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A new ergostane-type cholesterol biosynthesis inhibitor isolated from *Hormoconis resinae*

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Abstract—A new ergostane-type steroid, 3β-hydroxy-1,11-dioxo-ergosta-8,24(28)-diene-4α-carboxylic acid (**1**) was isolated from the mold *Hormoconis resinae* as a cholesterol biosynthesis inhibitor in the Chang liver cell. The absolute stereostructure of **1** was established based on the spectroscopic analyses and modified Mosher's method.

Cholesterol is a major sterol in all the animal tissues and is synthesized from five-carbon isoprene units in the liver. Although it is an important precursor of specific biological products including bile acids, steroid hormones and vitamin D, higher blood cholesterol levels, more specifically, increased low density lipoprotein (LDL) cholesterol levels are major risk factors of coronary heart disease. Clinical studies have indicated that the lowering of total and LDL cholesterol levels reduces the risk factor of coronary heart disease.[1,2]

Among various strategies for lowering blood cholesterol level, use of the hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitior has been one of the most effective hypolipidemic measures. The target enzyme, HMG-CoA reductase, is a rate-limiting enzyme that controls the level of mevalonate in cholesterol biosynthesis.[3] However, since mevalonate is also the precursor of other metabolites that are essential for the proper functioning of the human body, the effects of reducing the mevalonate level by HMG-CoA reductase inhibitors is not restricted to the cholesterol biosynthetic pathway.[4] In this regard, much interest has been shown in the development of more specific inhibitors, which exert inhibition at the later stages of the cholesterol biosynthetic pathway. In the previous studies, we have established a modified assay system in vitro to screen cholesterol biosynthesis inhibitors using Chang liver cell.[5,6] The EtOAc extract of the mold *Hormoconis resinae* showed significant inhibiting activity in the assay system. Bioactivity-guided fractionation of the extract led to the isolation of a new ergostane-type steroidal analogue, 3β-hydroxy-1,11-dioxo-ergosta-8,24(28)-diene-4α-carboxylic acid (**1**) as an inhibitor of cholesterol biosynthesis. In the present study, isolation, structure elucidation and biological activity of the novel compound **1** are presented in detail.

Hormoconis resinae (KCTC 6966, identical with ATCC 22711) is a fungus which originated from jet fuel JP-4, and was obtained from the Korean Collection for Type Cultures (KCTC). *H. resinae* was grown in each 1 L

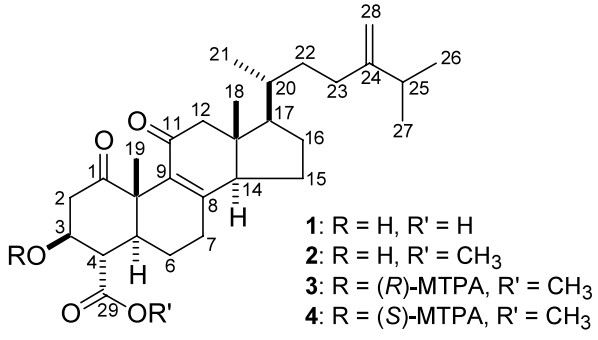

Figure 1. Structures of compounds **1**–**4**.

Keywords: *Hormoconis resinae*; cholesterol biosynthesis inhibitor; ergostane; 3β-hydroxy-1,11-dioxo-ergosta-8,24(28)-diene-4α-carboxylic acid.

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Erlenmeyer flask containing 400 mL YM media at 25°C, 200 rpm for 4 days in shaking incubator. Total 60 L of culture media was repeatedly extracted with EtOAc and the combined extract was concentrated in vacuo. The EtOAc extract (16.2 g) was successively subjected to Si gel column chromatography ($CHCl_3$/MeOH/H_2O mixtures) and reversed phase MPLC (53% MeOH) by bioactivity-guided fractionation. The further purification by preparative TLC ($CHCl_3$/MeOH/H_2O = 80:20:2) afforded compound **1** (11 mg) as amorphous powder.

The $[M+H]^+$ peak at m/z 471.3118 (calcd. m/z 471.3110) shown in HR FABMS of **1** was assigned the molecular formula $C_{29}H_{42}O_5$ with an index of hydrogen deficiency of nine. The UV and IR spectra showed absorption peaks for a hydroxyl group (ν_{max} 3447 cm^{-1}) and an α,β-unsaturated ketone (ν_{max} 1654 cm^{-1}, λ_{max} 250 nm). The ^1H NMR spectra (Table 1) showed an exomethylene signal at δ 4.67 (brs) and 4.73 (d, J = 1.0 Hz), two tertiary methyl signals at δ 0.71 and 1.51, a secondary methyl at δ 0.96 (d, J = 6.5 Hz), two isopropyl methyl signals at δ 1.02 (d, J = 7.0 Hz) and 1.03 (d, J = 7.0 Hz) and a hydroxymethine at δ 3.95 (ddd, J = 6.3, 10.3, 11.0 Hz). The ^{13}C NMR spectra (Table 1) indicated the presence of a conjugated enone including a tetrasubstituted double bond at δ 136.1, 160.7 and 201.0, a ketone at δ 211.8, a carboxyl group at δ 180.8, in addition to the exomethylene double bond at δ 107.0 and 157.1, which corresponds to five degrees of unsaturation. These findings suggested that **1** is an oxygenated tetracyclic steroid analogue having twenty nine carbons. The DEPT and HSQC spectra exhibited the correct carbon multiplicities and heteronuclear couplings between carbon atoms and their directly attached protons (Table 1).

Table 1. The NMR spectral data of **1** in CD_3OD[a,b]

Position	δ_H[c]	δ_C[d]	^1H–^1H COSY	HMBC (H→C)
1	–	211.8 s		
2α	2.46 (1H, dd, J = 6.0, 11.0 Hz)	47.6 t		C-1, C-3, C-4, C-10
2β	3.08 (1H, t, J = 11.0 Hz)			C-1, C-3
3α	3.95 (1H, ddd, J = 6.0, 10.3, 11.0 Hz)	75.3 d		C-29
4β	2.68 (1H, dd, J = 10.3, 12.3 Hz)	56.6 d	H-5	C-3, C-5, C-29
5α	1.42 (1H, dt, J = 2.5, 12.3 Hz)	45.0 d		C-19
6α	1.73 (1H, m)	22.0 t	H-7	C-8
6β	1.61 (1H, m)			C-19
7α	2.23 (1H, m)	30.3 t		
7β	2.35 (1H, dd, J = 4.5, 20.0 Hz)			C-5, C-8, C-9
8	–	160.7 s		
9	–	136.1 s		
10	–	51.7 s		
11	–	201.0 s		
12α	2.51 (1H, d, J = 14.5 Hz)	57.2 t		C-11, C-13, C-18
12β	2.74 (1H, d, J = 14.5 Hz)			C-9, C-11, C-13, C-14, C-18
13	–	49.2 s		
14α	2.79 (1H, dd, J = 7.0, 12.5 Hz)	53.8 d	H-15	C-8, C-9, C-18
15α	1.84 (1H, m)	24.2 t	H-14	
15β	1.50 (1H, m)			
16α	2.03 (1H, m)	28.7 t	H-15, H-17	
16β	1.40 (1H, m)			
17α	1.58 (1H, m)	56.3 d		
18	0.71 (3H, s)	12.3 q		C-12, C-13, C-14, C-17
19	1.51 (3H, s)	19.8 q		C-1, C-5, C-9, C-10
20	1.48 (1H, m)	37.0 d		
21	0.96 (3H, d, J = 6.5 Hz)	18.9 q	H-20	C-17, C-20, C-22
22a	1.19 (1H, m)	35.6 t	H-23	
22b	1.60 (1H, m)			
23a	1.93 (1H, ddd, J = 4.0, 11.0, 14.0 Hz)	32.0 t	H-22	C-22, C-24, C-28
23b	2.14 (1H, ddd, J = 5.5, 11.0, 15.0 Hz)			C-24, C-28
24	–	157.1 s		
25	2.24 (1H, sept, J = 7.0 Hz)	34.9 d		C-24, C-26, C-28
26	1.02 (3H, d, J = 7.0 Hz)[e]	22.3 q[e]	H-25	C-24, C-25, C-27
27	1.03 (3H, d, J = 7.0 Hz)[e]	22.4 q[e]	H-25	C-24, C-25, C-26
28a	4.67 (1H, brs)	107.0 t	H-23	C-23, C-25
28b	4.73 (1H, d, J = 1.0 Hz)		H-25	C-23, C-25
29	–	180.8 s		

[a] Assignments were confirmed by ^1H, ^{13}C, ^1H–^1H , NOE, NOESY, DEPT, HSQC and HMBC experiments.
[b] J values are given in Hz.
[c] The ^1H NMR spectra were measured at 500 MHz.
[d] The ^{13}C NMR spectra were measured at 125 MHz.
[e] Assignments are interchangeable.

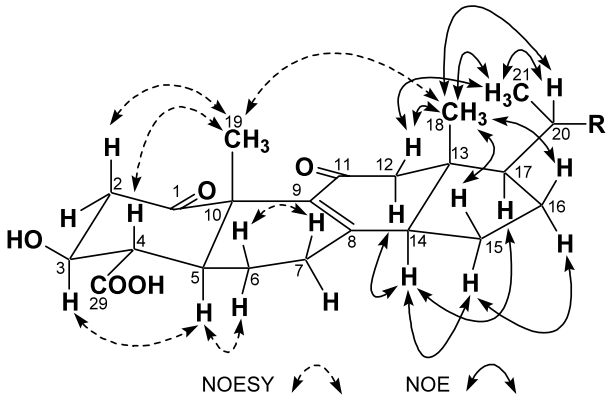

Figure 2. NOSEY and NOE correlations of compound **1**.

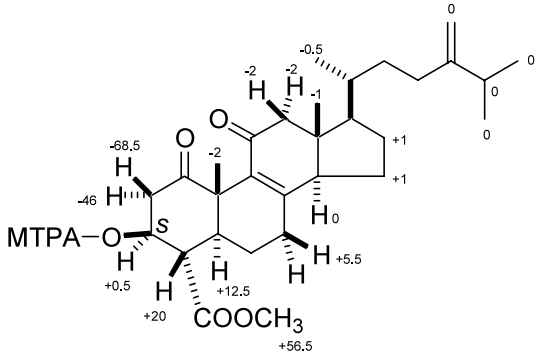

Figure 3. $\Delta\delta$ Values (δ_S–δ_R) from the MTPA esters **3** and **4** in Hz.

Further information regarding the structure of **1** was established by analysis of HMBC and ^{1}H–^{1}H COSY spectral data (Table 1). The exomethylene signals at δ 157.1 (C-24) and 107.0 (C-28) showed correlations with the proton signals of isopropyl group at δ 2.24 (H-25, sept, J=7.0 Hz), 1.02 (H-26, d, J=7.0 Hz) and 1.03 (H-27, d, J=7.0 Hz) and with the methylene protons at δ 1.93 (H-23a, ddd, J=4.0, 11.0, 14.0 Hz) and 2.14 (H-23b, ddd, J=5.5, 11.0, 15.0 Hz). Carbonyl group at δ 211.8 (C-1), quaternary carbon at δ 51.7 (C-10) and hydroxymethine carbon at δ 75.3 (C-3) were correlated to the methylene protons at δ 3.08 (H-2β, t, J=11.0 Hz) and 2.46 (H-2α, dd, J=6.0, 11.0 Hz). The carboxyl group at δ 180.8 assignable to C-29 showed long range couplings with the methine protons at δ 2.68 (H-4, dd, J=10.3, 12.3 Hz) and 3.95 (H-3, ddd, J=6.0, 10.3, 11.0 Hz). The tetrasubstituted olefinic carbons at δ 160.7 (C-8) and 136.1 (C-9), and the ketone signal at δ 201.0 (C-11) in a conjugated enone were correlated to the proton signals at δ 2.35 (H-7β, dd, J=4.5, 20.0 Hz) and 2.79 (H-14, dd, J=7.0, 12.5 Hz) and the methylene proton at δ 2.51 (H-12α, d, J=14.5 Hz) and 2.74 (H-12β, d, J=14.5 Hz), respectively. The tertiary angular methyl signal at δ 12.3 (C-18) showed correlations with the methylene (H-12α and H-12β) and two methines at δ 2.79 (H-14, dd, J=7.0, 12.5 Hz) and 1.58 (H-17, m). Another tertiary methyl at δ 1.51 (H-19, s) was correlated to the ketone at δ 211.8 (C-1), quaternary olefinic carbon signal at δ 136.1 (C-9) and two junctional carbons at δ 51.7 (C-10) and 45.0 (C-5). Two methines at δ 56.3 (C-17) and 37.0 (C-20) and methylene carbon at δ 35.6 (C-22) also showed long range couplings with the secondary methyl protons at δ 0.96 (H-21, d, J=6.5 Hz). The connectivities of C-14, C-15, C-16 and C-17 were clarified by the ^{1}H–^{1}H COSY correlations of H-14/H-15, H-15/H-16 and H-16/H-17 protons. Based on the 1D and 2D NMR data, the structure of **1** was assigned as a novel oxygenated ergostane-type steroid, 3-hydroxy-1,11-dioxo-ergosta-8,24(28)-diene-4-carboxylic acid.

The relative stereochemistry of **1** was determined by NOESY, selective NOE correlations and $J_{H,H}$ values. The NOESY data (Fig. 2) exhibited correlations between H-19 methyl and H-2β, H-4β, H-18 protons, as well as between H-5α and H-3α, H-6α protons. Moreover, the hydroxyl group at C-3 and the carboxyl group bound to C-4 were elucidated to be in equatorial position on the basis of $J_{H,H}$ values of H-3α (ddd, J=6.0, 10.3, 11.0 Hz) and H-4β (dd, J=10.3, 12.3 Hz). In selective NOE experiments (Fig. 2), irradiation of H-18 methyl caused NOE enhancement in the signals of H-12β, H-15β, H-16β, H-19, H-20 and H-21 protons. Additional irradiation of H-12β increased the intensities of H-18 and H-21 methyl signals, but not H-20 methine signal. Also, H-14α methine showed NOE correlations with H-12α, H-15α and H-17α protons. From these data, configuration of **1** was assigned as shown in Fig. 1.

The absolute stereochemistry at C-3 in **1** was determined using modified Mosher's method.[7] Methylation of **1** with CH_2N_2 gave methyl ester **2**,[8] which was transformed into the (*R*)- and (*S*)-MTPA esters, **3**[9] and **4**.[10] $\Delta\delta$ Values (δ_S–δ_R, Hz) calculated from the two esters (Fig. 3) indicated that the absolute stereochemistry at C-3 was *S*, and these results were in good agreement with those of ergosterol.[7] Therefore, the absolute structure of **1** was established to be a novel oxygenated ergostane-type steroid, 3β-hydroxy-1,11-dioxo-ergosta-8,24(28)-diene-4α-carboxylic acid based on the spectroscopic analyses and modified Mosher's method. To the best of our knowledge, combination of the 1,11-dioxo-8-ene moiety and the secondary carboxyl group at C-4 has never been yet reported in the naturally occurring steroids including ergostanes. The optical rotation of **1** was $[\alpha]_D$ +104.4° (*c* 0.2, MeOH).

The cholesterol biosynthesis inhibitory effect of **1** was evaluated on the Chang liver cells in vitro. The compound treated cells were continuously incubated with ^{14}C-labeled acetate in serum free media and then the amount of synthesized cholesterol was estimated using phosphor image analyzer after TLC development ($CHCl_3$/Et_2O=18:1).[5,6] Compound **1** exhibited moderate to significant cholesterol biosynthesis inhibitory activity with an IC_{50} value of 8.0 μg/mL when compared with that of mevastatin (0.3 μg/mL), commercially available HMG-CoA reductase inhibitor. **1** showed an inhibitory profile different from that of mevastatin. Analysis of the extracts of cells treated with **1** revealed the presence of accumulated lanosterol in the

sterol fraction as evidenced by TLC, whereas no labeled sterol intermediate was detected in case of mevastatin under the same condition. It is postulated that compound **1** may inhibit cholesterol biosynthesis at the later stages, i.e. post-lanosterol steps, in cholesterol biosynthetic pathway.

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9. (*R*)-MTPA ester (**3**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.40 (H-3, dt, $J=6.5$, 11.0 Hz, 1H), δ 4.73 (H-28b, brs, 1H), δ 4.65 (H-28a, brs, 1H), δ 3.56 ($COOCH_3$, s, 3H), δ 3.17 (H-2β, t, $J=11.5$ Hz, 1H), δ 3.12 (H-4, brt, $J=11.0$ Hz, 1H), δ 2.93 (H-2α, dd, $J=6.5$, 11.5 Hz, 1H), δ 2.84 (H-12β, d, $J=14.5$ Hz, 1H), δ 2.72 (H-14, dd, $J=7.0$, 12.5 Hz, 1H), δ 2.50 (H-12α, d, $J=14.5$ Hz, 1H), δ 2.28 (H-7β, dd, $J=4.7$, 20.3 Hz, 1H), δ 2.10 (H-23b, 1H), δ 2.01 (H-16α, 1H), δ 1.88 (H-23a, 1H), δ 1.77 (H-15α, 1H), δ 1.66 (H-5, brt, $J=11.0$ Hz, 1H), δ 1.52 (H-19, s, 3H), δ 1.03 (H-27, d, $J=6.7$ Hz, 3H), δ 1.02 (H-26, d, $J=6.7$ Hz, 3H), δ 0.94 (H-21, d, $J=6.0$ Hz, 3H), δ 0.68 (H-18, s, 3H).
10. (*S*)-MTPA ester (**4**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.40 (H-3, dt, $J=6.5$, 11.0 Hz, 1H), δ 4.73 (H-28b, brs, 1H), δ 4.65 (H-28a, d, $J=1.0$ Hz, 1H), δ 3.68 ($COOCH_3$, s, 3H), δ 3.16 (H-4, brt, $J=11.0$ Hz, 1H), δ 3.04 (H-2β, t, $J=11.5$ Hz, 1H), δ 2.84 (H-2α, dd, $J=6.5$, 11.5 Hz, 1H), δ 2.83 (H-12β, d, $J=14.5$ Hz, 1H), δ 2.72 (H-14, dd, $J=7.0$, 12.5 Hz, 1H), δ 2.50 (H-12α, d, $J=14.5$ Hz, 1H), δ 2.29 (H-7β, dd, $J=4.7$, 20.3 Hz, 1H), δ 2.10 (H-23b, 1H), δ 2.01 (H-16α, 1H), δ 1.88 (H-23a, 1H), δ 1.77 (H-15α, 1H), δ 1.68 (H-5, dt, $J=2.5$, 11.5 Hz, 1H), δ 1.52 (H-19, s, 3H), δ 1.03 (H-27, d, $J=6.7$ Hz, 3H), δ 1.02 (H-26, d, $J=6.7$ Hz, 3H), δ 0.94 (H-21, d, $J=6.0$ Hz, 3H), δ 0.68 (H-18, s, 3H).