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Styrylpyrone glucosides with antimicrobial activity from *Senecio mannii* Hook. (Asteraceae)

[Glucósidos de estirilpironas con actividad antimicrobiana aislados de *Senecio mannii* Hook. (Asteraceae)]

Jean Claude NDOM [1*] Joseph Tanyi MBAFOR [2*] Zanetsie KAKAM [2] Ngeufa HAPPI [1] Juliette Catherine VARDAMIDES [1] Luc Mbaze MEVA'A [1], Theophile Mpondo NGANDO [1], Zacharias Tanee FOMUM [2]

[1]*Department of Chemistry, University of Douala, Faculty of Science P.O. Box 2415, Douala, Cameroon.*
[2]*Department of Chemistry, University of Younde I, Faculty of Science, P.O. Box 812, Yaounde, Cameroon.*

*Contact: Tel: +237-694-71-51; E-mail 1: ndomjefr@yahoo.fr (JC Ndom); E-mail 2: Jtmbafor@yahoo.fr (JT Mbafor).

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Abstract

Phytochemical investigation of the methanol extract of the dried ground aerial plants of *Senecio manii* gave two new compounds 4-methoxy-6-(11-O-ß-D-glucopyranosylstyryl)-α-pyrone (1) and 4-methoxy-6-(11-O-α-L-rhamnopyranosylstyryl)-α-pyrone (2) as well as the four known compounds, namely 4-methoxy-6-(11-hydroxystyryl)-α-pyrone (3), 4-methoxy-6-(12-hydroxystyryl)-α-pyrone (4), 4-methoxy-6-(12-O-ß-D-glucopyranosylstyryl)-α-pyrone (5) and α-amyrin. Their structures were established based on spectroscometric analysis. The styrylpyrone derivatives showed a significant antimicrobial activity.

Keywords: Senecio mannii; Asteraceae; styrylpyrones; antimicrobial activity; antifungal activity.

Resumen

Estudios fitoquímicos del extracto metanólico de las partes aéreas secas y pulverizadas de *Senecio manii* dieron lugar al aislamiento y determinación de dos nuevos compuestos, 4-metoxi-6-(11-O-ß-D-glucopiranosilestiril)-α-pirona (1) y 4-metoxi-6-(11-O-α-L-ramnopiranosilestiril)-α-pirona (2) asi como cuatro compuestos conocidos: 4-metoxi-6-(11-hidroxiestiril)-α-pirona (3), 4-metoxi-6-(12-hidroxiestiril)-α-pirona (4), 4-metoxi-6-(12-O-ß-D-glucopiranosilestiril)-α-pirona (5) y α-amyrina. Sus estructuras fueron establecidas en base a análisis espectroscópicos. Las estirilpironas demostraron estar dotadas de una significativa actividad antimicrobiana.

Palabras clave: Senecio mannii; Asteraceae; estirilpironas; actividad antimicrobiana; actividad antifungica

INTRODUCTION

Senecio mannii (Asteraceae) is a perennial medicinal herb native to African mount. It can be found in the South West, West and Nord West provinces of Cameroon where it is used locally to treat microbial and fungal diseases (Dalziel, 1937; Hutchinson and Dalziel, 1958; Bouquet and Debray, 1974). Previous pharmacological studies presented antimicrobial and antifungal activity of the total extract of *S. inaequidens* DC, *S. vulgaris* and *S. boissieri* (Steenkamp *et al.*, 2001; Louzzo *et al.*, 2004). Several species of *Senecio* permit to isolate a large variety of secondary metabolites include flavonoids (Ragaa and Nabiel., 1981), chalcones (D'Agostino *et al.*, 1991), acetophenones (Urones *et al.*, 1987), xanthones (Catalano *et al.*, 1996), triterpenoids (Rücker *et al.*, 1999). Recently we reported the isolation of cacalolide and shikimic acid from this genus (Ndom *et al.*, 2006). As part of our continuing search of bioactive compounds from the plant genus *Senecio*, we have now investigated the constituents of *S. mannii*. We report here the isolation and structural elucidation of two new compounds as well as the antimicrobial activity of compounds **3 - 4** evaluation.

MATERIAL AND METHODS

Plant material

Air-dried and ground aerial plants of *S. mannii* Hook. were collected in November 2006 at Limbe

locality to mount Cameroon (2200 m), South - West of Cameroon. The sample was identified by Mr Ndive Elias from Botanical gardens, Limbe, Cameroon, where a voucher specimen (IC12394) is deposited.

Extraction and Isolation

The air-dried and ground aerial plants of *S. mannii* (1.5 kg) were immersed in MeOH at room temperature during 72 hours. After removing the solvents by evaporation under reduced pressure, the obtained crude extract (76.7 g) was chromatographed over silica gel 60 (230-400 mesh ASTM, Merck), using *n*-hexane (Hex) and ethylacetate (EtOAc) in increasing polarity order. A total of 75 subfractions (ca. 200 mL each) were collected and combined in ten fractions (A-J) based on TLC analysis. Fraction D (7.8 g) is the combined subfractions 30-46 eluted with mixture Hex-EtOAc (8.5:1.5). Fraction F (5.2 g) was set up of subfractions 62-77 eluted with mixture Hex-EtOAc (4:1). Fraction G (4.0 g) was set up of subfractions 78-82 eluted with mixture Hex-EtOAc (7.5:2.5). Main fraction D was chromatographed over silica gel 60C (20-40 µm) column with mixture hex-EtOAc gradient. 25 fractions (*ca*. 100 mL each) were collected and combined based on TLC. Fractions 5-24 were further chromatographed over preparative TLC (Silica gel 60C, 20-40 µm) using mixture Hex-EtOAc gradient (8.5:1.5) to give 4-methoxy-6-(11-hydroxystyryl)-α-pyrone (**3)** (15.6 mg) and 4-methoxy-6-(12-hydroxystyryl)-α-pyrone (**4)** (31.2 mg). Fraction F was chromatographed over silica gel 60C (20-40 µm) column with mixture Hex-EtOAc gradient. 28 fractions of (*ca*. 100 mL each) were collected and combined based on TLC. Fractions 1-12 and 16-26 were successively further chromatographed over silica gel 60C (20-40 µm) with mixture Hex-EtOAc (4:1) to give α-amyrin (40 mg) and 4-methoxy-6-(12-O-ß-D-glucopyranosylstyryl)-α-pyrone **(5)** (20.3 mg).

Main fraction G was chromatographed over silica gel 60C (20-40 µm) column with a gradient. 45 fractions of (*ca*. 100 mL each) were collected and combined based on TLC. Fractions 7-29 were chromatographed using preparative TLC on silica gel 60C (20-40 µm) with a mixture Hex-EtOAc as eluent to give 4-methoxy-6-(11-O-ß-D-glucopyranosylstyryl) -α-pyrone **(1)** (26.8 mg) and 4-methoxy-6-(11-O-α-L-rhamnopyranosylstyryl)-α-pyrone **(2)** (29.4 mg).

Identification of Compounds

Melting points were determined on a Buchi apparatus and were uncorrected. UV spectra were obtained on a Shimadzu- 265 Spectrophotometer and recorded in methanol. IR spectra were recorded on a Perkin-Elmer 727B spectrometer in KBr discs. The HR-ESI-TOF-MS were obtained in the positive ion mode on pulsar mass spectrometer. ^{1}H and ^{13}C-NMR spectra were obtained with a Brucker model equipped with a 5 mm ^{1}H and ^{13}C (ATP) probe operating at 300 and 75 MHz, respectively, with TMS as internal standard. Homonuclear ^{1}H connectivities were determined by using the COSY and NOESY (mixing time 500 ms experiments) experiments. One-bond ^{1}H-^{13}C connectivities were determined with HMQC (Heteronuclear Multiple Quantum Connectivity by 2D-multiple) gradient pulse factor selection. Two - and three-bond ^{1}H-^{13}C connectivities were determined by HMBC (Heteronuclear Multiple Bond Connectivity by 2D-multiple Quantum) experiment. Chemical shifts were reported in δ (ppm) and coupling constants (J) were measured in Hz. Precoated aluminium sheets silica gel 60 F_{254} TLC (Thin Layer Chromatographic) plates was used to check the purity of compounds and preparative chromatography. Spots were visualised by UV lamp (254 nm and 365 nm) or by 50% H_2SO_4 reagent. All reagents used were of analytical grades.

4-methoxy-6-(11-O-ß-D-glucopyranosylstyryl)-α-pyrone (1)

White crystals; (CH_2Cl_2); mp 262-264° C; UV λ^{MeOH}_{max} (logε) nm: 224 (4.03), 264 (3.85), 309 (3.33), 328 (3.28), 360 (3.23); IR (KBr) υ_{max} 3450, 1750, 1631, 1517 cm^{-1}; ^{1}H NMR and ^{13}C NMR see table 1; HR-ESI-TOF-MS $[M+H]^+$ m/z. 407.1263 (calc. for $C_{20}H_{22}O_9$ 407.1264); EIMS *m/z* (rel. int.): 406 (100) $[M]^+$.

Acid hydrolysis of 4-methoxy-6-(11-O-ß-D-glucopyranosylstyryl)-α-pyrone:

The sample (25 mg) was dissolved in 7% H_2SO_4 and refluxed on a water bath for 4 hours. The reaction mixture was diluted with 20 mL of H_20 and extracted with CH_2Cl_2. Evaporation of solvent followed by purification of the residue by prep. TLC over silica gel with toluene-Me_2CO (10:3) as eluente gave a white compound identified as 4-methoxy-6-(11-hydroxystyryl)-α-pyrone by comparison with its physical and spectral data (Keith *et al.*, 1967).

Identification of the sugar moiety: the aqueous phase after extraction with CH_2Cl_2 was neutralised with 1M NaOH and evaporated in vacuum. H_2O was added to the residue and the mixture was again evaporated in vacuum to remove all the impurities. The residue obtained was compared to standard sugars by TLC using n-BuOH-toluene-pyridine-H_2O (5:1:3:3)

(BTPW).The sugar was detected with aniline hydrogen phalate, and shown to consist to D-glucose. For GLC analysis, the residue was dissolved in TRISIL (0.05 ml: N-(trimethylsilyl)-imidazole in pyridine), left at room temp. for 15 min, and analysed by GLC on a SHIMADZU GC-GA gas chromatograph, glass column 2.6 mm x 2mn packed with 1.5 % SE-30 on chromosorb W, detector FID injection temp. 150° carrier gas N_2 (40 mL min^{-1}). The GLC peaks of the silylated derivative of the residue and glucose had the same retention time (R_t 4.9 min).

4-methoxy-6-(11-O-α-L-rhamnopyranosylstyryl)-α-pyrone (2)

White crystals, (CH_2Cl_2); mp 252-254°C; UV λ^{MeOH}_{max} (logε) nm 228 (4.01), 247 (3.81), 252 (2.64), 266 (3.87), 300 (3.36), 307 (3.50), 328 (3.30), 360 (3.25), IR (KBr) υ_{max} 3450, 1750, 1631,1517 cm^{-1}; ^1H NMR and ^{13}C-NMR see table 3; HR-ESI-TOF-MS $[M+H]^+$ at m/z 390.1314 (calc.$C_{20}H_{22}O_8$ 390.1315). EIMS m/z (rel. int.): 390 (100) $[M]^+$.

Identification of the sugar moiety: the sample (18.7 mg) was hydrolysed as described above and the aglycone was identified as 4-methoxy-6-(11-hydroxystyryl)-α-pyrone by comparison with its physical and spectral data (Keith *et al.*, 1967). The sugar was identified as L-rhamnose by comparison of its trimethylsilylated derivative to that of standard sugars using GLC.

Antibacterial activity

An aliquot of the crude extract of *S. mannii* was serially diluted to stand a range of 1.0 - 0.01 mg/mL in 2% acetone final concentrations. Compounds **3** and **4** were diluted to final concentrations of 100, 10.0, 5.0 and 0.5 µg/mL in 2% acetone. The plant extract and isolated pure compounds (sterilised by filtering through a 0.22 µm filter) were added to 5 mL of sterilised nutrient agar in Petri dishes and swirled carefully before solidifying. The organisms were streaked in radial patterns on the agar plates (Mitsher *et al.*, 1976). Plates were hatched at 37°C in the dark and examined after 24h and 48h. Complete inhibition of growth of bioactive compounds was required to be considered active.

The controls consisted of Petri dishes containing only nutrient agar and others containing nutrient agar in 2% acetone. Each treatment was analysed in triplicate. The extract and purified active principles from *S. mannii* were tested against five randomly - selected bacteria by agar dilution method (Turnbull and Kramer., 1991). A gram-positive bacteria tested showed significant activity in Compounds **3** and **4** (Table 1). But, the minimum inhibitory concentration (MIC) was very significant with compound **3** than **4**. Compounds **3** and **4** were not active on gram-negative bacteria except for *Pseudomonas aeruginosa* which was significantly inhibited at an MIC of 0.1µg/mL. These results are agreeing with previously reported of similar antimicrobial activity to *Senecio* genus (Louzzo *et al.*, 2004).

Antifungal activity.

The plant extract as well as compounds **3** and **4** were subjected to the same treatment as noted above except that instead of streaking bacteria into the agar, cultured fungal inocula discs were carefully deposited at the centre of each Petri dish. Plates were incubated at 25 °C in the dark and examined after 24 and 48 hours. Complete inhibition of growth was similarly required for compounds to be declared active. Controls were also prepared containing only nutrient agar on nutrient agar in 2% acetone. The growth of three fungal species *Fusarium solani*, *Aspergillus flavus* and *Candida glutamate* were significantly inhibited at a low MIC's by compound **4** than compound **3**.

In this study, we observed that compounds **3** and **4** have the ability to inhibit the growth of all fungal species tested. These results confirm the use of these compounds as broad spectrum antimicrobial agent. This probably explains the use of extracts from this plant by traditional healers against a certain number of infections because antibacterial activity seems to be related to the presence of phenolic compounds.

RESULTS AND DISCUSSION

The perennial plant of *S. manii* was extracted with methanol. The extract was subjected to silica gel column chromatography, eluting with increased parts of ethylacetate in hexane to give six compounds numbered **1-6**.

Compound **3-6** were identical with 4-methoxy-6-(11-hydroxystyryl)-α-pyrone (Keith *et al.*, 1967), 4-methoxy-6-(12-hydroxystyryl)-α-pyrone (Dharmaratne *et al.*, 2002; Whitton, *et al.*, 2003), α-amyrin (Loucam *et al.*, 1973) and 4-methoxy-6-(12-O-ß-D-glucopyranosylstyryl)-α-pyrone (Wang *et al.*, 2004).

Compound **1** obtained as white crystals from a solution in CH_2Cl_2, was found to be a glucoside by the Molish test. The positive high resolution electrospray-TOF mass spectrum running on an API QSTAR pulsar mass spectrometer (HR-ESI-MS) showed a

Figure 1. Styrylpirones isolated from *S. manii*

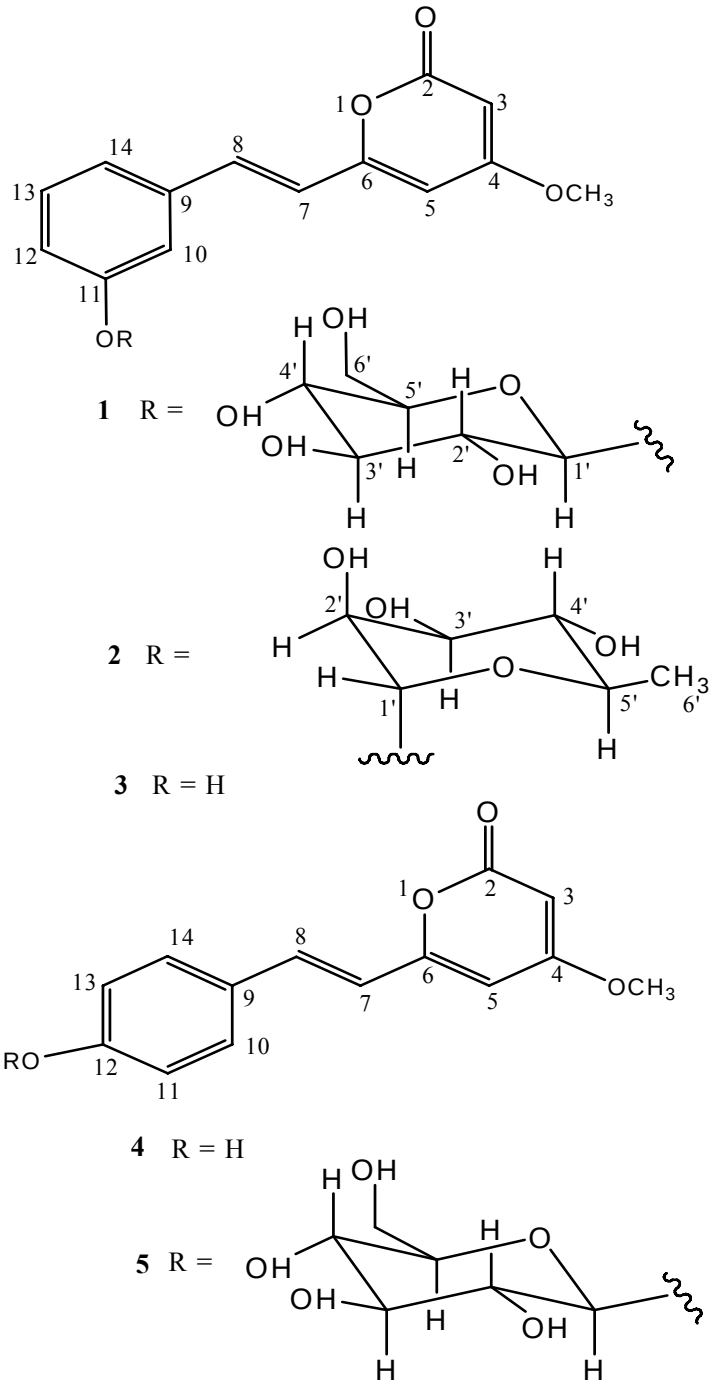


Table 1. Antibacterial activity of the crude acetone extract from the ground aerial plants of S. mannii and isolated compounds 3 and 4.

Bacteria species	**Gram (+/-)**	**Minimum inhibitory concentration**		
		Crude extract (mg/ml)	**3 (µg/mL)**	**4 (µg/mL)**
Straphylococcus aureus ATCC13709	+	1.0	0.1	0.5
Escherichia coli. ATCC 25922	-	-	-	-
Escherichia coli. ATCC 35218	-	1.0	-	-
Klebsiella pneumonia. ATCC 10031	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	1.0	0.1	0.1

(-) Not active

Table 2. Antifungal activity of the crude acetone extract from the ground aerial plants of *S. mannii* and isolated compounds **3** and **4.**

Fungal species	**Minimum inhibitory concentration**		
	Crude extract (mg/ml)	**3** (µg /mL)	**4** (µg /mL)
Fusarium solani	1.0	0.1	0.5
Aspergillus flavus	0.01	1.0	0.5
Candida glutamate	0.05	1.0	1.0

Table 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) assignments for 4-methoxy-6-(11-O-α-L-rhamnopyranosylstyryl) -α-pyrone **(1)** and 4-methoxy-6-(11-O-α-L-rhamnopyranosylstyryl)-α-pyrone **(2)** in DMSO-d_6. Assignments were based on HMQC, HMBC and NOESY experiments.

Attribution	**(1)**			**(2)**		
	^{1}H	**^{13}C**	**HMBC**	**^{1}H**	**^{13}C**	**HMBC**
2		162.6	H-3		162.7	H-3
3	5.60 (d, J = 2.2)	88.6	H-5	5.61(d, J = 2.2)	88.4	H-5
4		170.6	OCH_3-15		170.8	OCH_3-15
5	6.15 (d, J = 2.2)	101.8	H-3, H-7	6.25(d, J = 2.2)	100.7	H-3, H-7
6		157.9	H-8		158.6	H-8
7	6.75 (d, J = 13.0)	117.4	H-5	6.83 (d, J = 16.0)	117.7	H-5
8	6.50 (d, 13.0)	131.1	H-10, H-14	6.76 (d, J = 16.0)	133.8	H-10, H-14
9		134.3	H-13		127.7	H-13
10	6.85 (dd, 2.0, 1.9)	114.9	H-12, H-14	6.83 (dd, 8.7, 1.8)	128.7	H-12, H-14
11		158.8	H-13		116.5	H-13
12	6.66 (ddd, 8.7, 1.9, 1.8)	115.6	H-10, H-14	6.70(ddd, 8.5, 1.9, 1.7)	158.3	H-10, H-14
13	7.30 (dd, 8.7, 8.6))	131.1		7.25 (dd, J = 8.4, 8.5)	117.7	
14	6.80 (ddd, J = 8.6, 2.0, 1.9)	116.7	H-10, H-12	7.20 (ddd, J = 8.4, 1.9, 1.7)	128.9	H-10, H-12
15-OCH_3	3.5 (s)	56.3		3.5 (s)	56.1	
1’	4.9 (d, 7.4)	100.2		4.25 (d, 1.4)	102.1	
2’	3.27(m)	73.1		3.85 (t, J = 3.7)	71.9	
3’	3.30 (m)	76.5		3.65 (t, J = 10)	69.8	
4’	3.10 (m)	69.6		3.40 (t, 9.7)	66.6	
5’	3.37 (m)	77.0		3.3 (t, 9.7)	75.1	
6’	3.45 (dd, J = 11.7. 1.8)	60.6		3.5 (m)	66.6	
	3.60 (dd, J = 11.7, 1.8)			0.95 (d, J = 6)	16.1	

pseudomolecular ion peak $(M+H)^+$ at *m/z.* 407.1263 (calc.$C_{20}H_{22}O_9$ 407.1264). In the EIMS, the mass fragment arise at *m/z.* 244 (M^+- glucosyl) indicated the presence of glucosyl moiety.

The UV spectrum exhibited absorption maxima of a typical styrylpyrone at λ_{max} (MeOH) 224, 264, 309, 328, 360 nm (Dharmaratne *et al.*, 2002) suggesting the presence of a conjugated chromophore.

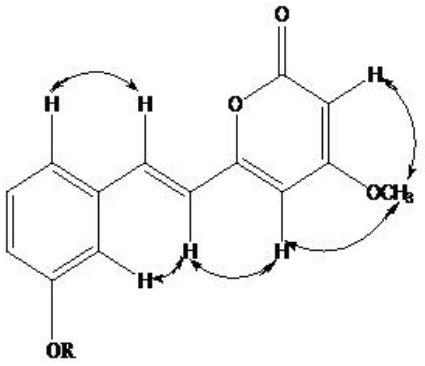
The IR spectrum of **1** showed characteristic bands for olefin groups (1631, 1517, 820 cm^{-1}) and carbonyl functionalities (1709, 1630 cm^{-1}).

The ^1H NMR spectra revealed characteristic signals at δ5.60 (d, J = 2.2 Hz, H-3) and at δ6.15 (d, J = 2.2 Hz, H-5) fixed in meta position and one methoxy group at δ3.5 (s) (Table 3). The ^{13}C NMR spectrum (Table 3) presented signals with a carbonyl group at δ162.6 (C-2), a quaternary oxygenated aromatic carbon at δ157.9 (C-6), a methoxy group at δ56.3 (C-15), a quaternary oxygenated aromatic carbon bearing an methoxy group at δ170.6 (C-4) and two aromatic methine at δ101.8 (C-5) and δ88.6 (C-3). All these data confirmed the presence of a α- pyrone ring.

On the other hand the ^1H NMR spectra of **1** revealed a typical AB system of two protons, one at δ6.75 (d, J= 13.0 Hz, H-7) and another at δ6.50 (d, J = 13.0 Hz, H-8). The value of the coupling constant indicated the trans configuration; four aromatic protons in which the ^1H NMR signals for H-10 and H-14 appeared close together (δ6.85 and 6.80 respectively) and a part of H-14 (ddd, J= 8.6, 2.0, 1.9 Hz) overlapped with the H-10 (dd, J = 2.0, 1.9 Hz) signal to give a broad band and two others at δ6.66 (ddd, J = 8.7, 1.8, 1.9 Hz, H-12) and δ7.30 (dd, J = 8.7, 8.6 Hz, H-13). This hypothesis was confirmed by the ^{13}C NMR Jmod spectrum of **1** which showed two olefin carbons at δ117.4 (C-7) and δ131.1 (C-8) four aromatic methyne carbons at δ114.9 (C-10), δ115.6 (C-12), δ131.1 (C-13), δ116.7 (C-14) and two quaternary aromatic carbons one bearing an glucosyl group at δ158.8 (C-11) and another at δ134.3 (C-9) (Table 3). All these data showed that compound **1** possess a styryl moiety. From this evidence, the structure of **1** resembled that of styrylpyrone derivatives (Rezende *et al.*, 1971; Dutta *et al.*, 1972; Veit *et al.*, 1993, 1995; Dharmaratne *et al.*, 2002).

HMBC correlations between C-2 (δ162.6) / H-3, C-4 (δ170.6) / OCH_3, C-3 (δ88.6) / H-5, C-5 (δ 101.8) / H-3, C-5 (δ101.8) / H-7, C-6 (δ157.9) / H-8, C-7 (δ117.4) / H-5 allow us to fixe the methoxy group at position 4. On the other hand, HMBC correlations between, C-8 (δ131.1) / H-14, C-8 (δ131.1) / H-10, C-9 (δ134.3) / H-13, C-9 (δ134.3) / H-7, C-10 (δ114.9) / H-14, C-11 (δ158.8) / H-13 aid to locate the glucosyl group in position 11.The coupling (COSY) relationship established completely different patterns in compound **1.** NOESY correlations between H-3 (δ 5.60) / OCH_3-4), H-5 (δ6.15) / OCH_3-4, H-5 (δ6.15) / H-7(δ6.75), H-8 (δ6.50) / H-14 (δ6.80), H-7(δ6.75) / H-10 (δ6.85) (Fig 1) and the various observed coupling constants (Table 3) indicated the close spatial proximity of particular protons and the site of O-methylation in position 4. The presence of a *E*-styrylpyrone moiety was evident by the characteristic shifts and couplings in these spectra (Table 3) and by comparison with data on compound **3** (Franca *et al.*, 1973) and styrylpyrones published earlier (Benerji *et al.*, 1980; Ganzeran and Khan., 1999).

Fig.2: Selected NOESY Correlations



The compound was subjected to acid hydrolysis with 7% H_2SO_4 to yield aglycone that was identified as 4-methoxy-6-(11-hydroxystyryl)-α-pyrone **3** from its physical and spectral data ^1H, ^{13}C NMR (Keith *et al.*, 1967)

The sugar moiety was identified by TLC and GLC of its TMSi derivative as glucose. This was confirmed ^{13}C NMR signals at δ100.2, 77.0, 76.5, 73.1, 69.6, and 60.6 agreeing with published data for D-glucose (Veit *et al.*, 1995). A β-D-glucopyranosyl configuration was deduced from the coupling constant (J = 7.9 Hz) of the anomeric proton signal at δ4.9 (H-1') in the ^1H NMR (Veit *et al.*, 1995).

The site of attachment of the glucose moiety was confirmed by correlation between H-1' and C-11, the absence of NOESY correlations between pyrone protons and glucosyl protons in the NOESY spectra and 2D HMBC, ^1H-^1H COSY, ^{13}C-^1H, optimized for long range couplings. Thus, the structure of **1** was clearly defined as 4-methoxy-6-(11-O-ß-D-glucopyranosylstyryl)-α-pyrone.

Compound **2** obtained as white crystals from a solution in CH_2Cl_2 was found to be a glucoside similar to compound **1** from its positive response to the Molish test and its ^1H NMR and ^{13}C NMR data. The positive high resolution electrospray-TOF mass spectrum running on an API QSTAR pulsar mass spectrometer (HR-ESI-MS) showed a pseudomolecular ion peak $(M+H)^+$ at (*m/z*. 390.1314 calc.$C_{20}H_{22}O_8$ 390.1315).

Acid hydrolysis under the same conditions as mentioned above for compound **1** gave for compound **2** a glycone identified as L-rhamnose by TLC and GLC of its TMSi derivative, confirmed by ^{13}C NMR signals at δ102.1, 75.1, 71.9, 69.8, 66.6 and 16.7 matching with those published for L-rhamnopyranose (Muzitano *et al.* 2006)

Mass fragment peak at (*m/z.* 244, M^+- rhamnosyl) indicated the presence of rhamnose moiety. In the ^1H NMR spectrum, a doublet at δ4.25 (J = 1.4 Hz) was assigned to the anomeric proton and confirmed the α-rhamnose configuration (Mamdouh *et al.*, 1999). An additional secondary methyl group at δ16.1 replaced the signal for the carbon of the hydroxymethylene group of the glucose in compound **1** (Table 3) further suggesting that the glycone in compound **2** is α-L-rhamnose. The rhamnose moiety was placed at C-11 from close similarity observed between the carbon signals in compounds **1** and **2** and the 2D COSY experiment. The structure of compound **2** was therefore elucidated as 4-methoxy-6-(11-O-α-L-rhamnopyranosylstyryl)-α-pyrone.

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