 1210, 1184 cm.^{-1} .
 ν_{max} (1.22 M CHCl_3): 1768 cm.^{-1} (ϵ 522, $\Delta \nu_{\frac{1}{2}}$ 28 cm.^{-1}), 1737 cm.^{-1} (shoulder) (ϵ 220), 1700 cm.^{-1} (ϵ 396, $\Delta \nu_{\frac{1}{2}}$ 20 cm.^{-1}).

u.v. λ_{max} . (EtOH) : 214 n.m. (ϵ 5,900).

n.m.r. 100 Mc/s. (CDCl₃) τ : 3.45 ($\frac{1}{2}$ H, S), 3.48 ($\frac{1}{2}$ H, S),
4.04 ($\frac{1}{2}$ H, S) and 4.09 ($\frac{1}{2}$ H, S) (all olefinic protons),
5.50 (1H, m, H-4), 6.34 (1H, dd, J = 9.0 and 11.0 Hz,
H-2), 7.3 - 8.2 (2H, m, 2H-3), 8.3 (2H, m, 2H-5),
8.6 (4H, m, sat. -CH₂-), 9.10 (3H, t, J = 6.0 Hz.,
CH₃ -CH₂-).

Analysis Found: C, 62.55; H, 7.43%; M⁺ at m/e 212.

C₁₁H₁₆O₄ requires C, 62.25; H, 7.60%; M.W. 212.

Methyl Canadensate: Mixture of C-2 Epimers (57).⁵⁴

(a) The mixture of canadensic acid C-2 epimers (21.2 mg.) was boiled in a slurry of potassium carbonate (35 mg.) and 'analar' acetone (10 ml.). Methyl iodide (0.2 ml.) was added with stirring under reflux, and heating continued for 4 hours. The reaction mixture was cooled, filtered (glass paper) and the solvent removed under reduced pressure. The residue was taken up in chloroform (10 ml.) and washed with fresh aqueous sodium bicarbonate solution, water, dried and concentrated in vacuo to give a 1:1 mixture of the methyl canadensate C-2 epimers (57) as an oil (21 mg., 93%) which failed to crystallise, and distilled at 115°/0.3 mm.

T.l.c. 0.59 and 0.58, CHCl₃ (brown).

i.r. ν_{max} . (thin film): 1765 (δ -lactone), 1716 (unsat. carbomethoxyl), 1635 (-C=C-), 1195, 840 cm.⁻¹

ν_{max} . (1.48 m MCHCl_3): 1767 cm^{-1} (ϵ 540), 1721 cm^{-1} .
(ϵ 430), 1633 cm^{-1} (ϵ 80).

u.v. λ_{max} . (Et.OH): 211 n.m. (ϵ 6,040).

n.m.r. 60 Mc/S. (CDCl_3) τ : 3.59 ($\frac{1}{2}\text{H}$, S) 3.62 ($\frac{1}{2}\text{H}$, S), 4.02
($\frac{1}{2}\text{H}$, S), 4.15 ($\frac{1}{2}\text{H}$, S) (olefinic H's), 5.50 (1H, m, H-4),
6.23 (3H, S, -OMe), 6.33 (1H, dd, $J = 11.0, 9.0$ Hz.,
H-2), 7.20 - 8.1 (2H, m, 2H - 3), 8.25 (2H, m, 2H-5),
8.55 (4H, m, sat. $-\text{CH}_2-$), 9.10 (3H, t, $J = 6.0$ Hz.,
 CH_3 $-\text{CH}_2-$).

Analysis. Found : C, 63.59; H, 7.95%; M^+ at m/e 226.

$\text{C}_{12}\text{H}_{18}\text{O}_4$ requires C, 63.70; H, 8.02%; M.W. 226.

(b) The mixture of canadensic acid C-2 epimers (22mg.) was left in methanol (3ml.) and concentrated sulphuric acid (0.1 ml.) for 7 days at room temperature. After this time the reaction mixture was diluted with water (5 ml.) concentrated in vacuo and extracted with chloroform (2 x 5 ml.). The organic layer was washed thoroughly with aqueous sodium bicarbonate solution (2 x 10 ml.), water (2 x 10 ml.) and dried. Removal of the solvent yielded an oil (20 mg.) which was identical with a sample of the 1:1 mixture of methyl canadensate C-2 epimers prepared in method (a), by comparison of i.r. and n.m.r. spectra, on t.l.c. and g.l.c.

Quaternary Mixture of Dihydrocanadensic Acid C-2, C-10 Diastereoisomers (56).⁵⁴

The mixture of canadensic acid C-2 epimers (24 mg.) were shaken with platinum oxide (6 mg.) in ethanol (3ml.) in an atmosphere of hydrogen for 50 minutes. The catalyst was separated on glass paper and the ethanol removed in vacuo to give the quaternary mixture of dihydrocanadensic acid C-2, C-10 diastereoisomers (56) as an oil (22 mg., 93%) which crystallised from benzene - light petroleum as an amorphous powder, m.p. 103-105° (softening at 90°).

O.R.D. in methanol (C 0.9): $[\Phi]_{400} + 350$; $[\Phi]_{250} + 1370$, $[\Phi]_{204} + 204$.

T.l.c. 0.6l,(35) benzene-(5)dioxan(1) acetic acid (dark brown).

i.r. ν_{\max} . (KBr): 3,300-2,600 (COOH), 1755 (γ -lactone), 1690 (CO₂H), 1195, 1119, 1004, 950 cm.⁻¹.
 ν_{\max} . (2.27 mM.CHCl₃), 1764 cm.⁻¹ (ϵ 712, $\Delta v_{\frac{1}{2}}$ 57 cm.⁻¹), 1711 cm.⁻¹ (ϵ 665, $\Delta v_{\frac{1}{2}}$ 38 cm.⁻¹).

u.v. λ_{\max} . (EtOH): No absorption > 200 nm.

n.m.r. 100 Mc/s. (CDCl₃) τ : 5.58 (1H, m, H-4), 6.92 (2H, m, H-2 and H-10), 7.7 - 8.0 (2H, m, 2H - 3), 8.2 (2H, m, 2H-5), 8.6 (4H, m, sat. -CH₂-), 8.77 (3H, d, J = 7.0 Hz., 3H - 11), 9.08 (3H, t, J = 6.5 Hz., CH₃-CH₂-).

Analysis Found : C, 61.45; H, 8.29%; (M⁺ - 57) at m/e 157.

C₁₁H₁₈O₄ requires C, 61.66; H, 8.47%; M.W. - 57, 157.

EXPERIMENTAL.CHAPTER 2.Hydrogenation of Canadensolide (51): 10-epidihydrocanadensolide 61

(a) Canadensolide (30mg.) in acetic acid (5ml.) was stirred with 10% palladium-on-charcoal. (10mg.) for 2 hours. The solution was filtered, diluted with chloroform (20ml.) and the organic solution extracted with aqueous sodium bicarbonate solution, water and dried. Evaporation of the solvent gave 10-epidihydrocanadensolide (28 mg., 92%) which crystallised from benzene - light petroleum as needles, m.p. 70°.

O.R.D. in methanol (C 0.43): $[\alpha]_{350}$, - 50; $[\alpha]_{240}$, - 1,470;

$[\alpha]_{200}$, 14,300.

T.l.c. 0.49, CHCl_3 (purple - brown).

i.r. ν_{max} . (KBr): 1780 (γ -lactone), 1762 (γ -lactone), 1203,

1165, 1132, 1098, 1069, 1055, 1034, 1010 cm^{-1}

ν_{max} . (CCl_4): 1805, 1795, 1180, 1155. cm^{-1}

u.v. λ_{max} . (EtOH): no absorption > 200 nm.

n.m.r. 60Mc/s. (CDCl_3) τ : 4.97 (1H, dd, J = 6.0, 4.0 Hz., H-3),

5.50 (1H, m, H-4), 6.53 (1H, dd J = 6.0, 10.5 Hz., H-2),

6.93 (1H, dq, J = 10.5, 7.5 Hz., H-10), 8.23 (2H, m, 2H-5),

8.5 (4H, m, sat.- CH_2 -), 8.53 (3H, d, J = 7.5 Hz., 3H -11),

9.06 (3H, t, J = 6.0 Hz. CH_3 - CH_2).

m.s. m/e (Rel. abundance): M^+ 212 (4), 166 (3), 141 (4), 140 (4),

125 (6), 112 (5), 99 (12), 98 (100), 70 (20), 69 (40), 57 (6),

56 (4), 55 (8), 41 (26).

Analysis Found : C, 62.36 ; H, 75.1% ; M^+ at m/e 212,

$C_{11}H_{16}O_4$ requires C, 62.25 ; H, 76.0% ; M.W. 212.

(b) Canadensolide (20 mg.) in ethanol (3 ml.) was stirred with 5% palladium-on-charcoal (10 mg.) overnight at R.T. The solution was filtered, and evaporated in vacuo to give 10-epidihydrocanadensolide (18 mg., 90%), identical in m.p., mixed m.p., and on t.l.c. to an authentic sample. No polar material could be detected.

Treatment of Canadensolide (51) with Alkali.

Canadensolide (20 mg., 0.095mM) was stirred under reflux with $N/10$ sodium hydroxide (5 ml.) for $\frac{1}{2}$ hour. The cooled solution was titrated with $N/10$ hydrochloric acid (3.2ml.) to the phenolphtholein endpoint (i.e. 0.18mM sodium hydroxide consumed). The solution was acidified with 5N hydrochloric acid (5 ml.) and extracted with chloroform. The organic layer was dried and evaporated to give after p.l.c. an oil (15 mg., 75%) identical to canadensolide on t.l.c. and in i.r. spectrum.

Methanolysis of Canadensolide (51) : Hydroxyester (63).

Canadensolide (35 mg.) in dry methanol (5 ml.) containing concentrated sulphuric acid (0.1 ml.) was refluxed fo 4 hours. The solution was diluted with chloroform (15 ml.) and extracted with aqueous sodium bicarbonate, watered and dried. Evaporation gave an oil (34 mg.) consisting of canadensolide (51) and hydroxyester (63).

Decomposition occurred on attempted preparative layer chromatography.

Hydroxyester (42) based on following spectra characteristics.

T.l.c. 0.43, $CHCl_3$ (brown).

i.r. ν (thin film): 3,490 (-OH), 1770 (γ -lactone), 1715 max. (unsaturated CO₂Me), 1638 (-C=C-), 1300, 1265, 1183, 1145, 998 cm⁻¹.

n.m.r. 60Mc/S (CDCl₃) τ : 3.33 (1H, S, olefinic H), 3.90 (1H, S, olefinic H), 5.20 (1H, m, H-4), 6.05 (1H, S, -OMe).

Cyclisation of Hydroxyester (63) to Canadensolide (41).

An approximately 1:1 mixture of canadensolide and hydroxyester (35 mg.) was refluxed under nitrogen in dry benzene for 24 hours. The solvent was evaporated and the oil subjected to p.l.c. on a 0.75m Kieselgel HF₂₅₄ plate. Elution of the major band with ethyl acetate gave canadensolide (25 mg., 71%) identical on t.l.c. in m.p. and i.r. spectrum with an authentic sample.

Hydrogenation of Hydroxyester (63).

Canadensolide (30 mg.) in dry methanol (10 ml.) containing concentrated sulphuric acid (0.1 ml.) was left at R.T. for seven days. The reaction solution was then stirred with 5% palladium-on-charcoal in an atmosphere of hydrogen for 2 hours. The methanol solution was filtered, concentrated in vacuo and diluted with chloroform (15 ml.). The organic solution was extracted with aqueous sodium bicarbonate solution and evaporated to give an oil (25 mg.). The two components were separated by p.l.c. using multiple elution in 20% light petroleum in chloroform to give 10-epidihydrocanadensolide (12 mg., 40%) and dihydrocanadensolide (8 mg., 25%) identical in m.p. and i.r. spectrum with authentic samples.

Conversion of 10-Epidihydrocanadensolide (61) to Dihydrocanadensolide (54).

Dihydrocanadensolide (33 mg.) in 2N methanolic potassium hydroxide (10 ml.) was left at R.T. overnight. The aqueous solution was acidified with 5N hydrochloric acid (15 ml.) and extracted with chloroform. Evaporation of the organic solvent gave an oil (30 mg.) shown by g.l.c. (1% SE 30, 4ft. column at 125°, flow rate 75 ml./min.) to consist of dihydrocanadensolide (R_t 11.0 min.) and 10-epidihydrocanadensolide (R_t 13.7 min.) in the ratio 4.5/1. Crystallisation from benzene-light petroleum gave dihydrocanadensolide (25 mg., 75%) as needles m.p. 94.5°, identical in m.p. and i r. spectrum to an authentic sample.

Diene (68) from Canadensolide (51).

Canadensolide (45 mg.) in dry dimethyl formamide (8 ml.) was stirred with silver oxide (30 mg.) and methyl iodide (0.2 ml.) at R.T. for 1 hour. Chloroform (15 ml.) was added, and the organic solution filtered through celite, and extracted with water (5x10 ml.) and dried. Evaporation of the solvent gave an oil which was purified by preparative layer chromatography on a Kieselgel HF₂₅₄ plate (0.75 mm. x 20 cm. x 20 cm.) in CHCl₃. Elution of the major dark-blue band under the u.v. (254 nm.) with ethyl acetate gave the diene (35 mg., 73%) as a slightly unstable oil, b.p. 78°/ 0.01 mm.

Q.R.D. in methanol (C 2.0) : [α]₃₅₀, - 450 ; [α]₂₇₀, -2,200 ;
[α]₂₄₄, -12,800 ; [α]₂₀₉, + 19,050.

T.l.c. 0.75, CHCl₃ (yellow).

i.r. ν (CCl₄), 1768 (γ -lactone), 1732 (CO₂Me), 1276, 1200, 1184, 1045, 1020, 968 cm.⁻¹.

u.v. λ (hexane) : 236nm. (ϵ 8, 500).

n.m.r. 60Mc/S. (CDCl₃) τ : 2.07 (1H, d, J=1.5Hz, H-3), 2.95 (1H, s, H-11 cis), 3.40 (1H, s, H-11 trans), 5.03 (1H, m, H-4), 6.15 (1H, s, -OMe), 8.5 (6H, m, sat. -CH₂-), 9.09 (3H, t, J = 6.0Hz, CH₃-CH₂-).

m.s. m/e (Rel. abundance) M⁺224 (6), 192 (20), 139 (21), 85 (71), 83 (100), 47 (28), 45 (17).

Analysis. Found : C, 64.55 ; H, 7.4 6% ; M⁺ at m/e 224.

C₁₂H₁₆O₄ requires C, 64.27 ; H, 7.1 9% ; M.W. 224.3.

Butenolide ester (69) from Dihydrocanadensolide (54).

Dihydrocanadensolide (32 mg.) in dry dimethyl formamide and methyl iodide (0.2 ml.) was stirred at R.T. overnight. Chloroform (20 ml.) was added to the reaction solution, and the organic solution filtered through celite, and extracted with water (5x10 ml.). The oil obtained on evaporation of the organic solvent was subjected to preparative t.l.c. using a Kieselgel HF₂₅₄ plate (0.75mm. x 20 cm. x 20 cm.) in CHCl₃. Elution of the major band observed with iodine in the usual way gave the butenolide ester (20 mg, 59%) as an oil, b.p. 80°/0.01 m.m.

O.R.D. in methanol (C 0.28) : $[\alpha]_{300}$, -970 ; $[\alpha]_{250}$, -4,800 ; $[\alpha]_{225}$, -16,400.

T.l.c. 0.69, CHCl₃ (light brown, on baking).

i.r. ν (thin film) : 1745 (γ -lactone), 1730 (CO₂Me), 1620 (wk.) (C=C), 1200, 1105, 1045, 960 cm.⁻¹.

$\nu_{\max.}$ (CCl_4) 1770, 1745, 1198, 1167, 1105, 1040, 960 cm.^{-1}

u.v. $\lambda_{\max.}$ (Et OH) : 215 nm. (ϵ 9,750).

n.m.r. 60Mc/S (CDCl_3) τ : 2.80 (1H, dd, $J_1 = J_2 = 2.0$ Hz., H-3), 5.07 (1H, dt, $J = 2.0, 6.0$ Hz., H-4), 6.30 (3H, s, -OMe), 6.47 (1H, dq, $J = 2.0, 7.5$ Hz., H-10), 8.30 (2H, m, 2H-5), 8.57 (3H, d, $J = 7.5$ Hz., 3H-11), 8.6 (4H, m, sat. $-\text{CH}_2-$), 9.10 (3H, t, $J = 6.0$ Hz., $\text{CH}_3 - \text{CH}_2-$).

m.s. m/e (Rel. abundance) ; M^+ 226 (6), 197 (9), 195 (16), 194 (22), 169 (19), 167 (31), 166(100), 141 (25), 138 (15), 137 (21), 124 (24), 113 (17), 110 (92), 109 (26), 95 (41), 92 (33), 91 (52), 67 (25), 59 (37), 57 (58), 55 (36), 54 (29), 53 (59).

Analysis. Found : C, 63.92 ; H, 8.01% ; M^+ at m/e 226.

$\text{C}_{12}\text{H}_{18}\text{O}_4$ requires C, 63.70 ; H, 8.02% ; M.W. 226.

Hydrogenation of Butenolide Ester (69) : Dihydro Ester (70).

Butenolide ester (42 mg.) in ethanol (5 ml.) was stirred with 5% palladium-on-charcoal (15 mg.) in an atmosphere of hydrogen for 2 hours. The solution was filtered (glass paper) and the solvent evaporated to give an oil, which was purified by p.l.c. on Kieselgel HF₂₅₄ using CHCl_3 . The major band using iodine gave the dihydro ester (35 mg., 83%) as a colourless oil, b.p. $115^\circ/0.3$ m.m.

O.R.D. in methanol (C 0.50) : $[\alpha]_{300}^0$; $[\alpha]_{250}$, - 800 ;
 $[\alpha]_{224}$, -4,200 ; $[\alpha]_{210}$, +3,280.

T.l.c. 0.72, CHCl_3 (dark brown).

i.r. $\nu_{\max.}$ (thin film) : 1775 (δ -lactone), 1730 (CO_2Me), 1190,

1075, 1025 cm^{-1} .

u.v. No absorption maximum $> 210\text{nm}$.

n.m.r. 100Mc/S. (CDCl_3) τ : 5.62 (1H, m, H-4), 6.27 (3H, s - OMe), 7.00 (2H, m, H-2 and H-10), 7.7 - 8.1 (2H, m, 2H-3), 8.3 (2H, m, 2H-5), 8.59 (3H, d, $J = 7.0\text{Hz}$, 3H-11), 8.6 (4H, m, sat. $-\text{CH}_2-$), 9.00 (3H, t, $J = 6.0\text{Hz}$, CH_3-CH_2-).

m.s. m/e (Rel. abundance) : M^+ 228 (9), 197 (30), 171 (91), 143 (23), 142 (68), 139 (47), 111 (78), 88 (100), 69 (40), 55 (95).

Analysis Found ; C, 63.22 ; H, 8.68% ; M^+ at m/e 214.

$\text{C}_{12}\text{H}_{20}\text{O}_4$ requires C, 63.14 ; H, 8.83% ; M.W. 214.3.

Reduction of Dihydrocanadensolide (54) to Tetrol (80).

Dihydrocanadensolide (48 mg.) in ether (10 ml.) was added dropwise to a stirred suspension of lithium aluminium hydride (40 mg.) in ether (20 ml.) and refluxed for 8 hours. After working up in the usual way the tetrol (40 mg., 80%) was obtained as a colourless oil b.p. $125^\circ/0.01\text{ m.m.}$

T.l.c. 0.35, 5% MeOH, 95% CHCl_3 (brown).

i.r. ν_{max} (thin film) : 3,450 (strong) ($-\text{OH}$), 1,270, 1,140, 1050 cm^{-1} .

u.v. No absorption $> 210\text{ nm}$.

n.m.r. 60Mc/S. (CDCl_3) τ : 6.3 (10H, m, $-\text{CH}-\text{OH}$), 8.2 - 8.8 (8H, m, sat. $-\text{CH}_2-$), 9.1 (6H, m, CH_3-CH). On deuteration integral at 6.3 τ is reduced to 6H.

m.s. m/e (Rel. abundance) 211 (0.3), 185 (1.0), 18.3 (0.5), 127 (4.0), 117 (16), 116 (13), 115 (100), 99 (5), 98 (32), 97 (20), 85 (33), 83 (16), 81 (9), 71 (14), 69 (49), 68 (14), 67 (13), 58 (10), 57 (29), 56 (10), 55 (24).

Analysis Found : C, 59.68 ; H, 10.74%.

$C_{11}H_{24}O_4$ requires C, 59.97 ; H, 10.98% ; M.W. 220.3.

The tetrakis-trimethylsilyl ether was prepared using hexamethyldiazane and chlorotrimethylsilane in pyridine at R.T. overnight (requires M.W. 508).

m/e (Rel. abundance) 349 (5), 259 (100), 197 (15), 170 (18), 169 (92), 159 (54), 148 (37), 103 (27), 97 (23), 75 (51), 73 (73).

Reaction of Dihydrocanadensolide (54) with one Equivalent of Sodium Methoxide.

Dihydrocanadensolide (76 mg., 0.36 mM) in dry methanol (10 ml.) containing sodium methoxide (19 mg., 0.35 mM) was refluxed for 14 hours.

Hydrochloric acid (0.1N, 4 ml.) was added, the solution concentrated in vacuo, and extracted with chloroform (3x5 ml.). The organic solution was evaporated to give the crude butenolide acid (82) as an oil.

Purification was achieved by extracting an ether solution (15 ml.) of this oil with aqueous sodium bicarbonate (3x5 ml.). The aqueous extract was acidified with 5N hydrochloric acid and extracted with chloroform to give the butenolide acid (45 mg., 59%) as an oil.

T.l.c. 0.61 , (35)benzene -(5)dioxan-(1)acetic acid (yellow-brown).

i.r. ν_{\max} (thin film) : 3,500 - 2,600 (CO_2H), 1740 (γ -lactone),
1700 (CO_2H), 1200, 1120, 1045, 990 cm^{-1} .

u.v. λ_{\max} (EtOH) ; 211 nm. (ϵ 9, 240).

n.m.r. 60 Mc/S. (CDCl_3) τ : 2.40 (1H, m, H-3), 5.02 (1H, m, H-4),
6.35 (1H, dq, $J = 6.0, 2.0\text{Hz.}$, H-10), 8.55 (3H, d, $J = 6.0\text{Hz.}$, 3H-11),
8.4-8.7 (6H, m, 2H-5 and sat. $-\text{CH}_2-$), 9.10 (3H, t, $J = 6.0\text{Hz.}$, CH_3-CH_2-).

m.s. m/e (Rel. abundance) : $(M+1)^+$ 213 (2.6), 212 (3.1), 194 (24),
183 (17), 167 (22), 166 (100), 155 (25), 138 (22), 137 (25), 127 (27),
125 (13), 124 (26), 123 (12), 111 (16), 110 (90), 109 (26), 99 (45), 85
(52), 81 (26), 80 (26), 69 (10), 67 (12), 57 (50), 55 (18), 53 (31).

Methylation of Butenolide Acid (82).

The butenolide acid (34 mg.) in analar acetone (10 ml.) and methyl iodide (0.2 ml.) was stirred with dry potassium carbonate (40 mg.) for 6 Hours. The solvent was evaporated, chloroform (10 ml.) added and the organic solvent was filtered, extracted with aqueous sodium bicarbonate water and evaporated to give the butenolide ester (69) (30 mg., 84 %) identical in i.r. and n.m.r. spectra to a sample prepared using Ag_2O as described previously.

Reduction of Dihydrocanadensolide (54) with an Excess of Sodium Methoxide

Dihydrocanadensolide (200 mg.) in dry methanol (10 ml.) containing sodium (150 mg.) was refluxed for 10 hours. After removal of the solvent 5N hydrochloric acid (10 ml.) was added, and the solution extracted with ethyl acetate. Purification of the extract was achieved by

extraction into aqueous sodium bicarbonate

give the keto half-ester (150 mg., 65%) as a colourless oil.

T.l.c. 0.50, benzene (35)-dioxan (5)-acetic acid (1), (yellow-brown).

i.r. ν (thin film) : 3,400 - 2,700 (CO₂H), 1720 (ketone),
max.
1210 (br), 1135, 850 cm⁻¹.

u.v. No absorption > 210 nm.

n.m.r. 60Mc/S. (CDCl₃) τ : 1.93 (1H, br.s, COOH), 6.30 (3H, s,
-OMe), 6.40 - 7.60 (6H, m, H-2, H-10, 2H-3, 2H-5), 8.50 (4H, m, sat.
-CH₂-), 9.10 (3H, t, CH₃-CH₂-).

Keto Dicarboxylic Ester (81, R=Me).

The keto half-ester (60 mg.) in ether (10 ml.) was briefly treated with an excess of ethereal diazomethane. Evaporation of the solvent gave an oil which was purified by preparative t.l.c. on Kieselgel HF₂₅₄ (0.75 mm. x 20 cm. x 20 cm.) using iodine detection to give the keto dicarboxylic ester (54 mg., 80%) as a colourless oil, b.p. 65°/0.005 m.m.

T.l.c. 0.45, CHCl₃ (yellow-brown).

i.r. ν (CCl₄) : 1745 (CO₂Me), 1725 (ketone), 1206, 1170,
max.
1132 (wk.) 1108 (wk.) 1078 (wk.).

u.v. No absorption > 210 nm.

n.m.r. 60Mc/S. (CDCl₃) τ : 6.31 (6H, s, -OMe), 6.40 - 7.60 (6H, m,
H-2, H-10, 2H-3 and 2H-5), 8.5 (4H, m, sat. -CH₂-), 8.83 (3H, d, J =
7.5Hz., 3H-11), 9.10 (3H, t, J = 6.0Hz., CH₃-CH₂-).

m.s. ^{m/e} (Rel. abundance) : M⁺258 (2), 227 (26), 216 (40), 201 (55),

184 (44), 173 (28), 167 (29), 166 (32), 156 (23), 141 (81), 139 (31),
124 (26), 113 (48), 88 (58), 85 (82), 59 (33), 57 (100), 55 (47).

$216^+ \longrightarrow 184^+$, m^* 156.7,

$201^+ \longrightarrow 173^+$, m^* 148.9,

$173^+ \longrightarrow 141^+$, m^* 114.9,

$141^+ \longrightarrow 113^+$, m^* 90.6.

Analysis Found : C, 60.74 ; H, 8.75%; M^+ at m/e 258.

$C_{13}H_{22}O_5$ requires C, 60.45 ; H, 8.58%; M.W. 258.3.

Ethylene Ketal of Keto Dicarboxylic Ester (81, R=Me).

The keto dicarboxylic ester (32 mg.) in dry benzene (15 ml.) containing *p*-toluenesulphonic acid (3 mg.) was refluxed for 18 hours. The organic solvent was extracted with aqueous sodium hydrogen carbonate, water and evaporated. The ethylene ketal which was purified by chromatography on Kieselgel (0.75 mm. x 20cm. x 20 cm.), was obtained as an oil, b.p. $85^\circ/0.04$ m.m.

T.l.c. 0.49 , $CHCl_3$ (brown).

i.r. ν max. (CCl_4) : 1740 (CO_2Me), 1192, 1159, 1040 cm^{-1} .

u.v. No absorption $> 200nm$.

n.m.r. 60Mc/S ($CDCl_3$) τ : 6.10 (4H, s, $-O-CH_2-CH_2-O-$), 6.31 (6H, s, $-OMe$), 7.30 (2H, m, H-2 and H-10), 8.0-8.5 (8H, m, 2H-3, 2H-5, 2H-6 and 2H-7), 9.13 (3H, t, $J = 6.0Hz.$, $\underline{CH_3}-CH_2-$).

m.s. m/e (Rel. abundance) : $(M+1)^+$ 303 (0.07), 302 (0.04), 271 (9), 246 (8), 245 (41), 243 (4), 185 (27), 157 (6), 130 (12), 129 (100), 113

(7), 101 (8), 99 (5), 86 (10), 73 (6), 57 (16), 55 (11),

$245^+ \rightarrow 185^+$, $m^* 140$.

Analysis Found : C, 59.44 ; H, 8.87%; M^+ at m/e 302.

$C_{15}H_{26}O_6$ requires C, 59.58 ; H, 8.67%; M.W. 302.4.

Attempted Cyclisation of Butenolide Acid (82).

(a) Butenolide acid (25 mg.) was dissolved in trifluoroacetic acid (5 ml.) and the solution left at R.T. for 7 days. The trifluoroacetic acid was removed in vacuo to give an oil (23 mg.) identified by its i.r. and t.l.c. properties as starting material.

(b) Butenolide acid (15 mg.) was refluxed in trifluoroacetic acid (5 ml.) for 8 hours, and the reaction worked-up as before to give an oil (13 mg.), identical in i.r. and t.l.c. properties to the starting material.

Attempted Cyclisation of Butenolide Ester (69).

Butenolide ester (45 mg.) in a mixture of concentrated sulphuric acid (1 ml.) and formic acid (3 ml.) was left at R.T. for 14 days. The solution was diluted with water (20 ml.), and the aqueous solution extracted with chloroform. The chloroform solution was then extracted with aqueous sodium hydrogen carbonate, watered and evaporated to give an oil (38 mg.), identical in i.r. and n.m.r. spectra to the butenolide acid (82).

Attempted Hydration of Butenolide Ester (69).

(a) Using Mercuric Acetate.

Butenolide ester (30 mg.) in tetrahydrofuran (1 ml.) was added to a stirred solution of mercuric acetate (50 mg.) in 50% aqueous tetrahydrofuran

(5 ml.), and the yellow suspension stirred for 1 hour. 5N sodium hydroxide (3 ml.) and sodium borohydride (100 mg.) were added, and the solution stirred at R.T. for 30 minutes. The solution was filtered through celite, acidified with 5N hydrochloric acid, and extracted with chloroform. Evaporation of the organic solvent gave an oil, shown to be acidic and consist of at least two components (R_f 0.05, 0.03 CHCl_3) by t.l.c. This material was recovered unchanged after treatment with 5N methanolic potassium hydroxide (5 ml.) overnight.

(b) Using Dilute Acid.

Butenolide ester (15 mg.) was refluxed on 0.1N hydrochloric acid (10 ml.) for 7 days. The solution was extracted with chloroform to give an oil, identical on t.l.c. and its spectrum, to butenolide acid (82).

(c) Using Hydrogen Bromide.

Butenolide ester (30 mg.) in a 50% solution of hydrogen bromide in acetic acid (10 ml.) was left at R.T. for 7 days. The solution was diluted with water (30 ml.) and extracted with ether. Evaporation of the organic solvent gave an oil (25 mg.), which was dissolved in 2N methanolic potassium hydroxide (5 ml.) and left overnight. The solution was acidified using 5N hydrochloric acid and extracted with chloroform to give an oil (22 mg.) shown by t.l.c. to contain no neutral product.

EXPERIMENTAL.CHAPTER 3.Canadensic Acid (95).

The compound was isolated as described earlier and crystallised from benzene - light petroleum as colourless needles m.p. 113-114°.

O.R.D. in methanol (C 0.58): $[\alpha]_{500}$, - 440; $[\alpha]_{400}$, - 405;

$[\alpha]_{300}$, - 990; $[\alpha]_{256}$, -2,390; $[\alpha]_{250}$, - 3,140; $[\alpha]_{222}$,
- 15,300; $[\alpha]_{217}$, -12,800.

T.l.c. 0.60, benzene (35)-dioxan (5)-acetic acid (1)- (brown).

i.r. ν_{\max} . (K.Br.): 3,200 - 2,600 (CO₂H), 1752 (γ -lactone),
1696 (CO₂H), 1624 (C = CH₂), 1195, 1172, 982, 930 (br.),
842, 800 cm.⁻¹.

u.v. λ_{\max} . (EtOH): 214 (ϵ 2,000).

n.m.r. 100 Mc/S. (CDCl₃) τ : 3.46 (1H, s, H-11c), 4.05 (1H, s,
H-11t), 5.60 (1H, m, H-4), 6.34 (1H, dd, J = 9.0, 12.0 Hz.,
H-2), 7.46 (1H, m, J = 6.0, 9.0, 12.0 Hz., irr. 6.34 \longrightarrow dd
(J = 12.0, 6.0 Hz.), irr. 5.60 \longrightarrow dd (J = 12.0, 9.0 Hz.),
H-3a), 8.03 (1H, m, J = 12.5, 12.0, 12.0 Hz., irr. 5.60 \longrightarrow dd
(J₁ = J₂ = 12.0 Hz.), H-3b), 8.30 (2H, m, 2H-5), 8.6 (4H, m,
sat. -CH₂-), 9.09 (3H, t, J = 6.0 Hz., CH₃-CH₂-).

m.s. m/e (Rel. abundance): M⁺212 (2), 168 (17), 155 (63),
137 (85), 111 (67), 99 (43), 98 (58), 81 (50), 57 (80),
55 (62), 41 (100).

Analysis Found : C, 62.33; H, 7.64%; M⁺ at m/e 212.

C₁₁H₁₆O₄ requires C, 62.25; H, 7.60%; M.W. 212.

Methyl Canadensate (100).

Canadensic acid (30mg.) in dry methanol (10ml.) containing

concentrated sulphuric acid (0.1ml.) was refluxed for 4 hours. The reaction solution was then diluted with water (20ml.), concentrated in vacuo and extracted with chloroform. The organic layer was washed with aqueous sodium hydrogen carbonate solution, water and dried. Removal of the solvent gave methyl canadensate as an oil (28mg., 87%), b.p. 80°/0.01 mm.

T.l.c. 0.58, CHCl₃ (brown).

i.r. ν_{\max} . (thin film): 1765 (γ lactone), 1720 (unsat. CO₂Me), 1635 (C=CH₂), 1180, 1010, 985, 832 cm.⁻¹.

u.v. λ_{\max} . (EtOH): 211 nm. (ϵ 5,900).

n.m.r. 100Mc/s. (CDCl₃) τ : 3.60 (1H, s, H-11c), 4.17 (1H, s, H-11t), 5.58 (1H, m, H-4), 6.24 (3H, s, -OMe), 6.35 (1H, dd, J = 11.0, 9.0 Hz., H-2), 7.48 (1H, m, H-3A), 8.04 (1H, m, H-/B), 8.30 (2H, m, 2H -5), 8.57 (4H, m, sat. -CH₂-), 9.07 (3H, t, J = 6.0 Hz., CH₃-CH₂-).

m.s. m/e (Rel. abundance): (M+1)⁺ 227 (1.9), 226 (1.3), 195 (14), 182 (30), 169 (38), 143 (31), 141 (35), 125 (100), 123 (51), 122 (37), 113 (33), 112 (54), 111 (31), 93 (33), 82 (42), 81 (65), 79 (57), 67 (49), 59 (42), 57 (81), 55 (81).

Analysis Found : C, 64.13 ; H, 8.06%; M⁺ at m/e 226.

C₁₂H₁₈O₄ requires C, 63.70 ; H, 8.02%; M.M. 226.

Treatment of Canadensic Acid with Diazomethane.

Canadensic acid (32mg) in ether (5ml.) was treated with an excess

of ethereal diazomethane at ice temperature for 10 minutes. Removal of the solvent yielded an oil (36mg.) which was purified by p.l.c. on Kieselgel HF₂₅₄ to give the mixture of C-10 pyrazoline epimers (96%).
T.l.c. 0.15, CHCl₃ (yellow-brown).

i.r. ν_{\max} . (thin film): 1760 (γ -lactone) 1735 (CO₂Me), 1550 (wk.) (N=N), 1452, 1430, 1350, 1250, 1180, 1002, 925, 887 cm.⁻¹.

u.v. λ_{\max} . (EtOH): No absorption > 200 nm.

n.m.r. 60Mc/S. (CHCl₃) τ : 5.37 (2H, t, J = 7.5 Hz., -CH₂-N=N-), 5.50 (1H, m, H-4), 6.27 (3H, s, -Ome), 6.7 - 8.0 (5H, m,), 8.6 (6H, m, 2H-S and sat. -CH₂-), 9.10 (3H, t, J = 6.0 Hz., $\underline{\text{CH}_3}$ - CH₂-).

m.s. m/e (Rel. abundance): M⁺ 268 (2), 240 (5), 225 (4.4), 209 (12), 208 (12), 181 (17), 163 (22), 157 (34), 139 (67), 126 (52), 125 (34), 123 (27), 111 (22), 107 (30), 95 (54), 94 (44), 93 (46), 81 (44), 79 (73), 67 (100), 59 (55), 55 (70), 53 (42).

Treatment of Canadensic Acid Mixture of C-2 Epimers (55) with Diazomethane.

The mixture of canadensic acid C-2 epimers (52mg.) in ether (10ml.) was treated with an excess of ethereal diazomethane at ice temperature for 10 minutes. Removal of the solvent gave an oil (64mg.) which appeared to consist of two main products from t.l.c. These were separated by p.l.c. using a Kieselgel HF₂₅₄ plate and multiple elution in chloroform.

Compound A was obtained as an oil (35 mg.)

T.l.c. 0.15, CHCl₃ (yellow-brown).

i.r. ν_{\max} . (CCl_4) : 1778 (δ -lactone), 1745 (CO_2Me), 1175, 905, 834 cm^{-1} .

u.v. λ_{\max} . (EtOH) : No absorption > 200 nm.

n.m.r. 60Mc/S. (CDCl_3) τ : 5.33 (2H, t, $J = 7$ Hz., $-\text{CH}_2-\text{N}=\text{N}-$), 5.50 (1H, m, H-4), 6.30 (3H, s, $-\text{OMe}$), 6.7 - 8.0 (5H, m), 8.6 (6H, m, 2H-5 and sat. $-\text{CH}_2-$), 9.07 (3H, t, CH_3-CH_2-).

Compound B was obtained as an oil (11 mg.).

T.l.c. 0.25, CHCl_3 (yellow-brown).

i.r. ν_{\max} . (CCl_4): 1768 (δ -lactone), 1740 (CO_2Me) 1680 (wk.), 1300, 1230, 1192, 1151, 1018, 968 cm^{-1} .

u.v. λ_{\max} . (EtOH): No absorption > 200 nm.

n.m.r. 60Mc/S. (CDCl_3) τ : 5.50 (1H, m, H-4), 6.20 (3H, s, $-\text{OMe}$), 8.6 (6H, m, 2H-5 and sat. $-\text{CH}_2-$), 9.10 (3H, t, CH_3-CH_2-).

m.s. m/e (Rel.abundance): M^+ 240 (0.5), 226 (17), 172 (22), 169 (100), 166 (23), 140 (38), 137 (40), 113 (27), 112 (16), 111 (26), 110 (17), 109 (22), 88 (31), 81 (33), 69 (23), 67 (21), 59 (30), 55 (50), 53 (44).

Methyl 11-methoxydihydrocanadensate (Mixture of C-10 enimers) (101).

Canadensic acid (52 mg.) in absolute methanol (15 ml.) containing concentrated sulphuric acid (0.3ml.) was refluxed for 3 days.

The solution was then diluted with water (30ml.), concentrated in vacuo and extracted with chloroform. The organic extract was washed with aqueous sodium hydrogen carbonate, water and evaporated to give an oil (64mg.). P.l.c. on Kieselgel H (0.7 mm. x 20 cm. x 20 cm.) using

iodine vapour for detection enabled methyl canadensate (100) (25 mg., 45%) (identity established by t.l.c., and n.m.r. spectra) and the more polar methyl 11-methoxydihydrocanadensate (28mg., 41%) to be separated. methyl 11-methoxydihydrocanadensate was obtained as an oil, b.p. 105°/0.5mm. T.l.c. 0.50, CHCl₃ (brown).

i.r. ν_{\max} . (thin film): 1768 (δ -lactone), 1735 (CO₂Me). 1455, 1433, 1384, 1352, 1185, (br.), 1118, 1005, 962 cm.⁻¹.

u.v. No absorption > 210 n.m.

n.m.r. 60Mc/s. (CDCl₃) τ : 5.60 (1H, m, H-4), 6.29 (3H, s, -CO₂Me), 6.30 (2H, m, -CH₂-O-Me), 6.63 (1H, s, -OMe), 7.70 (2H, m, H-2 and H-10), 8.5 (8H, m, 2H-3, 2H-5 and sat. -CH₂-), 9.10 (3H, t, J = 6.0 Hz., CH₃-CH₂-).

m.s. m/e (Rel. abundance): M⁺ 258 (1.0), 247 (19), 227 (6), 226 (3), 215 (22), 190 (17), 187 (24), 169 (20), 155 (16), 145 (26), 142 (17), 141 (22), 117 (100), 111 (24), 101 (33), 85 (54), 69 (31).

Analysis Found : C, 60.53; H, 8.68%; M⁺ at m/e 258.

C₁₃H₂₂O₅ requires C, 60.45; H, 8.58%; M.W. 258.3.

Methyl Canadensate C-2 Epimers (57).

Canadensic acid (30 mg.) was stirred in a slurry of potassium carbonate (50mg.), 'analar' acetone (10ml.) and methyl iodide (0.2ml.) for 6 hours. The reaction mixture was filtered (glass paper) and the solvent removed in vacuo. The residue was taken up in chloroform (10ml.) and washed with aqueous sodium hydrogen carbonate, water and the solvent

evaporated to give the mixture of methyl canadensate C-2 epimers (28mg., 87%), identical (t.l.c., n.m.r.) to a sample prepared by esterification of the mixture of canadensic C-2 acid epimers with methanol and sulphuric acid catalyst.

Dihydrocanadensic Acid (Mixture of C-10 Epimers) (107)

Canadensic acid (32mg.) in ethanol (10ml.) was stirred with platinum oxide (6mg.) in an atmosphere of hydrogen for 1 hour. The solution was filtered through celite and the ethanol evaporated to give the mixture of dihydrocanadensic acid C-10 epimers (30mg. 94%), which crystallised from benzene - light petroleum as needles, m.p. 116°.

T.l.c. 0.61, benzene (35) - dioxan (5) - acetic acid (1) (dark-brown).

i.r. ν_{\max} . (KBr) : 3,200 - 2,800 (CO₂H), 1758 (γ -lactone)

1695 (CO₂H), 1288, 1198, 1120, 1004, 952 cm.⁻¹.

u.v. λ_{\max} . (EtOH): no absorption > 200 nm.

n.m.r. 100Mc/S. (CCl₄) τ : 5.62 (1H, m, H-4), 6.90 (2H, m, H-2 and H-10), 7.6 - 8.0 (2H, m, 2H-3), 8.3 (2H, m, 2H-5), 8.5 (4H, m, sat. -CH₂-), 8.62 (3H, d, J = 8.0 Hz., 3H-11), 9.09 (3H, t, J = 6.0 Hz., CH₃-CH₂-).

m.s. m/e (Rel. abundance) : M⁺ 214 (0.8), 196 (2.0), 157 (45), 139 (1.0), 129 (15), 111 (64), 96 (65), 88 (22), 83 (39), 74 (65), 69 (53), 67 (25), 55 (95).

Analysis Found : C, 61.52; H, 8.42%; M⁺ at m/e 214.

C₁₁H₁₈O₄ requires C, 61.66; H, 8.47%; M.W. 214.3.

Methyl dihydrocanadensate (mixture of C-10 Epimers) (108)

The mixture of dihydrocanadensic acid C -10 epimers (25mg.)

in ether (5ml.) was treated with an excess of diazomethane at ice temperature for 15 minutes. The solvent was removed to give the mixture of epimeric methyl dihydrocanadensates as an oil (24mg., 90%), which distilled at 115°/0.3 mm.

C.r.d. in methanol (C 0.35): $[\Phi]_{300}$, -110; $[\Phi]_{250}$, -101; $[\Phi]_{225}$, -130; $[\Phi]_{200}$, +143.

T.l.c. 0.69, CHCl_3 (dark brown).

i.r. ν_{max} . (thin film): 1765 (γ -lactone), 1730 (CO_2Me), 1180, 1130, 1070, 1015, 955 cm^{-1} .

u.v. λ_{max} . (EtOH): No absorption > 200 nm.

n.m.r. 100 Mc/s. (CCl_3) τ : 5.62 (1H, m, H-4), 6.27 ($1\frac{1}{2}$ H, s, -OMe), 6.29 ($1\frac{1}{2}$ H, s, -OMe), 6.98 (2H, m, H-2 and H-10), 7.6 - 8.0 (2H, m, 2H - 3), 8.3 (2H, m, 2H - 5), 8.5 (4H, m, sat. $-\text{CH}_2-$), 8.60 ($1\frac{1}{2}$ H, d, $J = 7.0$ Hz., H-11), 8.74 ($1\frac{1}{2}$ H, d, $J = 7.0$ Hz., H-11), 9.03 (3H, t, $J = 6.0$ Hz., CH_3-CH_2-).

m.s. m/e (Rel. abundance): M^+ 228 (8), 197 (31), 171 (93), 143 (20), 142 (65), 129 (49), 111 (78), 88 (100), 69 (42), 55 (91).

Analysis Found : C, 63.24; H, 8.84% M^+ at m/e 228.

$\text{C}_{12}\text{H}_{20}\text{O}_4$ requires C, 63.14; H, 8.83%; M.W. 228.3.

Methyl Dihydrocanadensate (quaternary mixture of C-2, C-10 Diastereoisomers)
(109).

The quaternary mixture of dihydrocanadensic acid C-2, C-10 diastereoisomers (15mg.) in ether (5ml.) was treated with an excess of ethereal diazomethane at ice temperature for 15 minutes. The solvent was

evaporated to give the quaternary mixture of methyl dihydrocanadensate diastereoisomers (12mg., 75%) as an oil which distilled at 105°/0.2mm.

O.r.d. in methanol (C 0.24): $[\alpha]_{300}^{20} + 325$, $[\alpha]_{250}^{20} + 555$; $[\alpha]_{234}^{20} + 370$; $[\alpha]_{200}^{20} + 3,620$.

T.l.c. 0.69, CHCl₃ (dark brown).

i.r. ν_{\max} . (thin film): 1770 (γ -lactone), 1725 (CO₂Me), 1180, 1128, 1075, 1020, 960 cm.⁻¹.

u.v. λ_{\max} . (EtOH): No absorption > 200 nm.

n.m.r. 100Mc/S. (CDCl₃) τ : 5.60 (1H, m, H-4), 6.22 (3H, s, -OMe), 6.92 (2H, m, H-2 and H-10), 7.6 - 8.0 (2H, m, 2H-3), 8.3 (2H, m, 2H-5), 8.6 (4H, m, sat. -CH₂-), 8.56 (1 $\frac{1}{2}$ H, d, J = 7.0 Hz., H - 11), 8.67 ($\frac{3}{2}$ H, d, J = 7.0 Hz., H-11), 8.69 ($\frac{3}{2}$ H, d, J = 7.0 Hz., H-11), 9.00 (3H, t, J = 6.0 Hz., CH₃-CH₂-).

m.s. m/e (Rel. abundance): M⁺ 228 (13), 197 (29), 171 (73), 143 (35), 139 (39), 124 (15), 111 (77), 101 (27), 100 (23), 88 (100), 83 (28), 69 (43), 55 (94).

Analysis Found : C, 63.22; H, 8.68%; M⁺ at m/e 223.

C₁₂H₂₀O₄ requires C, 63.14; H, 8.83%; M.W. 228.3.

G.L.C. Analysis of the Quaternary Mixture of Methyl Dihydrocanadensate

C-2, C-10 Diastereoisomers (109).

The following 4 ft. columns were used on a Pye Argon chromatogram, with flow rate 40 ml./minute.

<u>Column</u>	<u>Temperature</u>	<u>Retention Time (min.)</u>	<u>No. of Peaks.</u>
1% SE30	140°	3.5	1
1% PEGA	135°	16.0	1
1% NGS	150°	4.8	1
5% APL	125°	64.0	1 (broad)
10% APL	163°	26.8, 29.0	2

Schmidt Reactions on Canadensic Acid (95) and Dihydrocanadensic Acid (Mixture of C-10 epimers) (107).

The reactions were carried out in an analogous manner to those described for the mixture of canadensic acid C-2 epimers and the quaternary mixture of dihydrocanadensic acid C-2, C-10 diastereoisomers (cf. Chapter 6), and gave similar yields of carbon dioxide and products.

Carboxy-inversion Reaction on Dihydrocanadensic Acid (Mixture of C-10 epimers) (95).

Dihydrocanadensic acid C-10 epimers (61 mg.) in thionyl chloride (2ml.) was allowed to stand at R.T. for 1½ hours, and then refluxed for ½ hour. Evaporation of the solvent gave the acid chloride as an oil, which was used without purification. The acid chloride in dry hexane (10ml.) under dry nitrogen, was cooled to ice temperature. *m*-chloroperbenzoic acid (55mg.) was added to the stirred solution. Freshly distilled dry pyridine (40mg.) in dry hexane (10ml.) was added dropwise to the stirred mixture in the reaction vessel. The reaction solution was allowed to heat to R.T., and was stirred for 24 hours when

a qualitative starch iodide test proved negative. The hexane solution was decanted from the gummy precipitate of pyridiniumhydrochloride. Methanol (20ml.) and concentrated sulphuric acid (0.1ml.) were added to the hexane solution which was heated under nitrogen to 70° for 1 hour using an oil bath. The reaction solution was concentrated, water (30ml.) added and the aqueous solution extracted with chloroform, and the organic extract washed with saturated aqueous sodium bicarbonate, water and evaporated to give an oil (45mg.), which appeared to consist of two main components, R_f 0.31 and 0.45, 1% MeOH in $CHCl_3$. These were separated by p.l.c. on Kieselgel HF₂₅₄ (0.75 mm. x 20 cm. x 20 cm.) using iodine vapour for detection.

Norcanadiensol A was obtained as an oil (26 mg., 49%) which distilled at 74°/0.2 mm.

T.l.c. 0.45, 1% MeOH in $CHCl_3$ (light brown).

i.r. ν_{max} . (CCl_4): 3,500 (-OH), 1758 (γ -lactone), 1268, 1182, 910 cm^{-1} .

u.v. λ_{max} . (EtOH); No absorption > 210 nm.

n.m.r. 100 Mc/s. ($CDCl_3$) τ : 5.66 (1H, m, H-4), 6.10 (2H, m, H-10 and -CH, 7.40 (1H, m, H-2), 7.70 (1H, m, H-3A), 8.34 (1H, m, H-3B), 8.4 (2H, m, 2H - 5), 8.6 (4H, m, sat.- CH_2 -), 8.67 (3H, d, J = 6.2 Hz., 3H-11), 9.09 (3H, t, J = 6.0 Hz., CH_3 - CH_2 -), On deuteration 6.10 resonance collapses to dq, J = 6.2, Hz.

m.s. m/e (Rel. abundance): (M-1)⁺ 185 (4), 171 (5), 153 (6), 142 (63)

129 (29), 124 (36), 113 (10), 111 (31), 100 (60), 96 (30), 85 (100).

Analysis Found: C, 64.56; H, 9.63%; (M-1)⁺ at m/e 185.

C₁₀H₁₈O₃ requires C, 64.49; H, 9.74%; M.W. 186.3.

Norcanadensol B was obtained as an oil (12 mg., 23%) which distilled at 85°/0.3 mm.

T.l.c. 0.31, 1% MeOH in CHCl₃ (light brown).

i.r. ν_{\max} . (CCl₄): 3,590 (-OH), 1765 (γ -lactone), 1185, 1170, 1010, 955 cm.⁻¹.

u.v. λ_{\max} . (EtOH): No absorption > 200 nm.

n.m.r. 100 Mc/S. (CDCl₃) τ : 5.80 (2H, m, H-4 and H-10), 7.50 (1H, m, H-2), 7.7 - 8.2 (2H, m, H-3), 8.3 (2H, m, 2H-5), 8.6 (4H, m, sat. -CH₂-), 8.80 (2H, d, J = 6.0 Hz., 3H-11), 9.09 (3H, t, J = 6.0 Hz., CH₃ -CH₂-).

m.s. m/e (Rel abundance): (M-1)⁺ 185 (1.3), 171 (6), 153 (3), 142 (34), 129 (27), 124 (18), 111 (17), 100 (28), 85 (100), 71 (27), 55 (24).

Analysis Found: C, 64.34; H, 9.72% (M-1)⁺ at m/e 185.

C₁₀H₁₈O₃ requires C, 64.49; H, 9.74%; M.W. at 186.3.

Treatment of the Mixture of Norcanadensol A (112) and Norcanadensol B (113) with Methanolic Potassium Hydroxide.

A mixture of norcanadensol A (10mg.) and norcanadensol B (5mg.) in 5N methanolic potassium hydroxide (1ml) was left at R.T. overnight. After dilution with water (10ml.) and acidification (5N hydrochloric acid), the aqueous solution was extracted with chloroform, and the organic

extract washed with water and evaporated to give an oil (12mg.). This was shown by t.l.c. to consist of four components, R_f 0.45, 0.43, 0.31 and 0.27, corresponding to the two starting norcanadensol alcohols and their C-2 epimers, and appeared identical on t.l.c. to the mixture of alcohols obtained from a carboxy-inversion reaction of the quaternary mixture of dihydrocanadensic acid C-2, C-10 diastereoisomers.

Oxidation of Norcanadensol A (112) using Jones Reagent.

Norcanadensol A (25 mg.) in 'analar' acetone (10ml.) was cooled to ice temperature. Jones reagent (0.4 ml., 266 mg./ml. CrO_3 , 405 mg./ml. H_2SO_4) was added dropwise and the solution allowed to stand 15 minutes. Methanol (2ml.) and water (15ml.) was added, and the aqueous solution extracted with aqueous sodium bicarbonate, water and evaporated to give the acyl lactone (116), an oil (20mg., 80%) which distilled at $70^\circ/0.3$ mm.

T.l.c. 0.50, CHCl_3 (yellow-brown).

i.r. ν_{max} . (CCl_4): 1780 (γ -lactone), 1728 (ketone), 1162, 1100, 930 cm^{-1} .

u.v. λ_{max} . (EtOH): No absorption > 210 nm.

n.m.r. 100 Mc/s. (CDCl_3) τ : 5.47 (1H, m, H-4), 6.27 (1H, m, H-2), 7.10 - 8.0 (2H, m, 2H-3), 7.40 (3H, s, 3H-11), 8.5 (6H, m, 2H-5 and sat. $-\text{CH}_2-$), 9.07 (3H, t, $J = 6.0$ Hz., CH_3-CH_2-).

m.s. m/e (Rel abundance) : M^+ 184 (5), 156 (6), 142 (24), 127 (11), 124 (15), 114 (10), 100 (24), 99 (10), 96 (11), 85 (9), 82 (21),

73 (14), 71 (14), 70 (13), 55 (23), 43 (100), 41 (19).

Analysis Found : C, 65.40; H, 8.79%; M^+ at m/e 184.

$C_{10}H_{16}O_3$ requires C, 65.19; H, 8.75%; M.M. 184.2.

Oxidation of Norcanadensol B (113).

(a) With Jones reagent.

Norcanadensol B (20mg.) in analar acetone (5ml.) was cooled to ice temperature, and Jones reagent (1.0ml.) was added dropwise. Complete oxidation of the alcohol had not occurred after 2 hours (t.l.c.) when the reaction was worked up in the usual way to give an oil (17mg.). P.l.c. on Kieselgel H (0.75 mm. x 20 cm. x 20 cm.) gave acyl lactone (116) (8mg.) identical in i.r. and n.m.r. spectra to an authentic sample.

(b) With Silver Carbonate.

Norcanadensol B (10mg.) in dry benzene (10ml.) was refluxed with silver carbonate on celite (50 mg., 0.57g = 1 m.mole Ag_2CO_3) for 2 days. The mixture was filtered, the filtrate extracted with water. Evaporation of the benzene gave norcanadensol B (9mg.) identical in i.r. and t.l.c. to the starting material. (A similar reaction using norcanadensol A was likewise unsuccessful).

(c) With Sarett Reagent.

Norcanadensol B (15mg.) in dry pyridine (5ml.) was treated dropwise with Sarett reagent (1ml.). The solution was left at R.T. for 2 days, then diluted with water (15ml.) and extracted with

chloroform. The organic extract was washed with 5N hydrochloric acid, water and evaporated to give an oil (12mg.) shown by t.l.c. and i.r. spectrum to be unreacted starting material.

Decarboxylation of the Acyl-lactone (116).

The acyl-lactone (25mg.) was dissolved in dioxan (2ml.) and 2.5N sulphuric acid (10ml.) added. The solution was heated to 90° on an oil bath under nitrogen, the effluent gas being monitored for carbon dioxide by bubbling it through a solution of barium hydroxide in the usual way. After 1 hour at this temperature the precipitated barium carbonate was obtained by centrifuging the solution, decanting the liquid, and was washed with water and acetone resp. (10mg., 37%).

The reaction solution was cooled and extracted with chloroform, and the organic extract washed with water and dried ($MgSO_4$). Evaporation of the solvent yielded an oil (15mg.), which appeared by t.l.c. to contain several components found to be inseparable by p.l.c. (0.75 mm. x 20 cm. x 20 cm.). Attempts to form a characterisable 2,4-dinitrophenylhydrazone using the normal procedure were likewise unsuccessful.

Cleavage of the Acyl-lactone (116) to Caprylolactone(111).

The acyl-lactone (30mg.) in ethanol (2ml.) was treated with 50% potassium hydroxide (4ml.) and the solution refluxed for 1 hour. The solution was cooled, acidified using 5N hydrochloric acid and the aqueous solution extracted with chloroform. The organic extract was washed with

water and evaporated to give an oil (25mg.) which was purified by p.l.c. on Kieselgel H (0.75 mm. x 20 cm. x 20 cm.) using iodine vapour for detection, to give an oil (18mg., 82%) whose i.r. spectrum was identical to that recorded for an authentic sample of capryloleone.

C.D. in methanol (C 0.47): ΔE_{300} , 0.0; ΔE_{250} , -0.01; ΔE_{216} , -0.17.

T.l.c. 0.48, CHCl_3 (pale grey).

i.r. ν_{max} . (thin film): 1768 (γ -lactone), 1215, 1188, 1130, 1030, 995, 931 cm.^{-1} .

n.m.r. 60Mc/S. (CCl_3) τ : 5.50 (1H, m, H-4), 7.4 (2H, m, 2H-2), 7.5 - 8.2 (2H, m, 2H - 3), 8.5 (6H, m, 2H-5 and sat. $-\text{CH}_2-$), 9.03 (3H, t, $\text{CH}_3 \cdot \text{CH}_2-$).

Attempted Isomerisation of the Methyl Canadensate C-2 Epimers (55).

The mixture of canadensate C-2 epimers (15mg.) in triethylamine (0.5ml.) was refluxed overnight. The solvent was removed, chloroform (10ml.) added, and the organic solution extracted with 5N hydrochloric acid, water and evaporated to give an oil (12mg.) identical in i.r. spectrum and on t.l.c. to starting material.

Isomerisation of the Mixture of Canadensic Acid C-2 Epimers (55).

The canadensic acid C-2 epimers (60 mg.) were stored in dimethylsulphoxide* (15ml.) at R.T. overnight. Chloroform (50ml.) was added and the solution extracted with water (3 x 10 ml.), dried (MgSO_4) and the solvent evaporated to give a solid (41mg.). This material was refluxed for 6 hours in analar acetone (15ml.) with dry potassium carbonate (40mg.) and methyl iodide (240 mg.). The solution

was filtered and the solvent removed in vacuo, chloroform (15ml.) added and the organic solution extracted with water, and evaporated to give an oil, (40mg.). This was shown by g.l.c. and spectral data to be a mixture consisting of 1 part of the methyl canadensate C-2 epimers (57) to 1 part of the methyl ester of isomeric acid (123, R=Me). Attempts to separate these by p.l.c. were unsuccessful.

T.l.c. 0.59, CHCl_3 (brown).

i.r. ν_{max} . (thin film): 1760 (γ -lactone), 1715 (CO_2Me), 1707 unsat. CO_2Me), 1680 (tetrasubs. $\text{C} = \text{C}$), 1615 (terminal methylene), 1300, 1235, 1190, 1158, 1025, 980 cm.^{-1} .

n.m.r. 60Mc/s. (CDCl_3) τ : 3.58 ($\frac{1}{2}\text{H}$, s), 3.60 ($\frac{1}{2}\text{H}$, s), 4.14 ($\frac{1}{2}\text{H}$, s), 4.19 ($\frac{1}{2}\text{H}$, s), 5.45 (1H, m), 6.15 ($1\frac{1}{2}\text{H}$, s, -OMe), 6.25 ($1\frac{1}{2}\text{H}$, s, -OMe), 7.4 - 8.0 (2H, m), 7.98 ($1\frac{1}{2}\text{H}$, $J = 1\frac{1}{2}\text{Hz.}$), 8.3 (2H, m, 2H-5), 8.5 (4H, m, sat. - CH_2 -), 9.08 (3H, t, $J = 6.0\text{ Hz.}$, $\text{CH}_3\text{-CH}_2$ -).

G.c.m.s. - see discussion.

G.l.c. of the above reaction mixture was carried out on a pye-argon chromatogram using 4 ft. columns at 141° with a flow rate of 45 ml./minute.

* This reaction did not work with redistilled DMSO. At least 5% water was present in the reagent utilised above.

<u>Column</u>	<u>Reaction Mixture</u>	<u>Methyl Canadensate C-2 Epimers (57)</u>
1% SE30	R _t . 4.78, 6.45 min.	R _t . 4.78 min.
1% QFI	R _t . 6.93, 11.41 min.	R _t . 6.93 min.
5% APL	R _t . 23.40, 33.04 min.	R _t . 23.40 min.
<u>Retention Index</u>	<u>1774, 1786</u>	<u>1774</u>

* This reaction did not work with redistilled DMSO. At least 5% water was present in the reagent utilised above.

Hydrogenation of Mixture of 1 part Isomeric ester (123, R=Me) to 1 part Methyl Canadensate C-2 Epimers (57).

The mixture of isomeric ester (30mg.) in ethanol was stirred with platinum oxide (10mg.) in an atmosphere of hydrogen for 1 hour. The solution was filtered (glass paper) and the solvent evaporated to give an oil (28 mg., 93%). This appeared identical in i.r. and n.m.r. spectra to the quaternary mixture of the dihydrocanadensic acid C-2, C-10 diastereoisomers methyl esters (109).
G.l.c. on 10% APL at 163° (Pye argon, 4 ft. columns).

<u>Solution</u>	<u>R_t Peaks</u>	<u>Ratio of Peak Areas</u>
Hydrogenation product	26.8 min.	3
	29.0 min.	1
Quaternary mixture (109)	26.8 min.	1
	29.0 min.	1

Attempted Desaturation of Methyl Canadensate (100).

Methyl canadensate (30mg.) in dry dioxan containing 2,3-dichloro

-4,5-dicyanobenzoquinone (10mg.) was refluxed for 24 hours.

The solution was filtered and the resulting oil purified by p.l.c. on Kieselgel H, to give unreacted starting material (22mg.) identified by t.l.c. and its i.r. spectrum.

Dihydrocanadensic Acid Amide (Mixture of C-10 Epimers) (139).

Dihydrocanadensic acid mixture of C-10 epimers (30mg.) in benzene (5ml.) containing oxalyl chloride (0.2ml.) was refluxed for 1½ hours. The solvent was evaporated, and a saturated solution of ammonia in benzene (2ml.) added to the reaction flask. After 5 minutes, the solvent was evaporated to give an oil (33mg.) purified by p.l.c. on Kieselgel H using iodine vapour for detection. The dihydrocanadensic acid amide C-10 epimers were obtained on elution of the major band as an oil (25 mg.) (78%).

T.l.c. 0.13, 0.14, 2% MeOH in CHCl₃ (brown).

i.r. ν_{max} . (thin film): 3,400 and 3,210 (CO.NH₂), 1750 (δ -lactone), 1660 (br.) (CO.NH₂), 1460, 1422, 1350, 1280, 1183, 1130, 1065, 1017, 964 cm.⁻¹.

n.m.r. 60Mc/3. (CDCl₃) τ : 3.87 (2H, m, -CO.NH₂), 5.5 (1H, m, H-4), 7.1 (2H, m, H-2 and H-10), 7.5 - 8.1. (2H, m, 2H-3), 8.2 (2H, m, 2H-5), 8.5 (4H, m, sat.-CH₂-), 8.80 (3H, d, J = 7.0 Hz., 3H -11), 9.07 (3H, m, CH₃-CH₂-).

Attempted Barton Reaction on the Mixture of Dihydrocanadensic Acid Amide C-10 Epimers (139).

To dihydrocanadensic acid amide C-10 epimers (40mg.) in analar

chloroform, was added iodine and then lead tetraacetate (320mg.), and the solution was irradiated with u.v. light from a mercury lamp for 5 hours at 15°. The solvent was then evaporated, 2N sodium hydroxide (10ml.) added and the solution allowed to stand at R.T. for 18 hours. After acidification, the aqueous solution was extracted with chloroform, and the organic extract washed with dilute aqueous sodium thiosulphate (2 x 10ml.), water and evaporated to give a crystalline solid shown by t.l.c., i.r. spectrum and m.p. to be dihydrocanadensic acid mixture of C-10 epimers (107) (35mg., 87%). A sample of the crude reaction product was methylated using ethereal diazomethane. G.l.c. of the resulting oil on a 1% SE30 column at 125° gave only one peak corresponding to the methyl dihydrocanadensate C-10 epimers, and no peak corresponding to dihydrocanadensolide (54) or related dilactonic compounds.

EXPERIMENTAL.

CHAPTER 4.

Hydroxyisocadenensic Acid (148).

The metabolite was isolated as described in Chapter 1, and crystallised from ether-light petroleum as colourless needles, m.p. 147°.

O.R.D. in methanol (C 0.56): $[\Phi]_{400}^0$, 0; $[\Phi]_{287}^0$, +1395, $[\Phi]_{266}^0$, +900, $[\Phi]_{256}^0$, +1800, $[\Phi]_{238}^0$, -4,460, $[\Phi]_{227}^0$, -3,280.

T.l.c. 0.40, (35) benzene-(5) dioxan-(1) acetic acid (brown).

i.r. ν_{\max} . (K.Br.): 3,600 and 3,420 (-OH), 3,300 - 2,600 (CO₂H), 1759 (δ -lactone), 1708 (CO₂H), 1770 (wk.) (C=C), 1224, 1093, 1035, 831, 722 cm.⁻¹.

u.v. ν_{\max} . (EtOH): 229 nm. (ϵ 7,900).

n.m.r. 100 Mc/s. (CDCl₃) τ : 4.91 (1H, m, H-3), 5.70 (1H, m, H-4), 6.1 (2H, m, D₂O exchangeable - OH), 7.76 (3H, s, 3H-11), 8.3 (2H, m, 2H-5), 8.6 (4H, m, sat. -CH₂-), 9.04 (3H, t, J=6.0 Hz., CH₃-CH₂-).

m.s. m/e (Rel. abundance): (M+1)⁺ 229 (1.4), 228 (100), 210 (6.6), 192 (6.5), 153 (23), 143 (15), 142 (100), 141 (50), 125 (12), 124 (50), 113 (78), 87 (17), 86 (22), 85 (35), 69 (62), 68(30), 67 (51), 59 (85), 58 (42).

Analysis Found: C, 57.68; H, 6.85%; M⁺ at m/e 228.

C₁₁H₁₆O₅ requires C, 57.89; H, 7.07%; M.W. 228.

Methyl Hydroxyisocanadesate (143).

Hydroxyisocanadensic acid (35 mg.) in methanol (2ml.) at ice temperature was treated with an excess of ethereal diazomethane and the solution allowed to stand 10 minutes. The solvent was then evaporated to give methyl hydroxyisocanadesate (32 mg., 86%) which crystallised from ether-light petroleum as long, colourless needles, m.p. 66°.

O.R.D. in methanol (C0.93): $[\Phi]_{400}^{20}$, 0; $[\Phi]_{286}^{20}$, + 1820;

$[\Phi]_{258}^{20}$, 0; $[\Phi]_{254}^{20}$, - 240; $[\Phi]_{227}^{20}$, -24,900.

C.D. in methanol (C0.56): ΔE_{274}^{20} , +1.13; ΔE_{243}^{20} , + 4.02, ΔE_{208}^{20} , -6.31.

T.l.c. 0.42, CHCl_3 (brown).

i.r. ν_{max} . (K.Br.): 3,400 (-OH), 1758 and 1745 (δ -lactone),

1728 (CO_2Me), 1228, 1070, 1036, 830 cm^{-1} .

ν_{max} . (thin film): 3,520 (-OH), 1760 (δ -lactone),

1725 (CO_2Me), 1760 (wk.) (C=C), 1225, 1105, 1047, 1028, 770 cm^{-1} .

ν_{max} . (CHCl_3): 3,590 cm^{-1} , 1763 cm^{-1} (ϵ 677), 1715 cm^{-1}

(ϵ 472).

u.v. λ_{max} . (EtOH): 230 nm. (ϵ 11,800).

n.m.r. 100 Mc/s. (CDCl_3) τ : 4.94 (1H, dq., J = 2.0, 1.5 Hz., irr.

5.86 \longrightarrow q (J = 2.0 Hz.), irr. 7.80 \longrightarrow d (J = 1.5 Hz.),

H-3), 5.86 (1H, dt, J = 6.0, 1.5 Hz., irr. 8.2 \longrightarrow (J = 1.5 Hz.),

H-4), 5.9 (1H, m, D_2O exchangeable, -OH), 7.80 (3H, d,

J = 2.0 Hz., irr. 4.94 \longrightarrow s, 3H-11), 8.2 (2H, m, 2H-5), 8.6

(4H, m, sat. - CH_2 -), 9.06 (3H, t, J = 6.0 Hz., CH_3 - CH_2 -).

m.s. m/e (Rel. abundance): M^+ 242 (0.08), 224 (1:1), 211 (0.2), 185 (1:7), 157 (14), 156 (100), 128 (7), 127 (36), 125 (19), 124 (48), 99 (15), 98 (9), 97 (14), 96 (14) 88 (9), 87 (7), 85 (11), 69 (25), 68 (17), 67 (40), 59 (18), 58 (20), 57 (16).
 M^+ at m/e 242.1105, $C_{12}H_{18}O_5$ requires 242.1154.

Analysis Found : C, 59.44; H, 7.57%; M^+ at m/e 242.

$C_{12}H_{18}O_5$ requires C, 59.49; H, 7.49%; M.W. 242.1.

Lithium Aluminium Hydride Reduction of Methyl Hydroxyisocanadensate (141).

Methyl hydroxyisocanadensate (47 mg.) in dry ether (20ml.) containing lithium aluminium hydride (75 mg.) was refluxed for 12 hours. Water (2ml.) was added dropwise, and the precipitate obtained dissolved in 6N NaOH (15 ml.). The ether was decanted, and the aqueous solution extracted with ethyl acetate. The combined organic extracts were washed with water, and evaporated to give the mixture of tetrols (141) as an oil (35 mg., 73%) which appeared as three spots on t.l.c.

T.l.c. 0.20, 0.23, 0.30, 10% MeOH in $CHCl_3$ (brown).

i.r. ν_{max} . (thin film): 3450 (-OH), 2995, 1465, 1380, 1250, 1125, 1040 cm^{-1} .

u.v. ν_{max} . (EtOH): No absorption > 200 nm.

n.m.r. 60Mc/s. ($CDCl_3$) τ : 4.4 (6H, m, - $\overset{|}{C}H-OH$), 4.7 (4H, m, D_2O exchangeable, -OH), 8.6 (8H, m, sat. - CH_2-), 9.07 (3H, m, $\overset{|}{C}H_3-CH-$).

The tetrol (141) (30mg) was oxidised with sodium periodate (0.30 g.) in water (10ml.) for 1 hour. The aqueous solution was then steam-

distilled into an ethenolic solution of 2,4-dinitrophenylhydrazine (30 mg. in 5 ml.), when n-valeraldehyde 2,4-dinitrophenylhydrazone (20mg.) was obtained, and purified on Kieselgel H and crystallised from ethanol as needles m.p. 106° , identical with an authentic sample.

Methyl Acetoxyisocanadensate (146).

Methyl hydroxyisocanadensate (40 mg.) in acetic anhydride (0.3 ml.) containing pyridine (0.05 ml.) was left at R.T. overnight. The solution was then concentrated in vacuo chloroform (15 ml.) added, and extracted with 5 N hydrochloric acid, saturated aqueous sodium bicarbonate and water. Evaporation of the solvent gave methyl acetoxyisocanadensate (35 mg., 74%) as an oil, b.p. 95° under 0.5 mm.

T.l.c. 0.75, CHCl_3 (brown).

i.r. ν_{max} . (thin film): 1765 (δ -lactone), 1740 (CO_2Me and $\text{CH}_3\text{CO}_2\text{O}$), 1670 (wk.) ($\text{C}=\text{C}$), 1343, 1222, 1105, 1050, 1025, 965 cm^{-1} .

ν_{max} . (CCl_4): 1778 (δ -lactone), 1745 (CO_2Me and $\text{CH}_3\text{CO}_2\text{O}$), 1668 (wk.) ($\text{C}=\text{C}$), 1237, 1092, 1048, 1021, 810, 760 cm^{-1} .

u.v. λ_{max} . (hexane): 228 nm. (ϵ 9,760).

n.m.r. 60Mc/S. (CDCl_3) τ : 4.68 (1H, t, $J=6.0$ Hz., H-4), 4.84 (1H, q, $J=2.0$ Hz., H-3), 6.12 (3H, s, -OMe), 7.83 (3H, d, $J=2.0$ Hz., 3H-11), 8.10 (3H, s, $\text{CH}_3\text{CO}_2\text{O}$ -), 8.2 (2H, m, 2H-5), 8.4 (6H, m, sat. $-\text{CH}_2-$), 9.10 (3H, t, $J=6.0$ Hz., CH_3-CH_2-).

m.s. m/e (Rel. abundance): (M+1)⁺ 285 (0.7), 284 (0.25), 225 (3.3),
224 (17), 199 (8), 198 (71), 157 (11), 156 (100), 129 (33),
124 (14), 123 (12), 69 (14), 67 (41), 59 (21), 55(31).
m/e 198 —————> m/e 156, m* at m/e 123.

Analysis Found : C, 59.10; H, 7.04%; M⁺ at m/e 284.

C₁₄H₂₀O₆ requires C, 59.14; H, 7.09%; M.W. 284.3.

Methyl Trifluoroacetoxisocanadensate.

Methyl hydroxyisocanadensate (25 mg.) was dissolved in trifluoroacetic anhydride (0.2ml.) and the solution left at R.T. overnight. The solvent was evaporated in vacuo to give methyl trifluoroacetoxisocanadensate (26 mg., 87%) as a slightly yellow oil.

T.l.c. 0.80, CHCl₃ (yellow-brown).

i.r. ν max. (CCl₄): 1790 (γ-lactone), 1745 (CO₂Me and F₃CO.O),
1340, 1161, 1048 cm.⁻¹.

n.m.r. 60 Mc/S. (CDCl₃) τ : 4.50 (1H, dt, J=7.0, 1.5 Hz., H-4),
4.80 (1H, dq, J=2.0, 1.5 Hz., H-3), 6.10 (3H, s, -OMe),
7.80 (3H, d, J=2.0 Hz., 3H-11), 8.3 (2H, m, 2H-5), 8.5
(4H, m, sat. -CH₂-), 9.08 (3H, t, J=6.0 Hz., CH₃ -CH₂-).

Oxidation of Methyl Hydroxyisocanadensate (143) to Ketone (152).

Methyl hydroxyisocanadensate (45 mg.) in 'analar' acetone (5ml.) at ice temperature was treated dropwise with Jones reagent until the red colour was not discharged, and the solution left 20 minutes. Methanol (0.1 ml.) was added, then water (10ml.) and the aqueous solution

extracted with chloroform. The organic extract was washed with saturated aqueous hydrogen carbonate, water and evaporated, and the resulting oil was then submitted once more to the above oxidation procedure. The ketone (152) (35 mg., 78%) was obtained as an unstable yellow oil b.p. $95^{\circ}/0.3$ mm.

T.l.c. Rapid decomposition occurs on attempted t.l.c.

i.r. ν_{max} . (thin film): 1770 (δ -lactone), 1722 (CO_2Me and $\text{C}=\text{O}$), 1225, 1080, 1015 cm.^{-1} .

ν_{max} . (CCl_4): 1780 (δ -lactone), 1732 (CO_2Me and $\text{C}=\text{O}$), 1218, 1075, 1025 cm.^{-1} .

u.v. λ_{max} . (1 hexane: 1 EtOH): 215 nm. (ϵ 9,280), 240 nm. (ϵ 5,190), 382 nm. (ϵ 721).

λ_{max} . (+ NaOH): 220 nm. (ϵ 10,420), 382 nm. (ϵ 14,850).

n.m.r. 60 Mc/S. (CDCl_3) τ : 4.53 (1H, q, $J=2.0$ Hz., H-3), 6.13 (3H, s, -OMe), 7.37 (2H, m, 2H-5), 7.77 (3H, d, $J=2.0$ Hz., 3H-11), 8.50 (4H, m, sat. $-\text{CH}_2-$), 9.10 (3H, t, $J=6.0$ Hz., CH_2-CH_2-).

m.s. m/e (Rel. abundance) M^+ 240 (0.6), 224 (2.5), 172 (8), 156 (4.5), 154 (3), 140 (11), 139 (4.3), 86 (8), 85 (100), 67 (25), 57 (86).

Analysis Found : C, 59.80; H, 6.83%; M^+ at m/e 240.

$\text{C}_{12}\text{H}_{16}\text{O}_5$ requires C, 59.99; H, 6.71%; M.W. 240.3.

Pseudo-acid (153) from Ketone (152).

(a) On P.L.C.

Attempted purification of ketone (152) (20 mg.) by p.l.c. on

Kieselgel HF₂₅₄ (0.75 mm x 20 cm. x 20 cm.) using chloroform as eluant resulted in the yellow band (under visible light) of the ketone giving rise to a dark-blue band (under u.v. light of 254 n.m.). Elution of this band with ethyl acetate gave the pseudo-acid (15 mg., 70%) as an oil, b.p. 85°/0.1 m.m.

T.l.c. 0.45, CHCl₃ (brown).

i.r. ν max. (thin film): 3,420 (-OH), 1780 (γ -lactone), 1735 (CO₂Me), 1670 (wk.)(C=C), 1235, 1162, 1130, 1078, 1044, 968, 755 cm.⁻¹.

ν max. (0.03M, CCl₄): 3,430 (-OH), 1790 (γ -lactone), (ϵ 521, $\Delta v_{\frac{1}{2}}$ 20 cm.⁻¹), 1739 (CO₂Me and C=O) (ϵ 504, $\Delta v_{\frac{1}{2}}$ 25 cm.⁻¹), 1675 (wk.)(C=C), 1230, 1162, 1131, 1078, 1045, 972 cm.⁻¹.

u.v. λ max. (hexane): 219 nm. (ϵ 10,300).

n.m.r. 60Mc/S. (CDCl₃) τ : 6.17 (3H, s, -OMe), 7.50 (2H, m, 2H-5) 7.70 (3H, s, 3H-11), 8.40 (4H, m, sat. -CH₂-), 9.10 (3H, t, J=6.0 Hz., CH₃-CH₂-).

m.s. m/s (Rel. abundance): M⁺ 256 (0.4), 225(3.0), 212 (11), 181 (10), 180 (12), 172 (10), 169 (17), 156 (53), 137 (46), 124 (63), 85 (80), 67 (85), 57 (100).

Analysis Found : C, 56.45; H, 6.47%; M⁺ at m/e 256.

C₁₂H₁₆O₆ requires C, 56.25; H, 6.29%; M.W. 256.

(b) Using Oxygen/Ethanol.

Ketone (152) (30 mg.) was dissolved in ethanol and oxygen

bubbled through the solution until the yellow colour disappeared (ca. 2 hours). The solvent was evaporated to give an oil (32 mg.) Purification of this by p.l.c. gave the pseudo-acid (22 mg., 64%) identical to an authentic sample (prepared by method (a)) on t.l.c., and in its i.r. and n.m.r. spectra.

Lactol Ether (159).

The pseudo-acid (32 mg.) in methanol (10ml.) and concentrated sulphuric acid (0.05 ml.) was refluxed for 4 hours. The solution was concentrated in vacuo , water (10 ml.) added and the aqueous solution extracted with chloroform. The organic extract was then washed with saturated aqueous sodium hydrogen carbonate, water and evaporated to give an oil, which was purified by p.l.c. on Kieselgel HF²⁵⁴. The lactol ether (22 mg., 68%) was obtained as an oil, b.p. 72⁰/0.01mm.

T.l.c. 0.45, CHCl₃ (yellow-brown).

i.r. $\nu_{\text{max.}}$ (CCl₄): 1790 (γ -lactone), 1735 (CO₂Me), 1330, 1240, 1195, 1130, 1078, 1000, 965, 945 cm.⁻¹.

n.m.r. 60 Mc/S. (CDCl₃) τ : 6.13 (3H, S, CO₂Me), 6.67 (3H, S, -OMe), 7.20 (2H, m, 2H-5), 7.70 (3H, S, 3H-11), 8.50 (4H, sat. -CH₂-), 9.10 (3H, t, J=6.0 Hz., CH₃ - CH₂-).

m.s. m/e (Rel. abundance): M⁺ 270 (0.04), 239 (5.3), 186 (67), 185 (23), 171 (11), 154 (32), 153 (37), 125 (25), 85 (100), 67 (42), 57 (93).

Analysis Found : C, 57.74; H, 6.49%; M⁺ at m/e 270.

C₁₃H₁₈O₆ requires C, 57.77; H, 6.71%; M.W. 270.3

Acetylation of Pseudo-Acid (153).

The pseudo-acid (33mg.) in acetic anhydride (2ml.) containing *p*-toluenesulphonic acid (2mg.) was refluxed for 30 minutes. The solvent was then evaporated, chloroform (15ml.) added and the organic solution extracted with aqueous sodium hydrogen carbonate, water and evaporated to give an oil. The oil was purified by chromatography on Kieselgel HF₂₅₄, when elution of the major band with ethyl acetate gave the tertiary acetate (152) (19 mg., 49%) as a colourless oil b.p. 82°/0.01 mm.

T.l.c. 0.70, CHCl₃ (brown).

i.r. ν_{\max} . (CCl₄) : 1800 and 1795 (gem acetoxy lactone), 1742 (CO₂Me and C=O), 1335, 1200 (br.), 1078, 1038, 982 cm.⁻¹.

n.m.r. 60Mc/S. (CDCl₃) τ : 6.13 (3H, s, -OMe), 7.30 (2H, m, 2H-5), 7.72 (3H, s, 3H-11), 7.80 (3H, s, CH₃CO.O-), 8.5 (4H, m, sat. -CH₂-), 9.08 (3H, t, J=6.0 Hz., CH₃-CH₂-).

m.s. m/e (Rel. abundance): M⁺ 298 (0.48), 256(12), 172 (36), 164 (15), 150 (16), 85 (100), 67 (35), 57 (85).

Analysis Found : C, 56.60; H, 6.36%; M⁺ at m/e 298.

C₁₄H₁₈O₇ requires C, 56.37; H, 6.06%; M.W. 298.3.

Attempted Cyclisation of Hydroxyisocanadensic Acid (148).

(a) using p-toluenesulphonic Acid.

Hydroxyisocanadensic acid (12mg.) was refluxed in dry benzene (10ml.) containing *p*-toluenesulphonic acid (3 mg.) for 2 days. The reaction was worked up in the usual way to give an oil (11 mg.) which contained no neutral products.

(b) using Sulphuric Acid

Hydroxyisocanadensic acid (6mg.) in concentrated 98% sulphuric acid (2ml.) was left at R.T. for 1 week, and worked up in the usual way to give an oil (4 mg.) which again contained no neutral products.

(c) using Polyphosphoric Acid

Hydroxyisocanadensic acid (15 mg.) in polyphosphoric acid (3 ml.) was left at 50° for 6 hours, and gave on work-up a reddish oil (12mg.) which appeared to consist of several acidic compounds by t.l.c.

Treatment of Hydroxyisocanadensic Acid (148) with Methyl Orthoformate.

Hydroxyisocanadensic acid (50mg.) in dry benzene (20ml.) and methyl orthoformate (0.5 ml.) was refluxed for 3 days. The solvent was removed in vacuo to give an oil (52 mg.), purified by p.l.c. on Kieselgel HF₂₅₄ to give methyl hydroxyisocanadensate (24 mg.) as the major component. This was identical in i.r. and n.m.r. spectrum to a sample prepared by methylation of hydroxyisocanadensic acid using diazomethane.

Treatment of Hydroxyisocanadensic Acid (148) with Anhydrous Copper Sulphate in Acetone.

Hydroxyisocanadensic acid (20 mg.) was stirred with anhydrous copper sulphate (50mg.) in acetone (5ml.) at R.T. for 3 weeks. The solution was then diluted with water (20ml.) and extracted with chloroform. The organic extract was washed with aqueous sodium hydrogen carbonate, water and evaporated to give a crystalline solid (16mg.) identical on t.l.c. and i.r. spectrum to the starting material.

U.V. Irradiation of Methyl Hydroxyisocanadensate (143).

Methyl hydroxyisocanadensate (32 mg.) in dry ether (10ml.) (under oxygen free nitrogen) was irradiated with light from a mercury lamp for 3 days. The solvent was removed to give an oil (30mg.), which although t.l.c. identical to the starting material, appeared to be a mixture of compounds (171) from its spectral data.

T.l.c. 0.41, CHCl_3 (brown).

i.r. ν_{max} . (thin film): 3,550 (-OH), 1770 (γ -lactone) 1730 (CO_2Me), 1205 (br.), 1110, 1020, 770 cm^{-1} .

u.v. λ_{max} . (EtOH): No absorption > 200 nm.

n.m.r. 60Mc/S. (CDCl_3) τ : 4.9 ($\frac{1}{2}$ H, m), 5.4 (1H, m), 6.17 (3H, br. s, -OMe), 6.9 (1H, m, -OH), 8.6 (9H, m, sat. $-\text{CH}_2-$), 9.10 (3H, τ , CH_3-CH_2-).

Preparation of Diene (172).(a) From Methyl Hydroxyisocanadensate (143) using Phosphorus Pentachloride.

Methyl hydroxyisocanadensate (45 mg.) in dry ether (10ml.) was stirred with phosphorus pentachloride (40mg.) for 36 hours. The solvent was removed in vacuo to give an oil (50 mg.), which was purified by p.l.c. on Kieselgel HF₂₅₄. Elution of the major band under the u.v. (254 nm) gave the diene (172) (20mg., 52%) as an unstable oil identical on t.l.c. and in i.r. spectrum to a sample prepared by method (C).

(b) From Methyl Hydroxyisocanadensate (143) using Thionyl Chloride.

Methyl hydroxyisocanadensate (50 mg.) in thionyl chloride (2ml.)

and pyridine (0.1 ml.) was refluxed for 2 hours. The solvent was evaporated, and the resulting oil (65mg.) purified by p.l.c. in the manner described in (a) to give the diene (172) (31 mg., 73%) as an oil, identical in t.l.c. and i.r. properties to a sample prepared by method (C).

(C) From Methyl Acetoxyisocanadensate (146).

Methyl acetoxyisocanadensate (43 mg.) was added to sodium hydride (20 mg.) (oil emulsion free by prior washing with light petroleum) in toluene (15 ml.) and the solution refluxed with stirring under nitrogen for 72 hours. The toluene solution was then extracted several times with water and evaporated to give the diene (172) (28 mg., 88%) as an unstable oil, which solidified on storing under nitrogen at ca. -20° .

T.l.c. 0.8l, 25% light petroleum in CHCl_3 (light brown).

i.r. ν_{max} . (CCl_4): 1735 (γ -lactone), 1730 (CO_2Me), 1660 ($\text{C}=\text{C}$), 1616 ($\text{C}=\text{C}$), 1335, 1220 (br.), 1075, 1030, 978, 920 cm^{-1} .

u.v. λ_{max} . (hexane): 234 nm. (ϵ 6,560), 308 nm. (ϵ 9,550).

n.m.r. 100Mc/s. (CCl_3) τ : 4.03 (1H, t, $J = 8.0$ Hz., irr. 7.60 \longrightarrow S, H-4), 6.10 (3H, S, -OMe), 7.60 (2H, m, 2H-5), 7.77 (3H, S, 3H-11), 8.6 (4H, m, sat. $-\text{CH}_2-$), 9.08 (3H, t, $J = 6.0$ Hz., $\text{CH}_3 - \text{CH}_2-$).

Attempted Hydrogenation of Hydroxyisocanadensic Acid (148).

Hydroxyisocanadensic acid (148) was found to be unaffected by hydrogenation at R.T. under the following conditions :

- (a) Platinum oxide in ethanol at atmospheric pressure.
- (b) 10% Palladium-on-charcoal in acetic acid at atmospheric pressure.
- (c) 10% Palladium-on-charcoal in acetic acid (99%) - perchloric acid (1%) at 5 atmospheres pressure.

Reduction of Methyl Hydroxyisocanadensate (143) with Zinc Dust.

The zinc dust was activated by washing with 5% hydrochloric acid (5X), water (5X), methanol (5X), ether (5X) and drying in vacuo for 24 hours.

To methylhydroxyisocanadensate (40 mg.) in glacial acetic acid (2ml.) and concentrated hydrochloric acid (2ml.) was added activated zinc dust (40mg.), and the solution refluxed (with the addition of a further amount of zinc dust (10mg.) after 1 hour) for 2 hours. The solution was then diluted with water (30,ml.) and extracted with chloroform. The organic extract was washed with aqueous sodium hydrogen carbonate, water and evaporated to give an oil (28mg.), shown by t.l.c. to consist of two components R_f s 0.52 and 0.49, CHCl_3 . These were separated by p.l.c. on Kieselgel HF₂₅₄ (0.75 mm. x 20cm. x 20 cm.) in a CHCl_3 (3)/light petroleum (1) mixture employing multiple elution, and using iodine vapour for detection.

Component of R_f 0.52. This was identical on t.l.c., in i.r. spectrum, m.p. and mixed m.p. to an authentic sample of dihydrocanadensolide. Its optical rotation ($[\alpha]_D = -46^\circ$) was similar to that recorded for

dihydrocanadensolide ($[\alpha]_D = -33^{054}$).

Component of R_f 0.49. This was identical on t.l.c., in i.r. spectrum, m.p. and mixed m.p. to an authentic sample of 10-epidihydrocanadensolide (61) prepared by hydrogenation of canadensolide (51). Its o.r.d. curve was identical to that recorded for 10-epidihydrocanadensolide.

EXPERIMENTAL.CHAPTER 5.Treatment of Canadensolide (51) with Diazomethane.

Canadensolide (45 mg.) in dry ether (5ml.) was treated with an excess of ethereal diazomethane (prepared from 0.5g. nitrosan), and the yellow solution stored at ice temperature overnight.

Evaporation of the solvent then gave an oily solid, shown by n.m.r. to consist of a 1:1 mixture of pyrazoline compounds. Crystallisation from chloroform gave needles, m.p. 107° , of Pyrazoline A (18mg., 33%).

P.l.c. of the oil obtained on evaporation of the mother liquor on Kieselgel HF₂₅₄ using iodine vapour for detection gave Pyrazoline B (15 mg., 27%) which crystallised from ether-light petroleum as needles, m.p. 100° .

Pyrazoline A crystallised from benzene-light petroleum as colourless needles m.p. 107° .

O.r.d. in methanol (C 1.6): $[\Phi]_{331} + 40, 600$; $[\Phi]_{306} - 26, 950$;
 $[\Phi]_{239} + 11,250$; $[\Phi]_{204} - 25,600$.

T.l.c. 0.14, CHCl_3 (yellow).

i.r. ν_{max} (KBr): 1798 (δ -lactone), 1775 (δ -lactone), 1550 (wk.)
 (N=N), 1368, 1208, 1094, 1054, 1045, 1000, 832, 812 cm^{-1} .

u.v. ν_{max} (EtOH): 213 nm. (ϵ 2,950), 293 nm. (ϵ 1,460).

n.m.r. 60 Mc/s. (CDCl_3) τ : 4.63 (1H, dd, $J=6.0, 8.0$ Hz., H-3), 5.17
 (2H, t, $J=8.0$ Hz., $-\text{CH}_2-\text{N}=\text{N}-$), 5.20 (1H, m, H-4), 6.43 (1H, d, $J=8.0$ Hz.,

H-2), 7.90 (2H, m, $-\underline{\text{CH}}_2-\text{CH}_2-\text{N}=\text{N}-$), 8.3 (2H, m, 2H-5), 8.5 (4H, m, sat. $-\text{CH}_2-$), 9.05 (3H, t, $J=6.0\text{ Hz.}$, $\underline{\text{CH}}_3-\text{CH}_2-$).

m.s. m/e (Rel. abundance): M^+ 252 (0.2), 224 (4); 180 (16); 165 (5), 150 (8), 138 (24), 137 (35), 124 (70), 123 (67), 110 (110), 82 (90), 81 (39), 71 (21), 67 (25), 57 (17), 55 (27), 54 (38), 53 (53), 43 (42), 41 (53).

Analysis Found: C, 57.40; H, 6.53; N, 11.37%; M^+ at m/e 252.

$\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_4$ requires C, 57.13; H, 6.39; N, 11.10%. M.W. 252.3.

Pyrazoline B crystallised from ether-light petroleum as colourless needles, m.p. 100° .

O.r.d. in methanol (see below).

T.l.c. 0.27, CHCl_3 (yellow).

i.r. ν_{max} (K.Br.): 1790 (δ -lactone), 1775 (δ -lactone), 1555 (wk.) ($\text{N}=\text{N}$), 1350, 1304, 1190, 1130, 1052, 1010, 990, 915, 838 cm.^{-1} .

u.v. 60 Mc/s. (CDCl_3) τ : 4.37 (1H, dd, $J=4.0, 8.0\text{ Hz.}$, H-3), 5.16 (2H, dd, $J=6.5, 7.0\text{ Hz.}$, $-\text{CH}_2-\text{N}=\text{N}-$), 5.35 (1H, m, H-4), 6.63 (1H, d, $J=6.0\text{ Hz.}$, H-2), 7.60 (2H, m, $-\underline{\text{CH}}_2-\text{CH}_2-\text{N}=\text{N}-$), 8.3 (2H, m, 2H-5), 8.5 (4H, m, sat. $-\text{CH}_2-$), 9.04 (3H, t, $J=6.0\text{ Hz.}$, $\underline{\text{CH}}_3-\text{CH}_2-$).

m.s. m/e (Rel. abundance): M^+ 252 (4), 224 (4), 180 (10), 163 (14), 138 (24), 137 (35), 124 (60), 123 (73), 110 (96), 82 (100),

80 (52), 57 (30), 55 (40), 54 (42), 53 (55).

Analysis Found : C, 57.14; H, 6.35; N, 11.00%; M^+ at m/e 252.

$C_{12}H_{16}N_2O_4$ requires C, 57.13; H, 6.39; N, 11.10%; M.W. 252.3.

O.r.d. in methanol (C 0.6): $[\eta]_{332}$, -25,700; $[\eta]_{307}$, +17,200;
 $[\eta]_{242}$, -6,000; $[\eta]_{216}$, +13,900.

Treatment of Avenaciolide (58) with Diazomethane.

Avenaciolide (30mg.) in methanol (2ml.) was treated with an excess of ethereal diazomethane, and the yellow solution (15ml.) left overnight at ice temperature. Evaporation of the solvent gave an oil, shown by n.m.r. to be a 3:1 mixture of pyrazoline compounds (35mg.), which decomposed on attempted separation by p.l.c.

By fractional crystallisation from ether-light petroleum the major (H_B /azo trans) pyrazoline (7mg., 20%) was obtained as needles, m.p. 83-86°.

n.m.r. 60 Mc/S. ($CDCl_3$) τ : 4.57 (1H, d, $J=7.0$ Hz.), 5.20 (2H, m, -CH₂-N=N-), 5.57 (1H, m), 6.83 (1H, dd, $J_1=J_2=7.0$ Hz.), 7.8 (2H, m, -CH₂-CH₂-N=N-), 8.3 (2H, m), 8.7 (12H, m, sat. -CH₂-), 9.12 (3H, t, CH₃-CH₂-).

Analysis Found: C, 62.30; H, 7.65%; N, 7.95%.

$C_{16}H_{24}N_2O_4$ requires C, 62.32; H, 7.84%; N, 9.08%; M.W. 308.4.

Partial Resolution Experiments with Methyl Hydroxyisocanadensate (143).

Methyl hydroxyisocanadensate and (-) - α -phenylbutyric anhydride in quantities shown in Table A were dissolved in dry pyridine (0.5ml.)

and allowed to stand at ice temperature for 18 hours. Water (0.1ml.) was then added and the solution heated gently on the steam bath for $\frac{1}{2}$ hour. Benzene (3ml.) and water (2ml.) were then added and the mixture titrated to the phenolphthalein end point using N/10 sodium hydride. The aqueous layer was extracted twice with chloroform, acidified with hydrochloric acid, and extracted with chloroform (2x10ml.). The latter chloroform extract was evaporated to give a solid which was dissolved in benzene, and after adjusting to 2ml., this solution was used for measurement of rotation.

Table A :

Expt. No.	<u>1</u>	<u>2</u>
Alcohol (mg.)	22.2	41.4
Anhydride (mM.)	0.224	0.345
Acid on hydrolysis (mM.)	0.374	0.555
Esterification (%)	80.7	76.0
Conc. for rotation (mg./ml.)	6.03	11.1
α obtained (°)	+ 0.07	+ 0.11
Optical yield (%)	+ 11.9	+ 10.6

EXPERIMENTAL.CHAPTER 6.Kuhn Roth Oxidation of Canadensic Acid (Mixture of C-2 Epimers) (55).

The mixture of canadensic acid C-2 epimers (32mg.) was refluxed for 1½ hours with 5ml. of cold oxidising reagent (prepared by adding 20 ml. of concentrated sulphuric acid to 16.8g of chromic anhydride in 100 ml. of water) in a long necked flask using a cold finger condenser. The condenser was then removed and washed free of any acid with as little water as possible (ca. 2ml.). Magnesium sulphate (7.0g.) was added and the flask set up for steam distillation, a flame being held under the reaction flask during passage of the steam to concentrate the contents. When 50 ml. had been collected, the distillate was titrated against sodium hydroxide solution (N/100) (using a pH meter) and was found to require 13.5 ml. to the end-point. A blank solution under the same conditions required 0.5 ml. sodium hydroxide (N/100).

$$\begin{aligned} \text{Terminal methyl No.} &= \frac{\text{normality of alkali} \times \text{vol. (ml.)} \times \text{M.W.}}{\text{grams of sample} \times 1000} \\ &= 0.86 \end{aligned}$$

A similar reaction using the quaternary mixture of dihydrocanadensic acid (C-2, C-10) diastereoisomers (56) (20.1mg.) required 16.5 ml. N/100 sodium hydroxide to the end-point, and hence the terminal methyl number of these diastereoisomers is 1.7.

Ozonolysis of the Canadensic Acid (Mixture of C-2 Epimers) (55).

The mixture of canadensic acid C-2 epimers (42.6 mg.) in ethanol (10ml.) was cooled to -25° (carbon tetrachloride/solid carbon dioxide bath) and ozone was passed through for $1\frac{1}{2}$ hours, the temperature being maintained at -25° . Nitrogen was then passed through the solution at ice temperature for $\frac{1}{2}$ hour, water (1ml) added and the solution left overnight at R.t. The solution was then steam distilled into an ethanolic solution of dimedone (50mg./5ml.) until 50ml. distillate had been collected, and the distillate concentrated and allowed to cool when needle crystals, m.p. 191° were obtained, and found not to depress the m.p. of an authentic sample of formaldehyde dimedone (26mg., 45%). The aqueous reaction solution and the steam distillate were both extracted with chloroform to give an oil (25mg.) which appeared to consist of at least four polar components from t.l.c. (using benzene (35)-dioxan (5)-acetic acid (1) as eluant), and no component was satisfactorily isolated by p.l.c.

Schmidt Reaction of Canadensic Acid (Mixture of C-2 Epimers) (55).

The mixture of canadensic acid C-2 epimers (50mg.) was dissolved in analar 98% sulphuric acid (0.2ml.) and sodium azide (30mg.) added. The solution was heated under nitrogen from 35° - 65° during $\frac{1}{4}$ hour, and maintained at this temperature for $1\frac{1}{2}$ hours. The barium carbonate which collected in the trap (5ml. of a solution containing 2% Ba(OH)₂, 3% NaNO₂) was centrifuged, and the solvent was decanted.

After washing successively with distilled water (3X), and dry acetone (2X), the solid was dried under reduced pressure to give barium carbonate (5mg., 5%). The acid solution was diluted with water (20ml.) neutralised with 1N sodium hydroxide, and subjected to constant extraction with chloroform for 24 hours, to give a yellow oil (7mg.) which consisted of several components more polar than the canadensic acid C-2 epimers by t.l.c. (benzene (35)-dioxan(S)-acetic acid (1)). This was not investigated further.

Schmidt Reaction of the Quaternary Mixture of Dihydrocanadensic Acid C-2, C-10 Diastereoisomers (56).

The dihydrocanadensic acid diastereoisomers (50mg.) were dissolved in analar 98% sulphuric acid (0.2ml.) and sodium azide (35mg.) added. The solution was heated under nitrogen to 60° over $\frac{1}{2}$ hour, and maintained at this temperature for one hour. The barium carbonate (10mg., 21%) which appeared in the CO₂ trap was collected as described previously. The acid solution was then diluted with water (20ml.) and extracted with chloroform (2X 10ml.) to yield an oil (10mg., 20% recovery), identical on t.l.c., and in its i.r. spectrum to the starting material. Constant chloroform extraction of the aqueous solution adjusted to pH7 (1N NaOH) for 5 days gave an oil (5mg.) which was shown to consist of material much more polar than the quaternary mixture of dihydrocanadensic acid diastereoisomers by t.l.c. (using benzene (35) dioxan (5)-acetic acid (1)).

10.11 - Dibromocanadensic Acid (Mixture of C-2, C-10 Diastereoisomers) (197)

The mixture of canadensic acid C-2 epimers (21mg.) in carbon tetrachloride (10ml.), was treated with a saturated solution of bromine in carbon tetrachloride (0.3ml.) and the solution was refluxed for 2 hours. The organic solution was then washed with water (3x10ml.), brine, dried and evaporated to give the dibromocanadensic acid as a yellow oil (37 mg., 95%).

T.l.c. 0.47, benzene (35)-dioxan (5)-acetic acid (1) (brown).

i.r. ν_{\max} . (CCl_4): 3,500 - 2,800 (CO_2H), 1775 (γ -lactone), 1710 (CO_2H), 1170, 1125 cm^{-1} .

u.v. λ_{\max} . (EtOH): No absorption > 210 nm.

n.m.r. 60 Mc/S. (CDCl_3) τ : 5.60 (3H, m, H-4 and 2H-11), 6.40 (1H, m, H-2), 7.2 - 8.1 (2H, m, 2H-3), 8.4 (2H, m, 2H-5), 8.6 (4H, m, sat. $-\text{CH}_2-$), 9.10 (3H, t, $J = 6.0$ Hz., CH_2-CH_2-).

Decarboxylation of Dibromocanadensic Acid (Mixture of C-2, C-10 Diastereoisomers) (208).

The mixture of bromoacids (30mg.) in analar ethyl acetate (15ml.) containing dry triethylamine (0.1ml.) was refluxed under nitrogen, the effluent gas being monitored for carbon dioxide by bubbling through a standard barium hydroxide solution (5ml.). This trap turned cloudy after 5 minutes reflux and the reaction was worked up after 30 minutes. The barium carbonate (8mg., 50%) was isolated in the usual way. On cooling the ethyl acetate solution, crystals of triethylamine hydrochloride separated and crystallised from chloroform-light petroleum

as needles (15mg., 70%), m.p. 123°. Identity with an authentic sample was established by mixed m.p. The mother liquor was washed with water (2 x 10ml.), dried and evaporated to give an oil (20mg.), containing at least 3 components all less polar than the starting material. (R_{f15} 0.60, 0.32 and 0.30 by t.l.c. in CHCl_3). Attempts to separate these by p.l.c. were unsuccessful. G.l.c. of the oil gave only a broad peak on all columns used, for example :

1% SE 30 at 70° R_t 31.4 min. R.I. 1390

This could not be satisfactorily recorded by g.c.m.s.

Dihydrocanadensol (Mixture of C-2, C-10 Diastereoisomers) (210).

The quaternary mixture of dihydrocanadensic acid C-2, C-10 diastereoisomers (60mg.) in dry benzene (15ml.) containing oxalyl chloride (0.5ml.) was refluxed for 1 hour. The solvent was evaporated and the crude acid chloride dissolved in dry dioxan (5ml.), sodium borohydride (50mg.) added and the solution stirred in a stoppered flask overnight. The dioxan solution was diluted with chloroform (30ml.) and the organic solution extracted with water, saturated aqueous sodium bicarbonate solution, dried and evaporated to give an oil (50mg.). This was purified by p.l.c. on Kieselgel (0.75 mm x 20cm. x 20cm.) to give the mixture of dihydrocanadensols as an oil (35mg., 59%).

T.l.c. 0.25, CHCl_3 (brown).

i.r. ν_{max} (thin film): 3,550 (-OH), 1760 (γ -lactone), 1195, 1130, 1055, 1030, 975 cm^{-1} .

u.v. λ_{max} . (EtOH): No absorption > 200 nm.

n.m.r. 60 Mc/S. (CDCl_3) τ : 5.60 (1H, m, H-4), 6.35 (3H, m, $-\text{CH}_2-\text{OH}$), 7.20 (1H, m, H-2); 7.4 - 8.0 [2H, m, (1H D_2O exchangeable), 2H-3 and $-\text{OH}$], 8.1 - 8.8 (7H, m, H-10, 2H-5 and sat. $-\text{CH}_2-$), 9.05 (6H, m, $\text{CH}_3 - \overset{1}{\text{C}}\text{H}-$).

Attempted Dehydration of Dihydrocanadensol (Mixture of C-2, C-10 Diastereoisomers) (210).

(a) The dihydrocanadensol mixture (20mg.) in dry benzene (15ml.) containing *p.*-toluenesulphonic acid (5mg.) was refluxed for 3 hours, the reaction being monitored by t.l.c. After evaporation of the solvent, the resulting oily solid in chloroform (15ml.), was extracted successively with saturated aqueous sodium bicarbonate, water and dried. Evaporation of the solvent then gave an oil, which was shown by t.l.c. to contain at least 6 components all less polar than the starting material.

(b) The dihydrocanadensol mixture (10mg.) in dry benzene (15ml.) containing a crystal of iodine (2mg.) was refluxed for 2 days without any reaction having occurred (by t.l.c.). Work-up in the usual way gave an oil (8mg.), identical on t.l.c. and in its i.r. spectrum to the starting material.

(c) The dihydrocanadensol mixture (5mg.) in dry benzene (10ml.) was passed down a column (10 cm. x $\frac{1}{2}$ cm.) of active basic (10g., Molm grade I). Evaporation of the solvent gave an oil (4mg.) shown to be starting material by t.l.c. and its i.r. spectrum.

Dihydrocanadensol p-Toluenesulphonates (Mixture of C-2, C-10 Diastereoisomers) (212).

The mixture of dihydrocanadensol C-2, C-10 diastereoisomers (45mg.) in dry pyridine (3ml), containing p-toluenesulphonyl chloride (43mg.) was left at ice temperature overnight. The solution was then diluted with chloroform (20ml.), and the organic solution extracted with 5N hydrochloric acid, saturated aqueous sodium bicarbonate and water. Evaporation of the solvent gave the crude p-toluenesulphonates which were purified by p.l.c. on Kieselgel H (20 cm. x 20cm. x 0.75mm.) to give dihydrocanadensol p-toluenesulphonates as an oil (58mg., 72%).

T.l.c. 0.60, CHCl₃ (brown).

i.r. max. (thin film): 1762 (γ-lactone), 1600, 1185, 1175, 1095, 985, 845, 822, 775 cm.⁻¹.

u.v. λ_{max} (EtOH): No absorption > 210 nm.

n.m.r. 60Mc/S. (CCl₄) τ: 2.17 (2H, d, J = 8.0 Hz., Ar-H), 2.64 (2H, d, J = 8.0 Hz., Ar-H), 5.60 (1H, m, H-4), 5.90 (2H, m, 2H-10), 7.15 (1H, m, H-2), 7.58 (3H, s, Ar-CH₃), 7.80 (2H, m, 2H-3), 8.3 (2H, m, 2H-5), 8.6 (7H, m, sat. -CH₂-and H-10), 9.10 (6H, m, CH₃-CH-).

Experiments with Labelled Precursors.

Growth and Extraction of Penicillium Canadense.

Penicillium canadense with labelled substrates was grown under identical conditions to those previously described for normal

cultures. The broth was extracted by means of the active charcoal-acetone technique.

Introduction of Radioactive Substrates into Cultures of *P. canadense*.

Radioactive precursors in sterilised water (0.5 hours steam at 242°F and 12 p.s.i.) were spread evenly over 5 roux bottles containing two day cultures of *Penicillium canadense*. These cultures were harvested in the usual way after 6 days growth.

Isolation of Labelled Canadensic Acid (Mixture of C-2 Epimers).

The dry broth extracts (ca. 0.5. from 5 roux bottles) were taken up in the minimum volume of chloroform (100 ml.), inactive canadensic acid (mixture of C-2 epimers) (ca. 150 mg.) added, and the solution extracted with freshly prepared aqueous sodium bicarbonate solution (2 x 50 ml.). The bicarbonate solution after cooling to ice temperature was acidified with 5N hydrochloric acid. The aqueous solution was then extracted with chloroform and the organic solution evaporated to give a solid, which on crystallising from benzene-light petroleum gave the mixture of canadensic acid C-2 epimers as needles m.p. 128-130°. Radiochemical purity was obtained by crystallising to constant activity (occurring usually after 4 crystallisations) and checked where possible by scanning for radioactivity on t.l.c.

Counting of Radioactive Material.

Radioactive assays were carried out using a Packard Tri-Carb Liquid Scintillation Spectrometer (Series 3000). Samples were counted

using a solution of 0.10g. dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazallyl)-benzene) and 3.0g. PPO (2,5-diphenyloxazole) in toluene (1 litre). Formaldehyde dimedone was counted in this solution but the canadensic acid C-2 epimers (being only slightly soluble in toluene) were counted in a mixture of the above solution (9ml) with dry ethanol (1ml.). Sodium acetate was counted by dissolving in water (1ml.) adding Bio Solv BBS-2 solution (2ml.) and the above toluene solution (10ml.). Efficiencies obtained for ^{14}C using these solutions were in the order of 92%, 70% and 85% respectively. The counting of radioactive samples was carried out for sufficient time to give a standard deviation of 2%.

Ozonolysis of Labelled Canadensic Acid (Mixture of C-2 Epimers).

This was carried out on canadensic acid⁺ (30mg.) in an identical manner to that described previously. The formaldehyde dimedone obtained on cooling the steam distillate was crystallised from ethanol-water to constant activity (usually ca. 3 times).

Kuhn Roth Oxidation of labelled Canadensic Acid⁺

This was carried out on canadensic acid (30mg.) in a similar manner to that described previously, except that after neutralisation of the aqueous acetic acid (ca. 50ml.) with N/100 sodium hydroxide, the aqueous solution was evaporated to dryness in vacuo. Water 2ml. was then added to the gummy sodium acetate obtained, and 1ml. used for counting by the Bio Solv-toluene scintillation technique.

+ Mixture of C-2 epimers.

Attempted Deuterium Enrichment of Canadensic Acid (Mixture of C-2 Epimers).

Attempts to use more sodium acetate and less glucose in the medium resulted in retarded fungal growth. Thus with the same glucose level mediums containing various concentrations of sodium acetate were used for incubation and gave the following result :

% Sodium Acetate in medium	0.001	0.01	0.05	0.1	1.0
Wt. of Mycelium after 10 days (g.)	11.5	11.5	10.5	9.0	3.5

To prevent retarded growth, a concentration of 0.07% sodium D₃-acetate was used in the medium (i.e. 2.5g. spread evenly over 20 roux bottles), and the fungus incubated for 6 days. The broth was then extracted with ethyl acetate in the usual way, and the gummy solid extracted subjected to chromatography on silicic acid in the usual way. However, no deuterium enrichment of samples of either canadensic acid (95) or dihydrocanadensolide (54) was detected by mass spectrometry.

Separation of the Metabolites in a 6 Day Culture of *P.canadense*.

The broth from a 6 day culture of *P.canadense* was extracted with ethyl acetate in the usual way, and after evaporation of the solvent the viscous oil (6.0g.) was absorbed onto silicic acid (10g.) and placed at

the top of a column of silicic acid (350g.) impregnated with silver nitrate (35g.). The column was eluted with 20% chloroform in light petroleum (2l.) and the polarity of the solvent increased by 10% each time till material was eluted (with 60% chloroform in light petroleum).

The distribution of metabolites is shown below.

<u>Eluting Solvent.</u>	<u>Fr. No.</u>	<u>Wt.</u>	<u>Components.</u>
60% CHCl ₃ in	15,16	50mg.	dihydrocanadensolide
light petroleum	17-21	100mg.	dihydrocanadensolide and canadensolide
	22-24	35mg.	canadensolide
	25,26	60mg.	isocanadensic acid and dihydroisocanadensic acid
	27-30	200mg.	canadensic acid.

Fractions 25 and 26, which contained acidic material slightly less polar than canadensic acid, was treated with an excess of ethereal diazomethane. Evaporation of the solvent gave an oil (65mg.), which by t.l.c. appeared to consist of two components of R_f 0.59 and 0.62, CHCl₃. These were separated by p.l.c. on Kieselgel HF₂₅₄ (20cm. x 20cm. x 0.75 mm.) with multiple elution in chloroform (2) -light petroleum (1), using u.v. light and iodine vapour for detection. to give Methyl isocanadensate (217) (12mg.) was obtained as an oil, b.p. 68°/0.05mm.

O.r.d. in methanol (C 0.35): $[\alpha]_{300}^D$, 0; $[\eta]_{244}^D$, + 5,700; $[\eta]_{217}^D$, - 9,550;
 $[\eta]_{200}^D$, - 5,080.

c.d. in methanol (C 0.28): ΔE_{300}^D , 0; ΔE_{283}^D , + 0.35; ΔE_{275}^D , + 0.32;
 ΔE_{228}^D , + 3.45; ΔE_{198}^D , -1.75.

T.l.c. 0.62, CHCl_3 (brown).

i.r. ν_{max}^D (CCl_4): 1770 (γ -lactone), 1728 (CO_2Me), 1218, 1117,
 1095, 1045 cm^{-1} .

u.v. λ_{max} (EtOH): 229 nm. (ϵ 13,500).

n.m.r. 100 Mc/S. (CDCl_3) τ : 4.94 (1H, m, irr. 7.83 \rightarrow dd ($J_1 = 8.0$,
 $J_2 = 1.5$ Hz.), H-3), 6.14 (3H, s, -OMe), 7.83 (3H; d, $J = 2.0$ Hz.,
 irr. 4.94 \rightarrow s, 3H-11), 8.3 (2H, m, 2H-4), 8.6 (6H, m, sat.
 - CH_2 -), 9.12 (3H, t, $J = 6.0$ Hz., CH_3 - CH_2 -).

m.s. M/e (Rel. abundance): M^+ 226 (15), 197 (53), 156 (100), 128 (48),
 127 (32), 124 (37), 123 (19), 99 (27), 71 (23), 67 (43), 55 (15).

Analysis Found : C, 63.88; H, 8.07%; M^+ at m/e 226.

$\text{C}_{12}\text{H}_{18}\text{O}_4$ requires C, 63.70; H, 8.02% M.W. 226.3.

Methyl dihydrocanadensate (218) (35 mg.) was obtained as an oil, b.p.
 110°/0.07 mm.

O.r.d. in methanol (C 0.53): $[\eta]_{232}^D$, + 3,880; $[\eta]_{216}^D$, 0; $[\eta]_{196}^D$, + 3,650;

c.d. in methanol (C 0.53): ΔE_{250}^D , 0; ΔE_{228}^D , + 1.38; ΔE_{205}^D , 0.

T.l.c. 0.59, CHCl_3 (brown).

i.r. ν_{max}^D (CCl_4): 1790 (γ -lactone), 1746 (CO_2Me), 1240, 1194, 1176,
 995, 925 cm^{-1} .

u.v. λ_{max} . (EtOH): No absorption > 200 nm.

n.m.r. 100 Mc/S. (CDCl_3) τ : 5.42 (1H, m, H-3), 6.28 (3H, s, -OCH₃),
6.90 (2H, m, H-2 and H-10), 8.4 (2H, m, 2H-4), 8.6 (6H, m, sat.
-CH₂-), 8.73 (3H, d, J = 6.0 Hz., 3H-11), 9.12 (3H, t, J = 6.0 Hz.,
CH₃ - CH₂-).

m.s. m/e (Rel. abundance): M⁺ 228 (2), 197 (11), 196 (9), 182 (12),
169 (20), 168 (13), 157 (55), 155 (22), 129 (95), 128 (21),
101 (50), 97 (54), 69 (100), 59 (23), 55 (36).

Analysis Found: C, 62.90; H, 8.58%; M⁺ at m/e 228.

$\text{C}_{12}\text{H}_{20}\text{O}_4$ requires C, 63.14; H, 8.83%; M.W. 228.3.

Dihydroisocanadensic Acid (219).

This compound was isolated in minor quantities from the broth of a 10 day culture, and crystallised from ether-light petroleum as colourless needles, m.p. 143°.

T.l.c. 0.62, benzene (35)-dioxan (5)-acetic acid (1) (brown).

i.r. ν_{max} . (KBr): 3,200 - 2,800 (CO₂H), 1758 (δ -lactone), 1742 (CO₂H), 1458, 1423, 1388, 1360, 1326, 1314, 1253, 1212, 1183, 1014, 988, 690 cm.⁻¹.

u.v. λ_{max} . (EtOH): No absorption > 210 nm.

n.m.r. 100 Mc/S. (CDCl_3) τ : -0.3 (1H, m, -COOH), 5.32 (1H, m, H-3),
6.82 (1H, m, irr. 8.66 \rightarrow d (J = 9.0 Hz.) H-10), 6.96 (1H, m,
irr. 5.32 \rightarrow d (J = 9.0 Hz.), H-2), 8.4 (2H, m, 2H-4),
8.6 (6H, m, sat. -CH₂-), 9.10 (3H, t, J=6.0 Hz., irr. 8.6
 \rightarrow s, CH₃ -CH₂-).

m.s.m/e (Rel. abundance) M^+ 214 (0.6), 196 (9), 168 (27), 155 (25),
143 (100), 115 (80), 114 (28), 97 (72), 87 (66), 86 (25)
69 (74), 55 (48).

Analysis Found : C, 61.57; H, 8.38%; M^+ at m/e 214.

$C_{11}H_{18}O_4$ requires C, 61.66; H, 8.47%; M.W. 214.3.

Attempted Desaturation of Methyl Dihydroisocanadensate(213) using D.F.S.

Methyl dihydroisocanadensate (10 mg.) in dry benzene (5ml.) containing 2,3-dichloro-5,6-dicyano-p.-benzoquinone (10mg.) was refluxed for 2 days. The solvent was then removed and the resulting oil (13mg.) subjected to p.l.c. on Kieselgel H. Elution of the major band detected by iodine vapour gave an oil (6mg.) identical on t.l.c. and in its i.r. spectrum to starting material. No methyl isocanadensate was detected by t.l.c. of the above reaction mixture.

Attempted Formation of Bisphenacyl Ester of Dihydroisocanadensic Acid (221).

To dihydroisocanadensic acid (40 mg., 0.187 mM) in ethanol (1ml.) was added N/10 sodium hydroxide (3.75ml., 0.375 mM) and the solvent evaporated on a steam bath. The residual solid was dissolved in dry acetone (10ml.) and stirred for 18 hours at RT with p.-bromophenacyl bromide (110mg.). The solvent was then evaporated and the resulting oil purified by p.l.c. on Kieselgel HF₂₅₄ (20 cm. x 20 cm. x 0.7 mm). Elution of the major band gave a solid (58mg., 75%) shown by its n.m.r. spectrum to be the monophenacyl ester (222) which was not investigated further.

T.l.c. 0.41, CHCl_3 (brown).

n.m.r. 60Mc/S. (CDCl_3) τ : 2.30 (AB q, $J = 10.0$ Hz., Ar - H), 4.60

(2H, s, CO- CH_2 - O.CO-), 5.27 (1H, m, H-3), 6.70 (2H, m, H-2 and H-10), 8.35 (2H, m, 2H-4), 8.6 (4Hz.m, sat. - CH_2 -), 8.64 (3H, d, $J = 6.5$ Hz., 3H-11), 9.08 (3H, m, CH_3 - CH_2 -).

An attempt to oxidise the hydroxyl group of any bisphenacyl derivative in situ was made by treating the above reaction solution with Jones reagent at ice temperature. Work-up in the usual way gave only the mono-phenacyl derivative.

Attempted Desaturation of 10-Epidihydrocanadensolide (61) using D.D.Q.

10-Epidihydrocanadensolide (10mg.) in dry benzene (5ml.) containing D.D.Q. (10mg.) was refluxed for 2 days. No canadensolide (51) was detectable by t.l.c. or g.l.c. of this reaction solution.

Evaporation of the solvent and purification of the resulting oil on Kieselgel H gave 10-epidihydrocanadensolide (6mg.) identical on t.l.c. and i.r. spectrum to the starting material.

EXPERIMENTALCHAPTER 7Isolation and Properties of 3-Chlorogentisyl alcohol.

This was isolated in minor quantities on one occasion by silica gel column chromatography of the charcoal-acetone extract of the broth of Penicillium canadense (see Chapter 1), but was detected on t.l.c. of fractions from several extracts. It was eluted from the column using 5% ethyl acetate in chloroform and crystallised as needles m.p. 144° from ethyl acetate-light petroleum.

$[\alpha]_D$ in methanol (C, 2.0), 0.

T.l.c. 0.25, 4% MeOH in CHCl₃ (black).

i.r. ν_{\max} . (KBr): 3,425 and 3,045 (-OH), 1597 (Ar-H), 1455, 1302, 1160, 1105, 1028, 978, 848, 843, 792 cm.⁻¹.

u.v. λ_{\max} . (EtOH): 294 nm. (ϵ 3,910)

λ_{\max} (EtOH + NaOH): 262 nm. (ϵ 3,900), 314 nm. (ϵ 7,060)
320 nm. (ϵ 8,120), 408 nm (ϵ 4,140), 433 nm. (ϵ 4,320)

λ_{\max} . (EtOH + NaOH under N₂) 280 nm. (ϵ 5,100).

n.m.r. 100 Mc/S. (D₆ D.M.S.O.) τ : 0.88 (1H, m, Ar.OH), 1.61 (1H, m, Ar.CH),
2.21 (1H, d, J = 3.0 Hz., Ar.-H), 3.35 (1H, d, J = 3.0 Hz., Ar.-H),
4.92 (1H, m, -CH₂.OH), 5.48 (2H, s, Ar.-CH₂-OH).

Spectrum plus D₂O: 0.88, 1.61 and 4.92 τ signals disappear.

Irradiation at 5.48 τ causes proton resonance at 3.21 τ to sharpen.

m.s. m/e (Rel. abundance): M^+ ^{37}Cl 176 (9.0), 174 (27), 159 (5),
158 (33), 157 (14), 156 (100), 130 (17), 128 (53), 110 (14),
103 (5), 101 (5), 100 (16), 99 (16), 65 (15).

M^+ ^{35}Cl at m/e 174.0075

$\text{C}_{17}\text{H}_{17}\text{ClO}_3$ requires 174.0084

3-Chlorogentisyl alcohol - triacetate.

The above chlorine containing metabolite (22mg.) in acetic anhydride (0.3ml.) and pyridine (0.05ml.) was left at R.T. overnight. The solution was concentrated in vacuo, chloroform (10ml.) added and the solution extracted with 5N hydrochloric acid, and evaporated to give the triacetate as an oil (28 mg., 74%).

T.l.c. 0.52, CHCl_3 (brown).

i.r. ν_{max} . (thin film): 3,180 (wk) (Ar.H), 1770 (phenolic $\text{CH}_3\text{CO.O}$),
1748 (benzylic $\text{CH}_3\text{CO.O}$), 1375, 1230, 1205, 1175, 1025, 1023, 765 cm.^{-1} .

u.v. λ_{max} . (EtOH): No absorption > 210 nm.

n.m.r. 60 Mc/s. (CDCl_3) τ : 2.78 (1H, d, $J = 3.0$ Hz., Ar.H), 2.89 (1H, d,
 $J = 3.0$ Hz., Ar.H), 4.98 (2H, s, Ar.- CH_2 -OH), 7.68 (3H, s, $\text{CH}_3\text{.CO.O-}$),
7.76 (3H, s, $\text{CH}_3\text{.CO.O-}$), 7.97 (3H, s, $\text{CH}_3\text{.CO.O-}$).

Attempted purification of the triacetate derivative by p.l.c. on Kieselgel HF₂₅₄ using multiple elution caused hydrolysis to the diacetate (245) (dark blue band under u.v. light).

T.l.c. 0.35, CHCl_3 (brown).

i.r. ν_{max} . (thin film): 3,500 (br.) (-OH), 1750 ($\text{CH}_3\text{CO.O}$), 1606, 1590, 1460,
1375, 1230, 1185, 1125, 1030, 903, 875, 815 cm.^{-1} .

u.v. λ_{\max} . (EtOH): 315 nm. (ϵ 2,900).

(EtOH) NaOH: 348 nm. (ϵ 3,400).

n.m.r. 60 Mc/S. (CDCl_3) τ : 3.15 (1H, d, $J = 3.0$ Hz., Ar.-H),
3.24 (1H, d, $J = 3.0$ Hz., Ar. H), 5.02 (2H, s, Ar. - CH_2 - CH),
7.68 (3H, s, $\text{CH}_3\text{CO.O-}$), 7.84 (3H, s, $\text{CH}_3\text{CO.O-}$).

Diacetyl- α -Resorcylic Acid (228).

α -Resorcylic acid (2.0g.) in acetic anhydride (10ml.)

containing sodium acetate (0.1g.) was left at R.T. for 24 hours. The solvent was concentrated in vacuo, 5N hydrochloric acid (20ml.) added and the aqueous solution extracted with chloroform. The organic extract was washed with aqueous sodium hydrogen carbonate, water and evaporated to give diacetyl- α -resorcylic acid (2.5g., 79%) which crystallised from chloroform - light petroleum as needles m.p. 162° (lit. m.p. $161-2^{0159}$).

i.r. ν_{\max} . (KBr):- 3,300 - 2,800 (CO_2H), 1770 ($\text{CH}_3\text{CO.O}$), 1695 (CO_2H),
1593 (wk) (Ar.-H), 1470, 1440, 1428, 1368, 1307, 1200 (br.),
1225, 1022, 945, 918, 911, 902, 890, 838 cm.^{-1} .

n.m.r. 60 Mc/S. (CDCl_3) τ : 0.03 (1H, m, $-\text{CO}_2\text{H}$), 2.67 (2H, d, $J = 2.5$ Hz.),
Ar.-H), 3.13 (1H, d, $J = 2.5$ Hz., Ar.-H), 7.80 (6H, s, $\text{CH}_3\text{CO.O-}$).

Diacetoxybenzyl Alcohol (229).

To diacetyl- α -resorcylic acid (1.49g., 6.3mM) in dry diglyme (20ml.) at ice temperature under nitrogen was added a 0.5M solution of diborane in dry tetrahydrofuran (8ml., 4 mM), and the solution left $\frac{1}{2}$ hour. Water (150 ml.) was added and the aqueous solution extracted with chloroform. The organic extract was then washed with aqueous sodium

hydrogen carbonate, water (10 x 20 ml.), dried and evaporated to give the diacetoxybenzyl alcohol (0.92g., (4%)) as an oil, b.p. 105°/0.005 mm.

T.l.c. 0.80, 3% MeOH in CHCl₃ (purple-brown).

i.r. ν_{\max} . (thin film): 3520 (-OH), 1760 (CH₃.CO.O), 1370, 1283, 1210, 1123, 1025, 970, 910 cm.⁻¹.

u.v. λ_{\max} . (EtOH): No absorption > 210 nm.

n.m.r. 60 Mc/S. (CDCl₃) τ : 3.06 (2H, d, J = 2.0 Hz., Ar.-H), 3.22 (1H, d, J = 2.0 Hz., Ar.-H), 5.19 (2H, s, Ar.-CH₂-OH), 5.80 (1H, m, -OH), 7.76 (6H, s, CH₃ CO.O-).

Spectrum plus D₂O: 5.80 τ resonance disappears.

Analysis Found: C, 58.87; H, 5.47%.

C₁₁H₁₂O₅ requires C, 58.93; H, 5.39%; M.W. 224.2.

Diacetoxybenzyl Acetate (230).

Diacetoxybenzyl alcohol (0.3g.) in acetic anhydride (5ml.) containing pyridine was left at R.T. for 24 hours. The solution was concentrated in vacuo, 5N hydrochloric acid (20ml.) added and the aqueous solution extracted with chloroform. The organic extract was washed with aqueous sodium hydrogen carbonate and evaporated to give an oil, which was purified by p.l.c. on Kieselgel HF₂₅₄ (100 cm. x 20 cm. x 20 cm. x 0.75 mm.) to yield the diacetoxybenzyl acetate (0.28g., 79%) as an oil, b.p. 105°/0.01mm.

T.l.c. 0.20, CHCl₃ (purple-brown).

i.r. ν_{\max} . (thin film): 3,025 (wk.) (Ar.-H), 1770 (phenolic $\text{CH}_3\text{CO.O}$) 1740 (benzylic $\text{CH}_3\text{CO.O}$), 1619, 1595, 1451, 1369, 1292, 1200 (br.), 1126, 1028, 755 cm.^{-1} .

u.v. λ_{\max} . (EtOH): 217 nm. (ϵ 5,800), 265nm. (ϵ 300).

n.m.r. 60 Mc/S. (CDCl_3) τ : 3.00 (2H, d, $J = 2.0$ Hz., Ar.-H), 3.07 (1H, d, $J = 2.0$ Hz., Ar.-H), 4.90 (2H, s, Ar.- $\text{CH}_2\text{-O.CO.CH}_3$), 7.71 (6H, s, $\text{CH}_3\text{CO.O-}$), 7.90 (3H, s, $\text{CH}_3\text{CO.O-}$).

Analysis Found : C, 58.56; H, 5.24%.

$\text{C}_{13}\text{H}_{14}\text{O}_4$ requires C, 58.65; H, 5.30%, M.W. 266.3.

Chlorine in Acetic Acid.

Chlorine gas after drying with concentrated sulphuric acid was passed into glacial acetic acid (100 ml.) to give a saturated solution. Titration of 0.1 ml. of this solution in 5 ml. of 0.5 N potassium iodide solution with starch indicator required 2.3 ml. N/10 sodium thiosulphate \equiv 0.12 mM. chlorine in acetic acid.

2-Chloro-3,5-diacetoxybenzyl Acetate (231).

Diacetoxybenzyl acetate (176 mg., 0.66 mM.) in acetic acid (1ml.) was treated with the above solution of chlorine in acetic acid (10ml., 1.2mM) and the solution left at R.T. for 24 hours. The solvent was then removed, and the oil obtained purified by p.l.c. on Kieselgel HF₂₅₄ (20 cm. x 20 cm. x 0.7 mm.) to give 2-chloro-3,5-diacetoxybenzyl acetate (120mg., 60%) as an oil, b.p. 115°/0.02 mm.

T.l.c. 0.28, CHCl_3 (yellow-brown).

i.r. ν_{\max} . (thin film): 3035 (wk) (Ar.-H), 1770 (phenolic $\text{CH}_3\text{CO.O}$),

1738 (benzylic CH_2CO_2), 1448, 1365, 1303, 1185 (br.), 1132, 1068, 1042, 1018, 960, 897, 719 cm^{-1} .

u.v. λ_{max} . (EtOH): 227nm. (ϵ 6,500), 276 nm. (ϵ 700).

n.m.r. 60 Mc/S. (CDCl_3) τ : 2.83 (1H, d, $J = 2.5$ Hz., H-6), 2.98 (1H, d, $J = 2.5$ Hz., H-4), 4.73 (2H, s, Ar.- CH_2 -OAc.), 7.70 (3H, s, CH_3CO_2 -), 7.77 (3H, s, CH_3CO_2 -), 7.82 (3H, s, CH_3CO_2 -).

Analysis Found : C, 52.08; H, 4.52%.

$\text{C}_{13}\text{H}_{13}\text{ClO}$ requires C, 51.92; H, 4.36; Cl, 11.79% M.W. 300.7.

Chlororesorcinol (II).

2-chloro-3,5-diacetoxybenzyl alcohol (30mg.) in methanol (10ml.) containing concentrated hydrochloric acid (0.05ml.) was refluxed under nitrogen for 6 hours. The solvent was removed in vacuo to give an oily solid. The chlororesorcinol (15 mg., 86%) was obtained as needles, m.p. 147° , on crystallising from ethyl acetate-light petroleum.

T.l.c. 0.24, 4% MeOH in CHCl_3 (dark brown).

i.r. ν_{max} . (KBr): 3,370 (-OH), 1590 (Ar.), 1438, 1237, 1148, 1064, 1003, 962, 843, 827 cm^{-1} .

u.v. λ_{max} . (EtOH): 283 nm. (ϵ 2,365).
 λ_{max} . (EtOH + NaOH): 303 nm (ϵ 3,840).

n.m.r. 60 Mc/S. (D_6 acetone) τ : 3.30 (1H, d, $J = 2.0$ Hz., Ar.H), 3.53 (1H, d, $J = 2.0$ Hz., Ar.H), 5.27 (2H, s, Ar.- CH_2 -OH).

m.s. m/e (Rel abundance): M^+ ^{37}Cl 176 (3), 174 (100), 159 (5), 157 (20), 156 (19), 147 (10), 145 (27), 139 (66), 130 (7), 128 (21), 111 (57),

110 (57), 93 (37), 81 (27), 69 (41), 65 (47).

Analysis Found : C, 48.32; H, 4.32%; M^+ ^{35}Cl at m/e 174.

$C_7H_7ClO_3$ requires C, 48.15, H, 4.04; Cl, 20.31%; M.W. 174.5.

U.V. Spectra of 3-Chloroanisyl Alcohol and p-Hydroquinone.

These were recorded in ethanol solution, their base shift being measured by the addition of 0.1 ml. of 5N sodium hydroxide giving peaks which decreased with time.

<u>Metabolite</u> (neutral)	nm.	-	295	-			
	ϵ		3,910				
(basic)	nm.	262 [•]	-	314 [•]	320 [•]	408 [•]	433 [•]
start	ϵ	3,900		7,060	8,120	4,140	4,320
(basic)	nm.	267	-	-	-	-	-
after 30 min.	ϵ	4,560					
<u>p-Hydroquinone</u> (neutral)	nm.	-	292	-	-	-	-
	ϵ		1,900				
(basic)	nm.	236 [•]	-	312 [•]	318 [•]	410 [•]	431 [•]
(start	ϵ	2,850		4,020	4,110	2,250	2,340
(basic)	nm.	238	-	311	-	-	-
after 30 min.	ϵ	3,200		1,805			

The u.v. solutions were 'deoxygenated' by bubbling nitrogen gas through for $\frac{1}{2}$ hour, and the 4N sodium hydroxide solution similarly treated by refluxing under nitrogen to give the following spectra.

Metabolite (neutral)	nm	-	295	-
	ϵ		3,910	
(basic)	nm	280	-	-
	ϵ	5,100		
p.Hydroquinone (neutral)	nm	-	294	
	ϵ		1,900	
(basic)	nm	284	-	-
	ϵ	2,750		

Diacetylgentisic Acid (239).

The sodium salt of gentisic acid (500mg.) in acetic acid (20ml.) containing acetic anhydride (15 ml.) and sodium acetate (100mg.) was left at R.T. for 24 hours. The solution was then diluted with water (100 ml.) and extracted with chloroform. The organic extract was washed with water and evaporated to give an oil hydrogen carbonate and precipitation with hydrochloric acid to give diacetylgentisic acid (380 mg., 56%) as rods, m.p. 120° (lit. m.p. 169° ²⁰⁵).

i.r. $\nu_{\max.}$ (KBr) : 3500 - 2,800 (CO_2H), 1759 and 1727 ($\text{CH}_3\text{CO.O}$),

1705 (CO₂H), 1488, 1434, 1380, 1250, 1205, 1175, 1125, 1065, 1010, 930,
850 cm.⁻¹.

u.v. λ_{max} . (EtOH): 273 nm. (ϵ 1,670).

(EtOH + NaOH): 294 nm. (ϵ 2,900).

n.m.r. 60Mc/S. (CDCl₃) τ : 2.25 (1H, d, J = 2.5 Hz., H-6), 2.77 (2H, m,
H-3 and H-4), 7.67 (6H, s, CH₃ CO.O-).

Analysis Found : C, 55.65; H, 4.27%.

C₁₁H₁₀O₆ requires C, 55.47; H, 4.23%. M.W. 238.2.

5-Acetoxy-salicylic Acid (240).

Diacetyl-gentisic acid (700 mg.) in dioxan (20ml.)-water (60ml.)
at pH 6.0 was refluxed for 2 hours. The acidified solution was then
extracted with chloroform, and the organic extract washed with water
and evaporated to give 5-acetoxy-salicylic acid (400 mg., 67%) which
crystallised as needles m.p. 130° from ether - light petroleum.

i.r. ν_{max} . (KBr):- 3420 and 3,200 (br.) (CO₂H and -OH), 1768 (CH₃CO.O),
1678 (CO₂H), 1625, 1488, 1444, 1369, 1340, 1215, 1192, 1134, 1015,
902, 838, 832, 804, 795 cm.⁻¹.

u.v. λ_{max} . (EtOH): 308 nm. (ϵ 3,850).

(EtOH + NaOH): 291 nm. (ϵ 3,920), 340 nm. (ϵ 2,290).

n.m.r. 60 Mc/S. (CDCl₃) τ : 2.36 (1H, d, J = 3.0 Hz., H-6), 2.73
(1H, dd, J = 3.0, 9.0 Hz., H-4), 3.01 (1H, d, J = 9.0 Hz., H-6),
7.73 (3H, s, CH₃CO.O-).

Analysis Found : C, 55.03; H, 4.13%.

$C_9H_8O_5$ requires C, 55.11; H, 4.11%; M.W. 196.2

Methyl 5-Acetoxy-salicylate (241).

5-Acetoxy-salicylate acid (50 mg.) in ether (10ml.) at ice temperature was treated with an excess of ethereal diazomethane for 1 minute. The solvent was removed to give methyl 5-acetoxy-salicylate (45 mg., 84%) which crystallised as needles m.p. 87° from methanol - water. T.l.c. 0.51, $CHCl_3$ (yellow-brown).

i.r. ν_{max} . (KBr): 3470 (wk) and 3,260 (-OH), 1754 ($CH_3CO.O$), 1670 (CO_2Me), 1612, 1480, 1435, 1282, 1200 (br.), 1078, 1002, 973, 927, 873, 834, 782 cm^{-1} .

u.v. λ_{max} . (EtOH): 315 nm. (ϵ 2,960).
(EtOH + NaOH): 344 (ϵ 3,100).

n.m.r. 60 Mc/S. ($CDCl_3$) τ : 2.25 (1H, d, $J = 3.0$ Hz., H-6), 2.80 (1H, dd, $J = 3.0, 9.0$ Hz., H-4), 3.97, (1H, d, $J = 9.0$ Hz., H-3), 6.06 (3H, s, -OMe), 7.73 (3H, s, $CH_3CO.O$).

Analysis Found : C, 57.01; H, 4.64%.

$C_{10}H_{10}O_5$ requires C, 57.14; H, 4.80%; M.W. 210.2

Methyl 3-Chloro-5-acetoxy-salicylate (242).

Methyl 5-acetoxy-salicylate (150mg.) in acetic acid (1ml.) was treated with a saturated solution of chlorine in acetic acid (3ml.) and

allowed to stand at R.T. for 1 hour. On dilution with water (10ml.), methyl 3-chloro-5-acetoxysalicylate (145mg., 84%) was precipitated and crystallised as needles, m.p. 124° from methanol - water.

T.l.c. 0.43, CHCl_3 (yellow).

i.r. ν_{max} . (KBr): 3,450 and 3,250 (wk.) (-OH), 1761 (CH_3CO_2), 1682 (CO_2Me), 1614, 1468, 1435, 1372, 1332, 1248, 1220, 1167, 1096, 1025, 990, 952, 908, 802, 790 cm^{-1} .

u.v. λ_{max} . (EtOH): 311 nm. (ϵ 3,010).

(EtOH + NaOH): 346 nm. (ϵ 3,430).

n.m.r. 60Mc/s. (CDCl_3) τ : 2.47 (1H, d, $J = 3.0$ Hz., H-6), 2.67 (1H, d, $J = 3.0$ Hz., H - 4), 6.03 (3H, s, - OMe), 7.78 (3H, s, CH_3CO_2).

Analysis Found : C, 49.27; H, 3.71 %.

$\text{C}_{10}\text{H}_9\text{ClO}_3$ requires C, 49.09; H, 3.71%; Cl, 14.49; M.W. 244.6.

Perchloroquinone (244).

An excess of chlorine gas was bubbled into a solution of methyl 5-acetoxysalicylate (350mg.) in methanol (15ml.) for 10 minutes, and the solution was found to increase in temperature. The yellow solution was then evaporated to give an oil (420 mg.). By fractional crystallisation from chloroform - light petroleum needle crystals, m.p. 119° , of perchloroquinone (180 m.g. 40%).

T.l.c. 0.46, CHCl_3 (bright yellow).

i.r. ν_{max} . (KBr): 2,950 (wk.), 1735 (CO_2Me), 1690, 1680, (quinone), 1622, 1570, 1435, 1328, 1250, 1230, 1118, 1003 (wk.), 955, 912, 865, 805, 776, 718, 690 cm^{-1} .

u.v. λ_{max} . (EtOH): 200 nm. (3,070), 272 nm. (ϵ 3,540).

n.m.r. 60 Mc/S. (CDCl_3) τ : 6.03 (s, -OMe).

m.s. m/e (Rel abundance): 275 (1), 271 (26), 269 (23), 241 (15),
239 (52), 237 (49), 213 (11), 211 (27), 209 (27), 185 (8), 183 (12),
181 (12), 89 (32), 87 (100).

Analysis Found : C, 36.1; H, 1.82%.

$\text{C}_8\text{H}_3\text{Cl}_3\text{O}_4$ requires C, 35.7; H, 1.1; Cl. 29.4%; M.W. 269.5.

$\text{C}_8\text{H}_5\text{Cl}_3\text{O}_4$ requires C, 36.2; H, 1.82; Cl. 39.18%; M.W. 271.5.

Reduction of Methyl 3-Chloro-5-acetoxysalicylate (242).

Methyl 3-chloro-5-acetygentisate (50mg.) in ether (10ml.) was added dropwise to a stirred suspension of lithium aluminium hydride (150 mg.) in dry ether (10ml.), and the solution stirred at R.T. for 1½ hours.

Ethyl acetate (20ml.) was carefully added and the ether allowed to evaporate. 5N hydrochloric acid (5ml.) was then added, and the solution stirred overnight. The ethyl acetate layer was decanted and the aqueous solution washed with ethyl acetate. The combined organic extracts were evaporated to give a red oil, which was purified by p.l.c. on Kieselgel HF₂₅₄ (20 cm. x 20 cm. x 0.75 mm.). Elution of the major dark-blue band under u.v. light with ethyl acetate, gave

3-chlorosentisyl alcohol (II) (24 mg., 68%), which crystallised as needles m.p. 144° from ethyl acetate - light petroleum, and was identical on t.l.c., in i.r. spectrum, and in mixed m.p. to the chlorine containing metabolite.

Analysis Found : C, 48.00; H, 3.94%

C₇H₇ClO requires C, 48.15; H, 4.04; Cl, 20.31%

EXPERIMENTALCHAPTER 8(a) Isolation of a Plasticiser.Acetyltributyl Citrate (247).

This compound was isolated in large quantity by silica gel column chromatography of the chloroform extract of the mycelium of Penicillium canadense¹⁷⁵, and was obtained as an oil, b.p. 140°/0.25mm.

T.l.c. 0.55, CHCl₃ (pale grey).

i.r. ν_{\max} . (thin film): 1745 (ester), 1285, 1180, 1065, 975 cm.⁻¹.

u.v. λ_{\max} . (Hexane): No absorption > 210 nm.

n.m.r. 60 Mc/S (CDCl₃) τ : 5.95 (6H, m, -CO.O-CH₂-), 6.94 (4H, AB quartet, J = 16.0 Hz. -CH₂-CO.O-), 7.94 (3H, s, CH₃CO.O-), 8.5 (12H, m, sat. -CH₂-), 9.10 (9H, t, J = 7.0 Hz., CH₃-CH₂-).

m.s. m/e (Rel. abundance): (M+)⁺ 403 (0.7), 402 (0.5), 347 (6), 342 (4), 329 (18), 301 (11), 260 (14), 259 (80), 212 (11), 186 (14), 185 (100), 157 (21), 129 (48), 111 (15), 57 (43), 56 (19), 55 (12).

Analysis Found : C, 59.77; H, 8.54%; M⁺ at m/e 402.

C₂₀H₃₄O₈ requires C, 59.68; H, 8.51% M.W. 402.5.

Molecular Weight Determination by Osmometry.

Standard 0.02, 0.04, 0.05 and 0.08M solutions of benzil in carbon tetrachloride were prepared and a graph of resistance (ΔR) against molar concentration (Cm) plotted. By preparing several solutions of the

plasticiser in carbon tetrachloride in the concentrations indicated below, it was possible to determine their molarity and hence the molecular weight (M) from the graph.

<u>Solution</u>	<u>Conc. (Mg./g)</u>	<u>R (measured)</u>	<u>Molarity (calc.)</u>	<u>M</u>
1	4.134	786	0.01625	405
2	8.450	1521	0.03340	404
3	10.830	1950	0.04280	404
4	23.330	4096	0.09380	398

Mean molecular weight of the plasticiser was 403 ± 5

Saponification of the Plasticiser.

A weighed amount of plasticiser dissolved in $N/10$ sodium hydroxide (10ml.) was refluxed for 2 hours. The solution was then cooled and titrated against $N/10$ hydrochloric acid, to give the molecular weight M calculated below (assuming the presence of 4 ester groupings). No product could be satisfactorily isolated from a constant chloroform extract of the acidified aqueous solution.

Weight of Plasticiser (mg.)	Vol. of $N/10$ NaOH (ml.)	Vol. of $N/10$ HCl (ml.)	$N/10$ NaOH Consumed (ml.)	M
58.9	10	4.3	5.7	414
52.2	10	4.9	5.1	410

Mean molecular weight was 412.

Selective Hydrolysis of Plasticiser.

The plasticiser (50mg.) and 3,5-dinitrobenzoic acid (30mg.) were

heated to 200° under nitrogen for 15 minutes. The reaction mixture was then cooled, diluted with chloroform (15ml.) and the organic solution washed with aqueous sodium bicarbonate, water and evaporated to give an oil. Purification was achieved by p.l.c. on Kieselgel (20 cm. x 20 cm. x 0.7 mm.) using iodine vapour for detection when tributylcitrate (32mg., 68%) was obtained as an oil, b.p. $150^{\circ}/0.1$ mm.

T.l.c. 0.40, CHCl_3 (pale grey).

i.r. ν_{max} . (thin film): 3,550 (-OH), 1740 (ester), 1185, 1065, 1022, 970 cm.^{-1} .

ν_{max} . (CCl_4): 3,520 (-OH), 1745 (ester), 1190, 1062, 1020, 960 cm.^{-1} .

u.v. λ_{max} . (EtOH): No absorption > 210 nm.

n.m.r. 60Mc/S. (CDCl_3) τ : 5.96 (6H, m, $-\text{CH}_2-\text{O}-\text{CO}$), 7.33 (4H, AB q., $J = 16.0$ Hz., $-\text{CH}_2-\text{CO}-$), 8.5 (12H, m, sat. $-\text{CH}_2-$), 9.03 (9H, t, $J = 7.0$ Hz. CH_3-CH_2-).

m.s. m/e (Rel abundance): $(M+1)^+$ 361 (0.1) 360 (0.2), 305 (2), 287 (5), 260 (11), 259 (65), 203 (6), 186 (15), 185 (100), 147 (9), 129 (67), 111 (15), 87 (13), 57 (39), 56 (21), 55 (13).

Analysis Found : C, 60.28; H, 8.91%; M^+ at m/e 360

$\text{C}_{18}\text{H}_{32}\text{O}_7$ requires C, 59.98; H, 8.95; M.W. 360.5.

Preparation of Tributyl Citrate.

Citric acid (10g.) in n-butanol (50ml.) at ice temperature was saturated with dry hydrogen chloride and the solution left at R.T. for seven days. The solution was then evaporated, and the resulting oil,

dissolved in chloroform (50ml.), extracted with saturated aqueous sodium bicarbonate, and water. Evaporation of the solvent gave tributyl citrate (15g., 80%) as an oil identical on t.l.c., and in i.r. and n.m.r. spectra to the alcohol obtained from selective hydrolysis of the plasticiser.

(b) Candipolin.

This compound was first isolated by silica gel column chromatography of the chloroform extract of the mycelium of Penicillium canadense¹⁷⁵. It was more conveniently obtained by concentrating the chloroform extract, and filtering the precipitated solid. Crystallisation from ethanol then gave candipolin as small needles, m.p. 210°.

$[\alpha]_D$ in ethanol (c 0.05), + 33.0.

T.l.c. 0.15, 1% MeOH in CHCl₃ (light grey).

i.r. ν_{\max} . (KBr): 3310 (CO.NH), 3060 (wk) (Ar.-H), 1750 (ester), 1640 (amide), 1604, 1580, 1530 (amide), 1450, 1390, 1030, 1000, 800, 744, 695 cm.⁻¹.

ν_{\max} . (CHCl₃): 3,435 cm.⁻¹ (ϵ 530), 1742 cm.⁻¹ (ϵ 400), 1660 cm.⁻¹ (ϵ 900).

u.v. λ_{\max} . (EtOH): 230 nm. (ϵ 10,000).

n.m.r. 100 Mc/S. (CDCl₃) τ : 2.27 (4H, m, $\underline{\text{O}}$ H of Ar.CO.O-), 2.5 - 2.8 (16H, m, Ar.- $\underline{\text{H}}$), 3.3 (2H, m, D₂O/F₃C.CO₂H exchangeable, irr. 5.05 \rightarrow s, -CO.NH-), 5.05 (1H, dt, $J_1 = J_2 = 6.5\text{Hz.}$, irr. 3.3 \rightarrow t ($J = 6.5\text{ Hz.}$), irr. 6.75 \rightarrow d, ($J = 6.5\text{ Hz.}$),

NH- $\underset{\text{H}}{\text{CH}}$ -CO.-), 5.4 (2H, m, irr. 5.9 \rightarrow br. S, - NH- $\overset{\text{H}}{\underset{\text{H}}{\text{C}}}$ -O-COR),
 5.9 (1H, dd, J = 5.0, 12.0 Hz., irr. 5.4 \rightarrow
 S, $\overset{\text{H}}{\underset{\text{H}}{\text{C}}}$ -O-COR, 6.75 (2H, d, J = 6.5 Hz., irr. 5.05 \rightarrow S, - $\underset{\text{H}}{\text{CH}_2}$ -Ar.),
 7.05 (2H, m, irr. 5.4 \rightarrow S, - $\underset{\text{H}}{\text{CH}_2}$ -Ar.).

m.s. m/e (Rel. abundance): M⁺ 506 (0.05), 415 (0.13), 294 (0.20),
 269 (1.5), 251 (3.3), 224 (3.3), 223 (2.2), 148 (33), 147 (17),
 146 (91), 118 (34), 105 (80), 91 (100), 77 (72),
 64 (17), 51 (27).

Analysis Found : C, 76.11; H, 6.20; N, 5.33%; M⁺ at m/e 506.

C₃₂H₃₀N₂O₄ requires C, 75.36; H, 5.97; N, 5.53%; M.W. 506.6.

Hydrolysis of Candipolin.

Candipolin (150 mg.) in a sealed glass tube (10 cm. x 0.4 cm. x 0.1 cm.) containing 5N hydrochloric acid (3ml.) was heated at 130° for 36 hours. The aqueous solution was then dried by azeotropic distillation using dry benzene and acetone (4X) to give an oily solid (A sample of this solid was methylated, using methanol and HCl, then trifluoroacetylated, using trifluoroacetic anhydride, and subjected to g.l.c. on a 1% NGS column at 150° to give two main peaks of R_t 5.4 and 9.6 min., which were identical to authentic samples of trifluoroacetylphenylalanine methyl ester and bistrifluoroacetylphenylalaninol). The oily solid was then washed with ether (3 x 5ml.), and the ether solution extracted with aqueous sodium bicarbonate. The aqueous solution was then acidified, extracted with chloroform

and the chloroform evaporated to give benzoic acid (46mg., 63%), which crystallised from methanol - water as needles, m.p. 122°, and was identical in mixed m.p. and i.r. spectrum to an authentic sample. Since attempts to separate the components of the ether extracted solid by solvent partition were unsuccessful, a solution in saturated methanolic hydrogen chloride (15ml.) was allowed to stand overnight. The solvent was then evaporated to give an oil (120mg.) which was treated with acetic anhydride (10ml.) and pyridine (0.1ml.) overnight. The solvent was removed in vacuo to give an oil (140mg.) which was separated into two components by p.l.c. on Kiesselgel HF₂₅₄ (20cm. x 20 cm. x 1.0 mm.) using multiple elution in 1% MeOH/99% CHCl₃.

Component A: L-Acetylphenylalanine Methyl Ester.

This compound (41 mg., 61%) crystallised from ether - light petroleum as needles, m.p. 90° (Lit. m.p. 91°¹⁸³).

[α]_D in chloroform (C 2.0), + 25° (lit. [α]_D in methanol, + 19.5°¹⁸³).

T.l.c. 0.20, 1% MeOH in CHCl₃ (pale brown).

i.r. ν_{\max} . (KBr.): 3330 (CO.NH), 3,200 (wk) (Ar.-H) 1748 (ester), 1645 (amide), 1598 (wk), 1527 (CO.NH), 1432, 1380, 1285, 1260, 1215, 1187, 1168, 1115, 1080, 1038, 995, 970, 948 cm.⁻¹.

n.m.r. 60Mc/S (CDCl₃) τ : 2.88 (5H, br. S, Ar. -H), 3.93 (1H, m, -CO.NH-), 5.05 (1H, dt, J = 7.0, 8.0 Hz., -NH-¹CH-CO.NH-), 6.20 (3H, s, -OMe), 6.80 (2H, d, J = 6.0 Hz., Ar. -CH₂-),

7.90 (3H, s, $\text{CH}_3\text{CO.NH}$).

Analysis Found : C, 65.40; H, 6.86; N, 6.39%.

$\text{C}_{12}\text{H}_{15}\text{NO}_3$ requires C, 65.14; H, 6.83; N, 6.33%; M.W. 221.3.

Component B: L-Diacetylphenylalaninol.

This compound (47 mg., 68%) crystallised from benzene - light petroleum as needles, m.p. 123° $[\alpha]_D$ in chloroform (C 1.5), -13° .
($[\alpha]_D$ of synthetic material (see below) in chloroform (C 2.25), -15.5°).

T.l.c. 0.18, 1% MeOH in CHCl_3 (pale brown).

i.r. ν_{max} . (KBr) 3,900 (CO.NH), 1725 (acetate), 1640 (amide), 1602 (wk), 1545 (CO.NH), 1385, 1370, 1305, 1265, 1255, 1193, 1040, 1028, 750, 698 cm^{-1} .

n.m.r. 60Mc/S. (CDCl_3) τ : 2.78 (5H, br. s, Ar.-H), 4.40 (1H, m, $-\text{CO.NH}-$), 5.60 (1H, m, $\text{NH}-\overset{|}{\text{CH}}-\text{CO.NH}-$), 5.97 (2H, d, $J = 5.0$ Hz., $-\text{CH}_2\text{OAc}$), 7.13 (2H, d, $J = 7.0$ Hz., Ar.- CH_2-), 7.90 (3H, s, $\text{CH}_3\text{CO.NH}-$), 8.07 (3H, s, $\text{CH}_3\text{CO.O}-$).

Analysis Found : C, 66.29; H, 7.10; N, 5.73%.

$\text{C}_{13}\text{H}_{17}\text{NO}$ requires C, 66.36; H, 7.28; N, 5.95%; M.W. 235.3.

L-Benzoylphenylalanine Ethyl Ester.

L-Phenylalanine ethyl ester hydrochloride (197mg.) in dry pyridine (5ml.) containing benzoyl chloride (120mg.) was left at ice temperature for 2 hours. The solution was then diluted with chloroform (20ml.) and the organic solution extracted with 5N hydrochloric acid, water and evaporated to give benzoylphenylalanine ethyl ester (205mg. 80%),

which crystallised from benzene - light petroleum as needles, m.p. 103°.

$[\alpha]_D$ in CHCl_3 (C 0.27), + 72.7°.

T.l.c. 0.70, CHCl_3 (faint grey).

i.r. ν_{max} . (KBr): 3,362 (CO.NH), 1752 (ester), 1645 (amide), 1605 (wk), 1570 (wk), 1525 (CO.NH) 1493, 1442, 1370, 1300, 1274, 1210, 1180, 1155, 1095, 1010 cm^{-1} .

n.m.r. 60Mc/S. (CDCl_3) τ : 2.27 (2H, m, ortho H of Ar.CO.O-), 2.5 - 2.8 (2H, m, Ar.-H), 3.20 (1H, m, -CO.NH-), 4.95 (1H, dt, J = 5.0, 7.0 Hz., -NH-CH¹-CO₂Et), 5.20 (2H, q, J = 7.0 Hz., CH₃.CH₂.O.CO). 6.73 (2H, d, J = 5.0 Hz., Ar.-CH₂-), 8.71 (3H, t, J = 7.0 Hz., CH₃.CH₂-O.CO-).

Analysis Found : C, 72.85; H, 6.54; N, 4.55%.

$\text{C}_{18}\text{H}_{19}\text{NO}_3$ requires C, 72.71; H, 6.44; N, 4.71%; M.W. 297.4.

L-Benzoylphenylalanine (252).

L-Benzoylphenylalanine ethyl ester (500mg.) in dioxan (17ml.) -water (25ml.) was refluxed for 2 hours. The solution on cooling to 5°, gave benzoylphenylalanine (350 mg., 77%) which crystallised from benzene - light petroleum as needles, m.p. 142°, (Lit. value 142° 203).

$[\alpha]_D$ in CHCl_3 (C 0.25), + 116°.

T.l.c. 0.40, benzene (35) - dioxane (5)-acetic acid (1) (faint grey),

i.r. ν_{max} . (KBr): 3380 (CO.NH), 3200 - 2,600 (CO₂H), 1705 (CO₂H), 1635 (CO.NH), 1598 (wk), 1575 (wk), 1520 (-CONH-), 1485, 1435, 1415, 1315, 1290, 1260, 1235, 1183, 1150, 1085, 1070, 1022, 745, 690 cm^{-1} .

n.m.r. 60Mc/s. (CDCl_3) τ : 1.47 (1H, s, D_2O exchangeable, $-\text{CO.OH}$),
 2.30 (2H, m, o Ar.-H), 2.6 (3H, m, m and p Ar.-H), 3.20
 (1H, d, $J = 7.0$ Hz., $-\text{CO.NH-}$), 4.87 (1H, m, $-\text{NH-CH-CO}_2\text{H-}$),
 6.67 (2H, d, $J = 5.0$ Hz., Ar.- CH_2-).

Analysis Found : C, 71.52; H, 5.73; N, 5.08%.

$\text{C}_{16}\text{H}_{15}\text{NO}_3$ requires C, 71.36; H, 5.61; N, 5.20%; M.W. 269.3

Preparation of L-Phenylalaninol (253) from Phenylalanine Ethyl Ester Hydrochloride

(a) Using Sodium Borohydride.

To a solution of sodium borohydride (2.5g.) in absolute ethanol (20ml.) was added dropwise a solution of phenylalanine ethyl ester hydrochloride (2g.) in absolute ethanol. The solution was refluxed for 24 hours, when phenylalanine (0.9g., 64%) crystallised on the surfaces of the flask, and was identified with an authentic sample by m.p. (283°) and on t.l.c. The ethanol solution was concentrated, water (20ml.) added, and the aqueous solution extracted with ethyl acetate. Evaporation of the organic solution gave phenylalaninol (42mg., 3%) identical in m.p. and i.r. spectrum to a sample prepared by method (b). This yield was not improved by using aqueous ethanol or purified sodium borohydride.

(b) Using Lithium Aluminium Hydride.

L-phenylalanine ethyl ester hydrochloride (1.0g.) in chloroform (20ml.) was extracted with 5N sodium hydroxide, water and evaporated

to give phenylalanine ethyl ester as an oil (0.85g. 94%). A solution of this oil in dry ether (20ml.) was added dropwise to a stirred suspension of lithium aluminium hydride (470mg.) in dry ether (20ml.) and the solution stirred at R.T. for 1 hour. 10% ammonium chloride (5ml.) was then carefully added, followed by wet ether (10ml.). After one hour the grey precipitate was filtered, washed with ether and the combined ethereal extracts evaporated to give a white solid. Crystallisation from benzene gave L-phenylalaninol (500 mg., 76%) as needles m.p. 92° (Lit. m.p. $91-3^{\circ}$ ²⁰⁴).

$[\alpha]_D$ in CHCl_3 (C 0.22), -22.7° (Lit. $[\alpha]_D$ in EtOH (C 1.0), -25.6°).

T.l.c. 0.2, 96% EtOH - 4% H_2O . Purple red on heating (ninhydrin).

i.r. ν_{max} . (KBr): 3,325 and 3,295 ($-\text{NH}_2$), 3,200 - 2,800 (br) ($-\text{OH}$), 1575, 1490, 1452, 1335, 1119, 1085, 1060, 988, 970, 958, 902, 850, 830, 750, 700, 693 cm^{-1} .

n.m.r. 60Mc/S. (CDCl_3) τ : 2.73 (5H, s, Ar.-H), 6.50 (2H, m, $-\text{CH}_2-\text{CH}$), 6.90 (1H, m, $-\overset{1}{\text{C}}\text{H}-\text{NH}_2$), 7.23 and 7.43 (2H, AB quartet $J = 6.0$ Hz., Ar.- CH_2 -).

Analysis Found : C, 71.56; H, 8.53; N, 9.03%

$\text{C}_9\text{H}_{13}\text{NO}$ requires C, 71.49; H, 8.67; N, 9.26%; M.W. 151.2.

N,O-Diacetyl-L-Phenylalaninol (251).

L-Phenylalaninol (60mg.) in acetic anhydride (10ml.) containing dry pyridine (0.2ml.) was left at R.T. overnight. The reaction solution was concentrated in vacuo, diluted with chloroform (20ml.) and the organic solution extracted with 5N sodium hydroxide, water and dried.

Evaporation of the solvent gave an oil. Crystallisation from benzene - light petroleum gave diacetyl-L-phenylalaninol, m.p. 128° identical in mixed m.p. and i.r. spectrum to component B of the candipolin hydrolysis.

$[\alpha]_D$ in CHCl_3 (C 2.25), - 15.5°

p-nitrophenyl ester of Benzoylphenylalanine

To L-benzoylphenylalanine (36.9mg.) in analar ethyl acetate (5ml.) at 0° was added p-nitrophenol (25mg.), followed by dicyclohexylcarbodiimide (28mg.) and the mixture stirred at ice temperature for $\frac{1}{2}$ hour, then allowed to warm to R.T. The precipitate of dicyclohexylurea was filtered, and the solution evaporated to give an oil. The p-nitrophenyl ester (45mg., 69%) crystallised from ethanol as needles, m.p. 159°.

$[\alpha]_D$ in CHCl_3 (C 0.25), 0°.

T.l.c. 0.70, CHCl_3 (reddish brown).

i.r. ν_{max} . (KBr): 3,320 (CO.NH), 1755 (ester), 1638 (amide), 1518 (-CONH), 1485 and 1344 (NO_2), 1225, 1200, 1145, 1035, 958, 890, 861 cm^{-1} .

n.m.r. 60Mc/s. (CDCl_3) τ : 1.73 (2H, d, J = 9.0 Hz.), 2.2 - 2.7 (10H, m, Ar.-H), 2.83 (2H, d, J = 9.0 Hz.), 3.30 (1H, m, -CO.NH-), 4.70 (1H, q, J = 7.0 Hz., -NH-CH-CO-O-), 6.60 (2H, d, J = 7.0 Hz., Ar.-CH₂-).

Analysis Found : C, 67.53; H, 4.64; N, 7.29%

$C_{22}H_{18}N_2O_5$ requires C, 67.69; H, 4.65; N, 7.18%; M.W. 390.4.

Reaction of p-nitrophenyl Ester with L-Phenylalaninol (253).

To L-Benzoylphenylalanine p-nitrophenyl ester (79mg.) in analar ethyl acetate (5ml.) was added L-phenylalaninol (30mg.) and the solution left at R.T. for 24 hours. The ethyl acetate solution was then washed with 5N sodium hydroxide, water and evaporated to give an oil (85 mg.). The two main components of this oil were separated by p.l.c. on Kieselgel HF₂₅₄ (20 cm. x 20 cm. x 1.0 mm.) to give

Amide Alcohol (254) (35 mg., 34%) was obtained as a glassy solid.

$[\alpha]_D^{25}$ in $CHCl_3$ (c 0.07), -26.2°

T.l.c. 0.20, 2% MeOH in $CHCl_3$ (brown).

i.r. ν_{max} . (KBr): 3,600 - 3,200 (br) (-OH), 3,380 (CO.NH), 3050 and 3020 (wk) (Ar.-H), 1655 (amide), 1630 (amide), 1530 (br) (CO.NH), 1485, 1440, 1375, 1320, 1250, 1075, 1028 cm^{-1} .

n.m.r. 60Mc/s. ($CDCl_3$) τ : 2.3 (2H, o H of Ar -CO.O-), 2.6 (3H, m. and p. H of Ar -CO.O-), 2.70 (5H, s, Ar.-H), 2.87 (5H, s, Ar.-H), 5.20 (1H, q, J = 7.0 Hz., -NH-CH - CO.NH-), 5.80 (1H, m, -NH-CH-CH₂OH), 6.47 (3H, m, 1H D_2O exchangeable \rightarrow d(J = 4.0 Hz.), -CH₂-OH), 6.78 (2H, m, Ar.-CH₂-), 7.20 (2H, d, Ar.-CH₂-).

m.s. m/e (Rel.abundance): (M-1)⁺ 401 (0.2), 400 (0.4), 382 (2), 293 (3), 252 (11), 224 (8), 190 (3), 182 (1), 134 (2), 131 (2), 120 (12), 105 (100), 91 (36), 77 (25), 60 (13), 51 (3).

Isomeric Amide Alcohol (255) (30mg., 29%)

crystallised from benzene as needles, m.p. 176°.

$[\alpha]_D$ in CHCl_3 (c 0.15), + 34.5°.

T.l.c. 0.18, 2% MeOH in CHCl_3 (brown).

i.r. ν_{max} . (KBr): 3,500 - 3,200 (br.) (-OH), 3,280 (CO.NH), 1650 (amide), 1630 (amide), 1600 (wk), 1575 (wk), 1530 (br) (CO.NH), 1485, 1435, 1370, 1315, 1276, 1238, 1180, 1070, 1038, 1023, 743, 695, 670 cm^{-1} .

n.m.r. 60Mc/s. (CCl_4) τ : 2.3 (2H, m, $\underline{\text{O}}$ H of Ar.-CO.O-), 2.6 (3H, m and $\underline{\text{O}}$ H of Ar.-CO.O-), 2.8 (1OH, br. S, Ar.- $\underline{\text{H}}$), 3.20 (1H, d, $J = 7.0$ Hz., -CO.NH-), 5.07 (1H, q, $J = 7.0$ Hz., -NH- $\overset{\text{I}}{\text{CH}}$ -CO.NH-), 5.80 (1H, m, -NH- $\overset{\text{I}}{\text{CH}}$ -CH₂OH), 6.40 (3H, m, 1H, D₂O exchangeable, -CH₂- $\overset{\text{I}}{\text{OH}}$), 6.93 (2H, d, $J = 7.0$ Hz., Ar.- $\overset{\text{I}}{\text{CH}_2}$ -), 7.32 (2H, d, $J = 7.0$ Hz., Ar. - $\overset{\text{I}}{\text{CH}_2}$ -).

Analysis Found: C, 74.47; H, 6.53; N, 6.91%.

$\text{C}_{25}\text{H}_{26}\text{N}_2\text{O}_3$ requires C, 74.60; H, 6.51; N, 6.96%; M.W. 402.5.

L-Benzoylphenylalanine Hydrazide

L-Benzoylphenylalanine ethyl ester (411 mg.) in ethanol (10ml.) was refluxed with 85% hydrazine hydrate (0.5 ml.) for 8 hours. On cooling to ice temperature L-benzoylphenylalanine hydrazide (311 mg., 80%) crystallised as needles m.p. 153°.

T.l.c. 0.12, 4% MeOH in CHCl_3 (brown).

i.r. ν_{max} . (KBr): 3,270 ($\overset{\text{I}}{\text{NH}}$), 3050 and 3020 (wk) (Ar.- $\overset{\text{I}}{\text{H}}$), 1630 (amide), 1595, 1575, 1520 (CO.NH), 1433, 1435, 1365, 1315, 1280, 1235, 1173, 1070, 1022, 920, 870, 795, 740, 690 cm^{-1} .

n.m.r. 60Mc/s. ($\text{CF}_3\text{CO}_2\text{H}$) τ : 2.40 (5H, m, Bz-H), 2.60 (5H, s, Ar.-H),
4.87 (1H, q, $J = 7.0\text{Hz.}$, $-\text{NH}-\overset{1}{\text{C}}\text{H}-\text{CO.NH}-$), 6.63 (2H, d, $J = 7.0\text{ Hz.}$,
Ar.- $\overset{2}{\text{C}}\text{H}_2$ -).

Analysis Found : C, 67.92; H, 6.02; N, 14.35%

$\text{C}_{16}\text{H}_{17}\text{N}_3\text{O}_2$ requires C, 67.33; H, 6.05; N, 14.33%; M.W. 283.3.

Reaction of L-Benzoylphenylalanine Azide and L-Phenylalaninol (253).

L-Benzoylphenylalanine hydrazide (100mg.) in glacial acetic acid (5ml.) was diluted with 5N hydrochloric acid (30ml.) and cooled with an ice-salt bath. Ethyl acetate (20ml.) was then added and the mixture stirred vigorously while an ice cold solution of sodium nitrate (90 mg.) in water (5ml.) was added all at once to the aqueous layer. After stirring for $\frac{1}{2}$ hour, the ethyl acetate layer was separated, washed with ice cold aqueous sodium bicarbonate, water and dried using MgSO_4 and CaCl_2 . L-Phenylalaninol (50mg.) was added to the dried solution and left at 5° for 24 hours. The solvent was then evaporated to give an oil (123 mg.) which was purified by p.l.c. on Kieselgel HF₂₅₄ (20 cm. x 20 cm. x 1.0 mm.). Elution of the major band under u.v. light (λ 254 nm) gave the amide alcohol (254) (102 mg., 72%) identical on t.l.c. and in i.r. and mass spectra to a sample prepared above from benzoylphenylalanine *p*-nitrophenyl ester.

Attempted Couplings of L-Benzoylphenylalanine (252) and L-Phenylalaninol 253

Attempts to couple L-benzoylphenylalanine (252) and L-phenylalaninol

(253) using the acid chloride and mixed anhydride techniques or with dicyclohexylcarbodiimide reagent were unsuccessful, only traces of the amide alcohol (254) being detected by t.l.c.

Benzoylated Amide (254).

The amide alcohol (254) (25 mg.) in dry pyridine (5ml.) containing benzoyl chloride (9mg) was left at ice temperature overnight. The solution was then diluted with chloroform (10ml.) and the solution washed with 5N hydrochloric acid, 5N sodium hydroxide and water.

Evaporation of the solvent gave the benzoylated amide as a solid (27 mg., 80%) which crystallised from ethanol as needles, m.p. 209°.

T.l.c. 0.15, 1% MeOH in CHCl₃ (light grey).

i.r. ν_{\max} . (KBr): 3,280 (CO.NH), 3050 and 3020 (wk) (Ar.-H), 1722 and 1710 (ester), 1652 (amide) 1630 (amide), 1530 (br) (CONH), 1490, 1448, 1376, 1312, 1280, 1135, 1065, 1023, 750, 710, 690 cm.⁻¹.

n.m.r. 100 Mc/S. (CDCl₃) τ : 2.00 (2H, o H of Ar.-CO.O-), 2.28 (2H, m, o H of Ar.CO.NH-), 2.55 (6H, m, Ar.-H), 2.86 (10H, br, s, Ar.-H), 3.16 (1H, d, J = 7.0 Hz., -CO.NH-), 3.75 (1H, d, J = 3.0 Hz., -CO.NH-), 5.21 (1H, dq, J = 7.0 Hz., 8.0 Hz., -NH-CH-CO.NH-), 5.50 (1H, m, NHCH-CH₂OBz), 5.88 (2H, m, -CH₂-OBz), 6.88 (2H, m, -CH₂-Ar), 7.16 (2H, d, J = 7.0 Hz., -CH₂-Ar.).

m.s. m/e (Rel. abundance): M⁺ 506 (0.01), 394 (10), 293 (12), 279 (4.6), 264 (2.5), 252 (3.5), 172 (8.5), 122 (8), 105 (100), 91 (17), 77 (30), 65 (3.5), 51 (8).

Analysis Found : C, 75.79; H, 6.11; N, 5.30% : M at m/e 506.

$C_{32}H_{30}N_2O_4$ requires C, 75.87; H, 5.97; N, 5.53%; M.W. 506.

Isomeric Benzoylated Amide (258).

The isomeric peptide alcohol (20mg.) in dry pyridine (3ml.) containing benzoyl chloride (8mg.) was left at ice temperature overnight. The solution was diluted with chloroform (10ml.) and worked up in the usual way. The isomeric benzoylated amide (18 mg., 72%) crystallised from ethanol as needles, m.p. 212°.

T.l.c. 0.15, 1% MeOH in $CHCl_3$ (light grey).

i.r. ν_{max} . (KBr): 3,285 (CONH), 3050 and 3020 (wk), (Ar.-H), 1730, 1718, 1708 (ester), 1655 (amide), 1630 (amide), 1540 (br.) (-CONH-), 1490, 1450, 1383, 1285, 1270, 1128, 1070, 1029, 790, 753, 717, 695 cm^{-1} .

n.m.r. 100 Mc/S. ($CDCl_3$) τ : 2.08 (2H, br. d, $J = 9.0$ Hz., $\underline{0}$ H of Ar.-CO.O-), 2.35 (2H, br. d, $J = 9.0$ Hz., $\underline{0}$ H of Ar.-CO.NH-), 8.5-8.6 (6H, m, Ar.-H), 8.74 (5H, s, Ar.-H), 8.78 (5H, s, Ar.-H). 3.26 (1H, d, $J = 7.0$ Hz., -CO.NH-), 3.68 (1H, d, $J = 7.0$ Hz., -CO.NH-), 5.20 (1H, dq, $J = 7.0, 8.0$ Hz., -NH-CH-CO.NH-), 5.50 (1H, m, -NH-CH-CH₂-O.Bz), 5.80 (2H, m, -CH₂-O-Bz), 6.90 2H, d, $J = 7.0$ Hz., Ar. - CH₂-), 7.20 (2H, m, Ar.-CH₂-).

Analysis Found : C, 75.84; H, 5.78; N, 5.66%.

$C_{32}H_{30}N_2O_4$ requires C, 75.87; H, 5.97; N, 5.53%; M.W. 506.6.

N,O-dibenzoyl-L-Phenylalaninol

L-Phenylalaninol (54mg.) in dry pyridine (5ml.) containing benzoyl chloride (94mg.) was left at ice temperature overnight. Chloroform (15ml.) was added and the organic solution washed with 5N hydrochloric acid, water and evaporated to give N,O-dibenzoylphenylalaninol as a solid (92 mg., 71%) which crystallised from benzene - light petroleum as needles, m.p. 170° (lit. m.p. 169°¹⁹⁸).

T.l.c. 0.46, CHCl₃ (pale grey).

i.r. ν_{\max} . (KBr): 3,310 (CO.NH), 1720 and 1710 (ester), 1635 (amide), 1602, 1578, 1538 (CONH), 1490, 1450, 1431, 1382, 1358, 1315, 1285, 1195, 1178, 1155, 1132, 1100, 1070, 1025, 975, 842, 797, 755, 708 cm.⁻¹.

n.m.r. 60Mc/S. (CDCl₃) τ : 1.98 (2H, m, oH of Ar.-CO, O-), 2.30 (2H, m, oH of Ar.-CO.NH-), 2.60 (6H, m, m and p H of Bz.-), 2.70 (5H, s, Ar.-H), 3.37 (1H, d, J = 8.0 Hz., -CO.NH-), 5.55 (2H, d, J = 5.0 Hz. -CH₂-OBz), 5.87 (1H, dd, J = 7.0, 8.0 Hz., -NH-CH-CH₂-O-), 6.90 (2H, m, Ar.-CH₂-).

Analysis Found : C, 77.10; H, 6.13; N, 3.79%.

C₂₃H₂₁NO₃ requires C, 76.86; H, 5.89; N, 3.90%; M.W. 359.4.

N-Benzoyl -L- phenylalaninol.

L-Phenylalaninol (40mg.) was added to a solution of benzoyl chloride (37mg.) in dry pyridine (4ml.) at 0° and the solution stirred for 1 hour. Chloroform (20ml.) was then added and the solution extracted with 5N hydrochloric acid, saturated aqueous sodium bicarbonate and water.

Evaporation of the solvent gave N-benzoyl-L-phenylalaninol as a solid, which crystallised from chloroform - light petroleum as plates, m.p. 173°.

T.l.c. 0.20, CHCl₃ (grey).

i.r. ν_{\max} . (KBr): 3,400 - 3,200 (br) (-OH), 3310 (CO.NH), 3020 (Ar.-H), 1635 (amide), 1600 (wk.), 1540 (CO.NH), 1485, 1446, 1328, 1282, 1215, 1082, 1050, 1030, 997, 850, 797, 755, 693 cm.⁻¹.

n.m.r. 60Mc/S. (CHCl₃) (weak soln.) τ : 2.3 - 2.7 (5H, m, Bz.-H), 2.73 (5H, Ar.-H), 5.6 (1H, m, -NHCH-), 6.21 (2H, d, J = 5.0 Hz., -CH₂-CH), 6.97 (2H, d, J = 7.0 Hz., Ar.-CH₂-).

Analysis Found: C, 75.10; H, 6.69; N, 5.53%.

C₁₆H₁₇NO₂ requires C, 75.27; H, 6.71; N, 5.49%; M.W. 255.3.

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