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Microbial Transformation of Cycloastragenol and Astragenol by Endophytic Fungi Isolated from *Astragalus* Species

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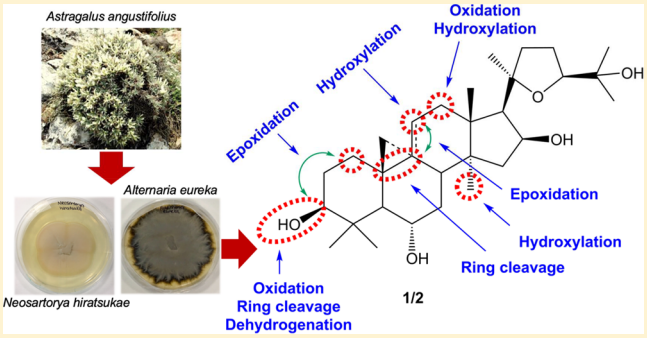
Supporting Information

ABSTRACT: Biotransformation of *Astragalus* sapogenins (cycloastragenol (**1**) and astragenol (**2**)) by *Astragalus* species originated endophytic fungi resulted in the production of five new metabolites (**3**, **7**, **10**, **12**, **14**) together with 10 known compounds. The structures of the new compounds were established by NMR spectroscopic and HRMS analysis. Oxygenation, oxidation, epoxidation, dehydrogenation, and ring cleavage reactions were observed on the cycloartane (9,19-cyclolanostane) nucleus. The ability of the compounds to increase telomerase activity in neonatal cells was also evaluated. After prescreening studies to define potent telomerase activators, four compounds were selected for subsequent bioassays. These were performed using very low doses ranging from 0.1 to 30 nM compared to the control cells treated with DMSO. The positive control cycloastragenol and **8** were found to be the most active compounds, with 5.2- (2 nM) and 5.1- (0.5 nM) fold activations versus DMSO, respectively. At the lowest dose of 0.1 nM, compounds **4** and **13** provided 3.5- and 3.8-fold activations, respectively, while cycloastragenol showed a limited activation (1.5-fold).



Biotransformation reactions offer several advantages over chemical synthesis due to their highly selective catalytic abilities, efficiency under mild conditions, and environmentally friendly nature.[1−3] These reactions can be performed using either isolated enzymes or whole-cell catalysts. Whole-cell systems are highly effective in multistep reactions with cofactor regeneration, provide a protective environment to enzymes, and are the cheapest form of biocatalyst formulation in comparison with isolated enzymes.[4−7] Microbial transformation is an effective tool to generate structural diversity in a chemical library due to its significant enantio-, regio-, and chemoselectivity. Fungal whole-cell systems have been studied extensively for the modification of natural and semisynthetic triterpenoids.[8−10] In particular, endophytic fungi deserve attention with their ability to modify complex natural products with a high degree of stereospecificity, making them promising sources of biocatalysts for biotransformation studies.[11−14]

Cycloartane-type triterpenoids isolated from *Astragalus* species have shown interesting biological properties (e.g., antiprotozoal activities, hepatoprotective, wound healing, antineoplastic, immunomodulatory, and antiviral).[15−17] Cycloastragenol (**1**), 20(*R*),24(*S*)-epoxy-3*β*,6*α*,16*β*,25-tetrahydroxycycloartane, the principal sapogenol of many cycloartane-type glycosides found in the genus *Astragalus*, was discovered as a small-molecule telomerase activator in 2000.[18] Telomerase is a ribonucleoprotein reverse transcriptase that catalyzes the addition of TTAGGG telomeric repeats to the ends of telomeres. Telomeres are dynamic nucleoprotein−DNA structures that cap and protect linear chromosome ends. In the absence of telomerase, telomeres progressively shorten with each cell division, and such telomere loss leads to cellular senescence. Telomere shortening has been proposed as a major cause of aging since short telomeres limit the proliferative capacity of stem cells, which creates conditions for the development of age-related diseases.[19−22] Also, one of the subunits of the telomerase enzyme, viz., hTERT, plays a key role in protection against Alzhemier's disease. Hippocampal neurons that expressed hTERT did not contain hyperphosphorylated tau protein, which occurs in the pathology of Alzhemier's disease. hTERT colocalizes with mitochondria both in the hippocampus of Alzheimer's patients and in

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cultured neurons under oxidative stress conditions. This colocalization and the presence of hTERT prevented ROS generation and oxidative damage in neurons induced by pathological tau, implying the significance of hTERT in Alzheimer's disease.[23] Thus, telomerase activators (TA) have been suggested as promising agents to enhance healthy aging, and they are projected to generate a large market in the future.[24−28]

Due to their activity on telomerase activation, the preparation of cycloastragenol derivatives with such activity could be of significance in the future. In a continuation of ongoing studies on cycloartanes from *Astragalus* species, the present work reports the microbial transformation of cycloastragenol (**1**) and its side product astragenol (**2**) using endophytic fungi isolated from species of *Astragalus*. Preparative-scale biotransformation reactions of the starting compounds with the fungal endophytes, namely, *Alternaria eureka*, *Neosartorya hiratsukae*, and *Camarosporium laburnicola*, resulted in the production of five new derivatives (**3**, **7**, **10**, **12**, and **14**) together with 10 known compounds. The structures of the new metabolites were elucidated by 1D and 2D NMR and HRMS analyses. Furthermore, the ability of the metabolites to increase telomerase activation in Hekn cells (neonatal human primary epidermal keratinocytes) was determined using the Telo*TAGGG* telomerase PCR ELISA kit.

RESULTS AND DISCUSSION

Altogether 15 endophytic fungi, isolated from the roots, stems, and leaves of two *Astragalus* species (*A. angustifolius* Lam. and *A. condensatus* Ledeb.) (Fabaceae), were screened for their ability to transform **1** and **2** into new derivatives. The endophytic isolates identified as *Alternaria eureka*, *Neosartorya hiratsukae*, and *Camarosporium laburnicola* were selected for further whole cell catalysis studies. Analytical-scale studies were performed to determine the ideal harvesting time of the fungi for transformation of the substrates. Subsequently, preparative-scale biotransformation of **1** and **2** with the endophytic fungi yielded five new metabolites (**3**, **7**, **10**, **12**, and **14**), together with 10 known compounds.

The biotransformation of **1** with the endophytic fungus *N. hiratsukae* for 10 days afforded three metabolites (**3**−**5**). The metabolite **3** gave a molecular formula of $C_{30}H_{48}O_6$ based on the HRESIMS data (*m*/*z* 527.3376 $[M + Na]^+$, calcd for $C_{30}H_{48}O_6Na$, 527.3348). The characteristic hydroxymethine proton at C-3 was not present in the ^1H NMR spectrum. Thus, in the ^{13}C NMR spectrum, the keto carbonyl signal observed at δ_C 220.8 was readily assigned to C-3, which was substantiated by the long-range correlations from C-3 to H_3-28 and H_3-29 in the HMBC spectrum. Apart from the characteristic low-field signals observed (H-6, H-16, and H-24), an additional oxymethine signal at δ_H 4.02 (dd, *J* = 9.2, 3.0 Hz) was apparent, corresponding to a carbon signal at δ_C 65.0 in the HSQC spectrum. Moreover, the ^1H NMR spectrum of **3** showed that one of the 9,19-cyclopropane ring signals (δ_H 1.23, H-19a) had undergone a significant downfield shift (ca. 0.6−0.7 ppm) when compared to that of **1**, a distinctive feature observed for C-11-hydroxylated cycloartanes.[29,30] The downfield shifts of C-9 and C-12 signals (ca. 6 and 12 ppm, respectively), together with the HMBC cross-peak between H-12b (δ_H 1.59) and the δ_C 65.0 signal, verified the hydroxy group as occurring at C-11. The β-orientation of OH-11 was established via the ROESY cross-peak between H_3-30 (δ_H 0.95) and H-11. Thus, the structure of **3** was elucidated as 20(*R*),24(*S*)-epoxy-6α,11β,16β,25-tetrahydroxycycloartan-3-one.

Metabolites **4** and **5** were identified as 20(*R*),24(*S*)-epoxy-6α,16β,25,30-tetrahydroxycycloartan-3-one and 20(*R*),24(*S*)-epoxy-6α,16β,25-trihydroxycycloartan-3-one, respectively, by comparison of their spectroscopic data with those previously reported.[30,31]

The preparative-scale incubation of **1** with *A. eureka* for 10 days yielded four derivatives (**6**−**9**). Compounds **6** and **8** were found to be monooxygenated derivatives of cycloastragenol. The ^1H and ^{13}C NMR spectra of **6** were in agreement with those reported for 20(*R*),24(*S*)-epoxy-3β,6α,12β,16β,25-pentahydroxycycloartane, previously reported from the biotransformation study of CA with *Cunninghamella blakesleeana*.[30] The spectroscopic data of **8** were identical to those described for 20(*R*),24(*S*)-epoxy-3β,6α,12α,16β,25-pentahydroxycycloartane, isolated from a *Cunninghamella elegans* biotransformation extract of cycloastragenol.[31]

The HRESIMS of **7** exhibited a sodium adduct ion at *m*/*z* 525.3168 $[M + Na]^+$ (calcd for $C_{30}H_{46}O_6Na$, 525.3192), supporting a molecular formula of $C_{30}H_{46}O_6$ with eight indices of hydrogen deficiency. The absence of low-field oxymethine signals due to H-3 and H-16 in the ^1H NMR spectrum, together with the observation of two keto carbonyl carbons (δ_C 217.7 and 219.4) in the ^{13}C NMR spectrum, suggested the C-3 and C-16 secondary alcohols had been oxidized. The carbon signal at δ_C 217.7 showed long-range correlations with H_2-15 and H-17, while the δ_C 219.4 signal displayed cross-peaks with H_3-28, H_3-29, and H_2-2, confirming the presence of the carbonyl groups at C-16 and C-3, respectively. On the other hand, a new proton signal observed at δ_H 4.15 indicated an additional oxymethine group in the structure. Examination of DQF-COSY and HSQC spectra suggested oxygenation at C-12, as in **6** and **8**. This assumption was confirmed by the HMBC experiment, which showed a cross-peak between the 73.1 ppm signal (C-12) and H_3-18. The relative configuration was established via the 2D-ROESY data, in which correlations of H-12 with the α-oriented H-17 and H_3-30 revealed that the hydroxy group at C-12 is β-oriented. Consequently, the structure of **7** was determined as 20(*R*),24(*S*)-epoxy-6α,12β,25-trihydroxycycloartan-3,16-dione.

Compound **9** was identified as 20(*R*),24(*S*)-6α,16β,25-trihdroxy-3β,10β;20,24-diepoxy-9,10-*seco*-cycloartan-9(11)-ene by comparison with literature data.[32]

The incubation of **2** with *A. eureka* for 10 days yielded five metabolites (**10**−**14**). The molecular formula of **10** was established as $C_{30}H_{48}O_6$ by HRESIMS analysis (*m*/*z* 527.3335 $[M + Na]^+$, calcd for $C_{30}H_{48}O_6Na$, 527.3348). The low-field characteristic signal of H-3 was absent, whereas eight tertiary methyl groups in the upfield region were readily apparent in the ^1H NMR spectrum of **10**. A broad singlet signal observed at δ_H 4.14, corresponding to a carbon at δ_C 74.0 in the HSQC spectrum, indicated an additional oxymethine group in the structure. A keto carbonyl signal at δ_C 220.0 was present in the ^{13}C NMR spectrum, implying the oxidation of C-3. The long-range correlations of H_3-28 (δ_H 1.32) and H_3-29 (δ_H 1.33) with the carbonyl carbon at δ_C 220.0 confirmed the oxidation of OH-3. A new hydroxy group was located at C-12 on the basis of the COSY correlation of H-12 (δ_H 4.14) with H-11 (δ_H 5.31) and the key long-range correlation between the 74.0 ppm signal and H_3-18 (δ_H 1.00). The relative configuration at C-12 was deduced based on the 2D-NOESY data. The H-12 (δ_H 4.14 bs) signal showed NOE correlations with the α-

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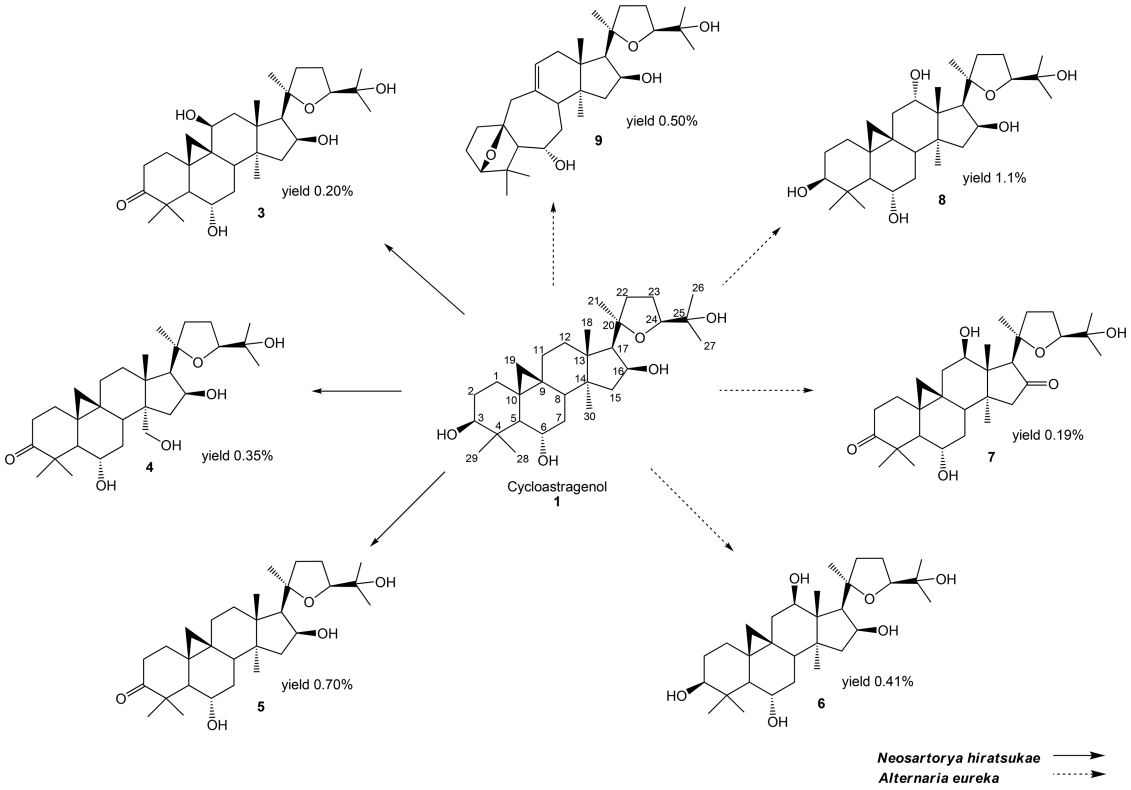

Figure 1. Transformation products of **1**.

oriented H-17 and H_3-30 and provided evidence for the β-orientation of the hydroxy group at C-12. Thus, the new metabolite was characterized as 20(*R*),24(*S*)-epoxy-6α,12β,16β,25-tetrahydroxylanost-9(11)-en-3-one.

The HRESIMS of **12** showed a major ion peak at *m*/*z* 545.3449 $[M + Na]^+$ ($C_{30}H_{50}O_7Na$). When compared to the starting compound, the absence of a tertiary methyl group was readily evident from the ^1H NMR spectrum. An AB system was observed at δ_H 3.88 and 4.01, corresponding to a methylene carbon at δ_C 61.1 in the HSQC spectrum and implying the presence of an oxygenated methylene group in the structure. Moreover, the characteristic olefinic signal (ca. 5.5 ppm) of H-11 in astragenol was lost in the ^1H NMR spectrum, whereas an additional doublet signal at δ_H 3.22 was evident. In the ^{13}C NMR spectrum, the olefinic carbon signals of C-11 and C-9 were replaced with two resonances at δ_C 57.9 and 67.5. A proton resonating at δ_H 3.22, which correlated with a resonance at δ_C 57.9 in the HSQC spectrum, showed a cross-peak with H-12a (δ_H 1.81) in the COSY spectrum, suggesting a modification on the ring C. In addition, the carbon and proton chemical shifts of H-11 were significant for the oxidation of the C-9(11) double bond, affording a C-9(11) epoxide.[33] The HMBC correlations of H-11 with C-12 and C-13, of H_3-18 with C-12, and of H-8 with C-30 were supportive of the proposed structure. In addition, the observation of an HMBC cross-peak from C-9 (δ_C 67.5) to a signal at δ_H 3.88 revealed mono-oxygenation of the methyl group at C-19. Thus, the structure of **12** was deduced as 9β,11;20(*R*),24(*S*)-diepoxy-3β,6α,16β,19β,25-pentahydroxycycloartane.

The HRESIMS of **14** (*m*/*z* 527.3350 $[M + Na]^+$, calcd for $C_{30}H_{48}O_6Na$, 527.3348) was consistent with a molecular formula of $C_{30}H_{48}O_6$. When the ^1H NMR spectrum of **14** was inspected, eight tertiary methyl groups in the upfield region and the characteristic oxymethine signals were observed unchanged when compared to the starting compound. In the ^{13}C NMR spectrum, the signal at δ_C 207.0 suggested a keto carbonyl group in the structure. The long-range correlations from the carbon at δ_C 207.0 to H-17/H_3-18 confirmed the location of the carbonyl group to be at C-12. On the basis of these results, the structure of **14** was elucidated as 20(*R*),24-(*S*)-epoxy-3β,6α,16β,25-tetrahydroxylanost-9(11)-en-12-one.

The 1D NMR spectra of **11** and **13** were identical to those reported for 20(*R*),24(*S*)-epoxy-3β,6α,12β,16β,25-pentahydroxylanost-9(11)-ene[33] and 20(*R*),24(*S*)-epoxy-3β,6α,12α,16β,25-pentahydroxylanost-9(11)-ene,[32] respectively.

Compounds **15** and **16**, the 3,4-*seco* derivatives of astragenol, were isolated previously from the biotransformation of this compound with *Glomerella fusarioides*.[33] Metabolite **17** was also identified as 20(*R*),24(*S*)-epoxy-6α,16β,25-trihydroxylanost-9(11)-en-3-one by comparing its NMR data with those previously reported in the literature.[18]

Cycloastragenol is currently the only natural telomerase activator in the antiaging market.[28] Thus, the purified metabolites were screened for their effects on telomerase activation by PCR-based ELISA testing. Prior to screening studies, cytotoxicity tests were performed (WST-1 assay as an end point test together and X-Celligence real-time cell analysis) to determine the dose range nontoxic to the used cell line (neonatal human primary epidermal keratinocytes; data not shown). Based on the preliminary telomerase activation tests in the dose range of 30−1000 nM, four compounds were selected for further screening (**3**, **4**, **8**, and **13**). The subsequent tests compared to the control cells treated with DMSO were performed by using lower doses (0.1−30 nM), which revealed telomerase activation ranging

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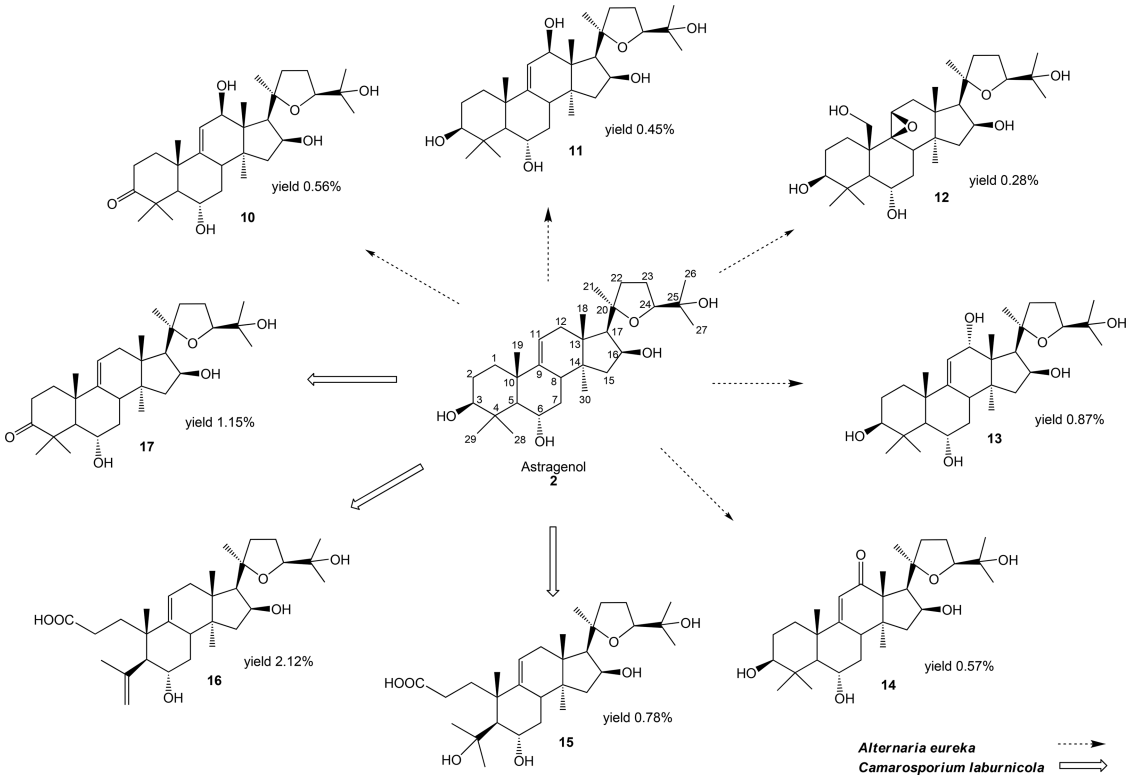

Figure 2. Transformation products of **2**.

from 1.5- to 5.2-fold (Figure S1, Supporting Information). Among the compounds, the positive control **1** and **8** were the most potent compounds, with 5.2- and 5-fold activations, respectively (at 2 and 0.5 nM doses, respectively). At the lowest dose of 0.1 nM, **4** and **13** were more active, with 3.5- and 3.8-fold activations, respectively, whereas **1** exhibited a moderate activation (1.5-fold).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a Rudolph Research Analytical-Autopol I polarimeter. The general experimental procedures were described previously for NMR, HRTOFMS, and chromatographic studies.[34]

Plant Material and Compounds. *Astragalus angustifolius* and *Astragalus condensatus* were collected from Spil Mountain (38°33.965′ N, 27°24.400′ E), Manisa, Turkey, in June 2013, and identified by Dr. Serdar Şenol (Department of Botany, Faculty of Science, Ege University, Turkey). Voucher specimens (IZEF 6620 for *A. angustifolius* and IZEF 6621 for *A. condensatus*) have been deposited at the herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy, Ege University, İzmir, Turkey. The plant samples were placed in plastic bags and transported to the laboratory within 24 h. The substrates (purity >98%), cycloastragenol (**1**) and astragenol (**2**), were donated by Bionorm Natural Products, Ltd. (İzmir, Turkey).

Fungal Strains and Culture Media. The fungal endophytes used in this study were isolated from fresh and healthy tissues of *Astragalus* species, as described previously.[34]

The endophytic fungi *Alternaria eureka* 1E1BL1 and *Camarosporium laburnicola* 1E4BL1 were identified by the identification service of the Leibniz-Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) using molecular data of the ITS, large subunit of rDNA (LSU), and TEF1 genes.[35,36] The endophytic isolate *Neosartorya hiratsukae* 1E2AR1-1 was also authenticated by molecular identification based on rDNA ITS sequence analysis. The ITS region was sequenced using primers ITS4[37] and ITS1F.[38] The assembled DNA sequences were compared with those in GenBank and MycoID databases. The original strains were deposited at the Bedir Laboratory with the deposit numbers 20131E1BL1, 20131E4BL1, and 20131E2AR1-1.[39]

Microbial Transformation Procedures. The microbial transformation processes of analytical and preparative scales were carried out as described previously.[34] Preparative-scale biotransformation studies were performed employing 1750 mg of **1** with *N. hiratsukae*, 1200 mg of **1** with *A. eureka*, 1140 mg of **2** with *A. eureka*, and 800 mg of **2** with *C. laburnicola* for 10 days (25 °C and 180 rpm).

Extraction and Isolation. After an incubation period, the fungal mycelia were filtered on a Buchner funnel, and the combined filtrate was extracted with EtOAc (×2), and the organic phase was dried over anhydrous Na_2SO_4, followed by filtration and concentration in vacuo. Compounds **3**–**5** were isolated from the EtOAc extract (2.1 g) of *N. hiratsukae* with **1**. This extract was chromatographed initially on a silica gel column (180 g) eluted with hexane/EtOAc (50:50) and a hexane/EtOAc/MeOH gradient (10:10:0.5, 10:10:1, 10:10:1.5, 10:10:2, 10:10:2.5, 10:10:3, 10:10:4, 10:10:5, 10:10:6, 10:10:8), to give three main fractions (A–C). Fraction A (20 mg) was purified by silica gel open-column chromatography (15 g) and eluted with hexane/EtOAc (50:50) and hexane/EtOAc/MeOH mixtures (10:10:2, 10:10:3, 10:10:4), to afford 12.3 mg of **5**. Fraction B (94 mg) was subjected to Sephadex LH-20 column chromatography (30 g) and eluted with MeOH, which provided 64 fractions. Fractions 37 to 55 were pooled for further purification. This fraction (40 mg) was applied to VLC (vacuum-liquid chromatography) loaded with reversed-phase silica gel (RP-18, 20 g), using a MeOH/H_2O gradient (50:50, 60:40, 70:30, 100:0), yielding 60 fractions. Fraction 40–42 (8.9 mg) was chromatographed over a silica gel column (10 g), with the solvent system cyclohexane/EtOAc/MeOH (10:10:2.5), to provide **3** (3.5 mg). Fraction C (153.8 mg) was submitted to silica gel column chromatography (35 g) to yield 95 fractions after elution with a $CHCl_3$/MeOH gradient (95:5, 93:7, 98:2, 91.5:8.5, 90:10, 88:12, 85:15). Fraction 68–90 (40 mg) was applied to VLC using reversed-phase silica gel (RP-18, 20 g) and eluted with a MeOH/H_2O gradient (50:50, 60:40, 70:30, 80:20, 90:10, 100:0), which provided 80 fractions. To purify metabolite **4** (6.2 mg), fraction 69–77 (12.3

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds 3, 7, 10, 12, and 14

	3[a]		7[b]		10[b]		12[b]		14[b]	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	30.2	1.91 m, 2.01 m	32.6	1.46 m, 2.15 m	36.4	2.10 m (2H)	23.9	1.04 m, 1.63 m	37.1	1.50 m, 1.98 m
2	36.7	2.66 m, 2.39 m	36.6	2.49 m, 2.65 m	34.2	2.39 m, 2.80 ddd (16.1, 10.9, 5.2)	27.2	1.54 m, 1.60 m	27.8	1.70 m
3	220.8		219.4		222.0		79.3	3.08 dd (11.3, 5.1)	79.1	3.12 dd (9.5, 6.1)
4	51.5		51.4		48.8		40.4		40.8	
5	55.0	2.01 m	54.0	1.98 d (9.9)	58.0	1.76 d (10.6)	56.8	1.42 m	57.4	1.08 m
6	69.7	3.55 m	69.8	3.50 td (10.0, 3.5)	70.0	3.93 td (10.8, 3.8)	69.8	4.10 td (4.1, 11.0)	69.4	4.20 td (10.7, 4.1)
7	38.6	1.43 m, 1.47 m	38.1	1.43 m (2H)	38.9	1.81 dt (4.0, 12.0)	38.9	1.69 m, 1.84 m	39.0	1.54 m, 1.99 m
8	49.0	1.97 m	46.9	1.92 dd (11.2, 5.0)	41.7	2.42 m	37.9	2.51 dd (13.5, 4.1)	43.5	2.99 m
9	28.7		22.2		148.6		67.5		168.2	
10	29.6		30.0		41.2		46.8		42.8	
11	65.0	4.02 dd (9.2, 3.0)	36.6	1.10 dd (11.2, 2.7), 2.63 m	122.6	5.31 s	57.9	3.22 d (6.0)	119.8	5.55 d (2.3)
12	48.2	1.59 dd (14.4, 3.2), 2.42 (13.6, 10.2)	73.1	4.15 dd (9.3, 2.6)	74.0	4.14 bs	36.5	1.81 m, 1.98 m	207.0	
13	47.0		44.4		46.7		45.5		59.2	
14	46.3		52.7		51.4		45.2		43.5	
15	46.7	1.98 m, 1.44 m	51.4	2.08 d (18.0), 2.35 d (18.0)	46.9	1.64 dd (12.9, 5.7), 2.05 dd (12.9, 8.0)	46.3	1.42 m, 1.88 m	44.3	1.80 m, 2.08 m
16	74.5	4.67 ddd (7.2, 7.9, 7.9)	217.7		73.2	4.52 ddd (7.7, 6.9, 7.7)	74.6	4.66 ddd (6.8, 7.6, 7.6)	74.0	4.66 ddd (8.0, 8.0, 7.5)
17	59.1	2.36 d (8.0)	69.4	2.97 s	59.6	2.31 d (7.9)	57.6	2.30 d (7.6)	51.0	2.79 d (8.4)
18	20.5	1.26 s	14.6	1.34 s	12.3	1.00 s	20.2	1.24 s	17.7	1.28 s
19	22.4	0.41 d (4.4), 1.23 m	32.0	0.58 d (4.5), 0.80 d (4.4)	24.4	1.01 s	61.1	3.88 d (11.0), 4.01 d (11.0)	23.4	1.21 s
20	88.2		85.2		87.8		88.2		88.15	
21	28.5	1.24 s	22.4	1.24 s	25.8	1.51 s	35.6	1.36 s	30.9	1.36 s
22	35.5	1.61 m, 1.65 m	42.8	1.75 m, 2.24 m	39.5	1.88 m, 2.00 m	36.6	1.61 m, 2.59 m	36.6	1.64 m, 2.45 m
23	26.8	2.04 m (2H)	26.4	1.86 ddd (13.0, 6.4, 3.9)	26.7	1.97 m, 2.09 m	26.8	2.02 m (2H)	26.0	1.97 m
24	82.7	3.77 m	85.3	3.87 dd (7.9, 6.4)	85.1	3.90 dd (8.1, 5.3)	82.6	3.76 m	83.3	3.77 t (7.6)
25	72.6		72.2		72.4		72.5		72.0	
26	26.7	1.13 s	25.9	1.15 s	26.7	1.17 s	26.6	1.12 s	26.7	1.12 s
27	27.6	1.28 s	26.2	1.18 s	25.8	1.19 s	27.6	1.25 s	27.4	1.24 s
28	28.5	1.34 s	28.3	1.33 s	31.9	1.32 s	31.7	1.31 s	31.5	1.30 s
29	20.5	1.22 s	20.6	1.21 s	20.7	1.33 s	16.7	0.95 s	16.5	1.05 s
30	21.6	0.95 s	19.8	1.14 s	19.5	0.85 s	20.0	0.99 s	19.7	0.79 s

[a]In $CDCl_3$. [b]In CD_3OD.

mg) was subjected to silica gel column chromatography (10 g) with the solvent system $CHCl_3$/MeOH/H_2O (85:15:05).

Compounds **6**–**9** were isolated from the EtOAc extract (1.79 g) of *A. eureka* and **1**. This crude extract was first chromatographed on a Sephadex-LH 20 column (75 g) and eluted with MeOH, which provided 70 fractions. Fractions 29 to 49 were pooled (1.38 g) and subjected to passage over a reversed-phase column (C_{18}, 100 g), using MeOH/H_2O mixtures (47.5:52.5, 50:50, 52.5:47.5, 55:45, 57.5:42.5, 60:40, 65:35, 67.5:32.5, 70:30, 72.5:27.5, 75:25, 80:20, 82.5:17.5, 85:15, 87.5:12.5, 90:10, 95:5, 100:0) for elution, yielding 17 fractions (A–R). Fraction B (5.3) and fraction C (32 mg) were combined and submitted to silica gel column chromatography (40 g), eluting with $CHCl_3$/MeOH (90:10, 88:12, 86:14), $CHCl_3$/MeOH/H_2O (85:15:0.5, 82.5:17.5:0.5, 80:20:1, 75:25:2.5, 70:30:3, 61:32:7), and MeOH, to give four fractions (BC1–4). Fraction H (32 mg) was applied to a silica gel column (40 g) using $CHCl_3$/MeOH/H_2O mixtures (85:15:0.5, 82.5:17.5:0.5, 80:20:1, 70:30:3) and MeOH for elution, which again provided four fractions (H1–4). Fraction H3 (10 mg) was subjected to passage over a silica gel column (7 g), using for elution cyclohexane/EtOAc/MeOH mixtures (10:10:2.5, 10:10:2.5), to afford 5 mg of **6**. Fraction I (52.4 mg) was further purified on a silica gel column (35 g) using $CHCl_3$/MeOH (95:5, 90:10, 88:12, 86:14) and $CHCl_3$/MeOH/H_2O (85:15:0.5, 82.5:17.5:0.5) for elution, to give metabolites **7** (2.3 mg) and **8** (13.2 mg). Fraction R (25.1 mg) was subjected to separation on a silica gel column (10 g) using $CHCl_3$/MeOH mixtures (98:2, 96:4, 95:5, 92:8, 90:10) to provide two fractions (R1 and R2). Fraction R1 (10.4 mg) was further purified on a silica gel column (7 g) and eluted with cyclohexane/EtOAc/MeOH mixtures (10:10:0.5, 10:10:1) to give fractions R1-1 (2.6 mg) and R1-2 (8.4 mg). Fraction R1-2 was then subjected to VLC using reversed-phase material (RP-18, 15 g), eluting with MeOH/H_2O (90:10, 95:5), to afford two fractions (R1-2a and R1-2b). To isolate metabolite **9** (6 mg), fraction R1-1 (2.6 mg) and fraction R1-2a (2.7 mg) were combined and further purified on a silica gel column (5 g) using cyclohexane/EtOAc/MeOH (10:10:0.75, 10:10:1). Fraction R2 (6.4 mg) was subjected to a silica gel column (5 g) using cyclohexane/EtOAc/MeOH mixtures (10:10:0.5, 10:10:1) to afford 4.5 mg of **8**.

Compounds **10**–**14** were isolated from the EtOAc extract (2.3 g) of *A. eureka* and **2**. This crude extract was chromatographed on a Sephadex-LH 20 column (75 g) and eluted with MeOH, which provided 65 fractions. Fractions 17 to 37 were pooled (1.5 g) and

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J. Nat. Prod. 2019, 82, 2979–2985

applied to a reversed-phase column (C_{18}, 100 g), using for elution mixtures of MeOH/H_2O (45:55, 50:50, 57.5:42.5, 60:40, 65:35, 70:30, 72.5:27.5, 75:25, 80:20, 85:15, 87.5:12.5, 90:10, 95:5, 100:0), yielding 13 fractions (A–M). Fraction D (47.7 mg) and fraction E (45 mg) were combined and further purified on a silica gel column (60 g) using $CHCl_3$/MeOH/H_2O mixtures (90:10:0.5, 87.5:12.5:0.5, 85:15:05, 82.5:17.5:0.5, 80:20:0.5) to give **10** (6.4 mg), **11** (5.3 mg), and fraction DE3 (5 mg). Fraction F (72 mg) was submitted to a silica gel column (50 g) using mixtures of $CHCl_3$/MeOH (90:10, 88:12) and $CHCl_3$/MeOH/H_2O (87.5:12.5:0.5, 85:15:0.5, 80:20:1), to give three fractions (F1–4). To isolate metabolite **12** (3.3 mg), fraction DE3 (5 mg) and fraction F3 (2.3 mg) were combined and subjected to a silica gel column (5 g), using $CHCl_3$/MeOH/H_2O (90:10.05). Fraction G (95 mg) was subjected to passage over a silica gel column (50 g) using mixtures of $CHCl_3$/MeOH/H_2O (90:10:0.5, 87.5:12.5:0.5, 85:15:0.5), to provide two fractions (G1 and G2). Fraction G1 (15.7 mg) was then applied to VLC loaded with reversed-phase silica gel (RP-18, 20 g), using MeOH/H_2O (75:25) to afford two fractions (G1-1 and G1-2). With the further purification of fraction G1-2 (10.2 mg) on a silica gel column (7 g) using gradient elution with cyclohexane/EtOAc/MeOH (10:10:2, 10:10:2.5), 6.6 mg of metabolite **14** was obtained. To isolate metabolite **13** (10 mg), fraction G2 (26.9 mg) was applied to VLC (RP-18, 20 g) and eluted with MeOH/H_2O (75:25, 80:20).

Compounds **15**–**17** were obtained from the EtOAc extract (1.42 g) of *C. laburnicola* and **2**. This crude extract was subjected to a reversed-phase column (C_{18}, 50 g) to yield metabolite **16** (17 mg) and two impure fractions (A and B) after elution with a MeOH/H_2O gradient (40:60, 45:55, 50:50, 55:45, 60:40, 70:30, 80:20, 90:10, 100:0). Fraction A (29.8 mg) was further fractionated over a silica gel column (15 g) with the solvent system $CHCl_3$/MeOH (97:3, 94:6, 95:5), to provide 6.3 mg of metabolite **15**. Fraction B (195 mg) was also subjected to separation over a silica gel column (56 g) using $CHCl_3$/MeOH (97:3, 94:6, 95:5), to give metabolite **17** (9.2 mg).

20(R),24(S)-Epoxy-6α,11β,16β,25-tetrahydroxycycloartan-3-one (**3**): $[\alpha]^{31}_{D}$ +23.0 (*c* 0.058, MeOH); ^{1}H NMR data ($CDCl_3$, 400 MHz), see Table 1; ^{13}C NMR data ($CDCl_3$, 100 MHz), see Table 1; HRESIMS (positive ion mode) *m*/*z* 527.3376 $[M + Na]^+$(calcd for $C_{30}H_{48}O_6Na$, 527.3348).

20(R),24(S)-Epoxy-6α,12β,25-trihydroxy-3,16-dioxo-cycloartane (**7**): $[\alpha]^{31}_{D}$ +7.3 (*c* 0.092, MeOH); ^{1}H NMR data (CD_3OD, 500 MHz), see Table 1; ^{13}C NMR data (CD_3OD, 125 MHz), see Table 1; HRESIMS (positive ion mode) *m*/*z* 525.3168 $[M + Na]^+$ (calcd for $C_{30}H_{46}O_6Na$, 525.3192).

20(R),24(S)-Epoxy-6α,12β,16β,25-tetrahydroxylanost-9(11)-en-3-one (**10**): $[\alpha]^{31}_{D}$ +40.0 (*c* 0.025, MeOH); ^{1}H NMR data (CD_3OD, 500 MHz), see Table 1, ^{13}C NMR data (CD_3OD, 125 MHz), see Table 1; HRESIMS (positive ion mode) *m*/*z* 527.3335 $[M + Na]^+$(calcd for $C_{30}H_{48}O_6Na$, 527.3348).

9β,11;20(R),24(S)-Diepoxy-3β,6α,16β,19β,25-pentahydroxycycloartane (**12**): $[\alpha]^{31}_{D}$ +14.9 (*c* 0.067, MeOH); ^{1}H NMR data (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR data (CD_3OD, 100 MHz), see Table 1; HRESIMS (positive ion mode) *m*/*z* 545.3449 $[M + Na]^+$ (calcd for $C_{30}H_{50}O_7Na$, 545.3454).

20(R),24(S)-Epoxy-3β,6α,16β,25-tetrahydroxylanost-9(11)-en-12-one (**14**): $[\alpha]^{31}_{D}$ +24.1 (*c* 0.083, MeOH); ^{1}H NMR data (CD_3OD, 400 MHz), see Table 1, ^{13}C NMR data (CD_3OD, 100 MHz), see Table 1; HRESIMS (positive ion mode) *m*/*z* 527.3350 $[M + Na]^+$(calcd for $C_{30}H_{48}O_6Na$, 527.3348).

Biological Assay. *Cell Line and Culture Condition.* Hekn (neonatal human primary epidermal keratinocytes) were obtained from the American Type Culture Collection (ATCC; PCS-200-010) and cultured in dermal basal cell medium (ATCC) supplemented with a keratinocyte growth kit (ATCC) containing the following growth supplements: bovine pituitary extract, rh TGFα, L-glutamine, hydrocortisone hemisuccinate, rh insulin, epinephrine, and apo-transferrin.

Telomerase Activity Assay. Measurements of telomerase activity were performed in Hekn cells (population doubling time: 4–6 days) using a PCR-based telomere repeat amplification protocol (TRAP) assay. Hekn cells were seeded in 12-well plates, and on the following day, the medium was refreshed and the day after this they were treated with the desired doses of the test compounds for 24 h. After a 24 h incubation, cells were harvested and counted using a hemocytometer. After transferring 2×10^5 cells to a prechilled and clean Eppendorf tube, this was centrifuged at 3000*g* for 5 min (+2 to +8 degrees). The supernatant was carefully removed without damaging the cell pellet. The cells in the pellet were suspended in 200 μL of lysis buffer and incubated on ice for 30 min. After completion of the incubation period, the lysates were centrifuged at 16000*g* for 20 min (+2 to +8 degrees). The supernatant was transferred carefully to a prechilled and clean Eppendorf tube. Then, telomerase activity was determined using a Telomerase PCR ELISA kit (Sigma-Aldrich, St. Louis, MO, USA) as recommended by the manufacturer. All solutions were included in the kit. Sample absorbance was measured with a Varioscan microplate reader (Thermo Fisher Scientific) at a wavelength of 450 nm (with a reference wavelength of approximately 690 nm) within 30 min after addition of the stop reagent. For prescreening studies, the assay was performed in two technical replicates with an internal standard (216 bp internal sequence used as an internal amplification control). Subsequent studies with the selected compounds were performed with three biological replicates. Values are expressed as the fold change relative to the control. Two-way ANOVA was used to determine the significance of the differences ($p^* \leq 0.05$, $p^{**} \leq 0.001$, $p^{***} \leq 0.005$).

■ ASSOCIATED CONTENT

Supporting Information

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Additional information (PDF)

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Notes

The authors declare no competing financial interest.

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