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NOTE

Diketopiperazines, inhibitors of sterol *O*-acyltransferase, produced by a marine-derived *Nocardiopsis* sp. KM2-16

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Sterol *O*-acyltransferase (SOAT, also known as acyl-CoA:cholesterol acyltransferase (ACAT), EC 2.4.1.26), an endoplasmic reticulum (ER) membrane protein, catalyzes the synthesis of cholesteryl ester (CE) from free cholesterol and long-chain fatty acyl-CoA. SOAT has been postulated as a target for modulation by a new type of antiatherosclerotic agent. Recent molecular biological studies revealed the existence in mammals of two different SOAT isozymes, SOAT1 and SOAT2.[1–4] SOAT1-selective inhibition may cause detrimental effects,[5–7] whereas SOAT2-selective inhibition has consistently shown antiatherosclerotic activity.[8,9] Therefore, it is important to determine the selectivity of inhibitors toward the two SOAT isozymes for development as new antiatherosclerosis agents. Our group has focused on the discovery of SOAT2-selective inhibitors of microbial origin in cell-based assay or an enzyme assay using microsomes prepared from SOAT2-expressing Chinese hamster ovary (CHO) cells. During the course of our screening program, two diketopiperazines, **1** and amauromine[10] (**2**) (Figure 1), were isolated as SOAT2 inhibitors from the culture broth of actinomycete strain *Nocardiopsis* sp. KM2-16. Amauromine was originally isolated as a vasodilator from the culture broth of the fungus *Amauroascus* sp. Yin *et al.*[11] reported that **1** was produced by bioconversion from cyclo-L-tryptophan-L-tryptophan using two recombinant enzymes involved in acetylaszonalenin biosynthesis.[12] Thus, **2** and structurally related acetylaszonalenin were fungal secondary metabolites. In this study, we showed that **1** and **2** were isolated as actinomycete secondary metabolites and that **2** selectively inhibited SOAT2 activity.

The strain KM2-16 was isolated from sea sediments collected off Iriomote Island in Okinawa, Japan in 2012. In a BLAST search, the 16S rRNA sequence of the strain KM2-16 indicated that it could be considered to belong to the actinomycete genus *Nocardiopsis*. The strain was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of production medium (1.0% starch, 0.40% yeast extract, 0.20% peptone, 0.10% $CaCO_3$, 0.010% KBr, 0.0040% $Fe_2(SO_4)_3 \bullet nH_2O$, 100 ml of natural seawater). Fermentation was carried out at 27 °C for 11 days under shaking conditions (180 r.p.m.). The culture broth (100 ml × 3) was extracted with acetone (300 ml). This extract was concentrated and extracted with EtOAc to yield the crude extracts (42 mg), which was purified by HPLC using a PEGASIL ODS column (10 × 250 mm^2, Senshu Scientific Co., Tokyo, Japan) under the following conditions: solvent, a 30-min linear gradient from 50 to 85% CH_3CN; flow rate, 3.0 ml min^{-1}; detection, UV at 285 nm. Compounds **1** and **2** were eluted as peaks with retention times of 14 and 29 min, respectively. Each peak was collected and concentrated to yield pure **1** (8.6 mg) and **2** (4.6 mg) as colorless solids. Interestingly, **1** and **2** were produced in the seawater-supplemented medium, whereas almost no production was observed in an analogous medium made with distilled water.

The physico-chemical properties of **1** and **2** are summarized in Table 1. The molecular formulas of **1** and **2** were determined to be $C_{27}H_{28}N_4O_2$ and $C_{32}H_{36}N_4O_2$ on the basis of HR-ESI-MS measurement, respectively. From ^1H and ^{13}C NMR and specific rotation, **2** was identified as amauromine previously reported as a fungal vasodilator.[10] Compound **1** had absorption maxima at 210, 219, 244, 282 and 290 nm in the UV spectrum. The IR absorption maxima of **1** at 3276 and 1664 cm^{-1} suggested the presence of amino and carbonyl moieties, respectively. Although **1** appeared to be a known compound reported by Yin *et al.*,[11] the structural determination has not been described in detail. The structure of **1** was mainly elucidated by analysis of NMR spectra, including 2D NMR. The ^{13}C NMR spectrum (in $CDCl_3$) showed 27 resolved signals, which were classified into two methyl carbons, two sp^3 methylene carbons, three sp^3 methine carbons, one sp^2 methylene carbon, 10 sp^2 methine carbons and 9 quaternary carbons, including two carbonyl carbons. The connectivity of proton and carbon atoms was established by the HMQC spectrum, as shown in Table 2. Analysis of the ^1H-^1H COSY spectrum gave the 7 partial structures **I** (NH-1 to C-2), **II** (C-4 to C-7), **III** (C-10 to C-11), **IV** (C-14 to C-17), **V** (C-19 to NH-20), **VI** (C-22 to C-25) and **VII** (C-1′ to C-2′) drawn with the bold lines in Figure 2. The ^1H-^{13}C long-

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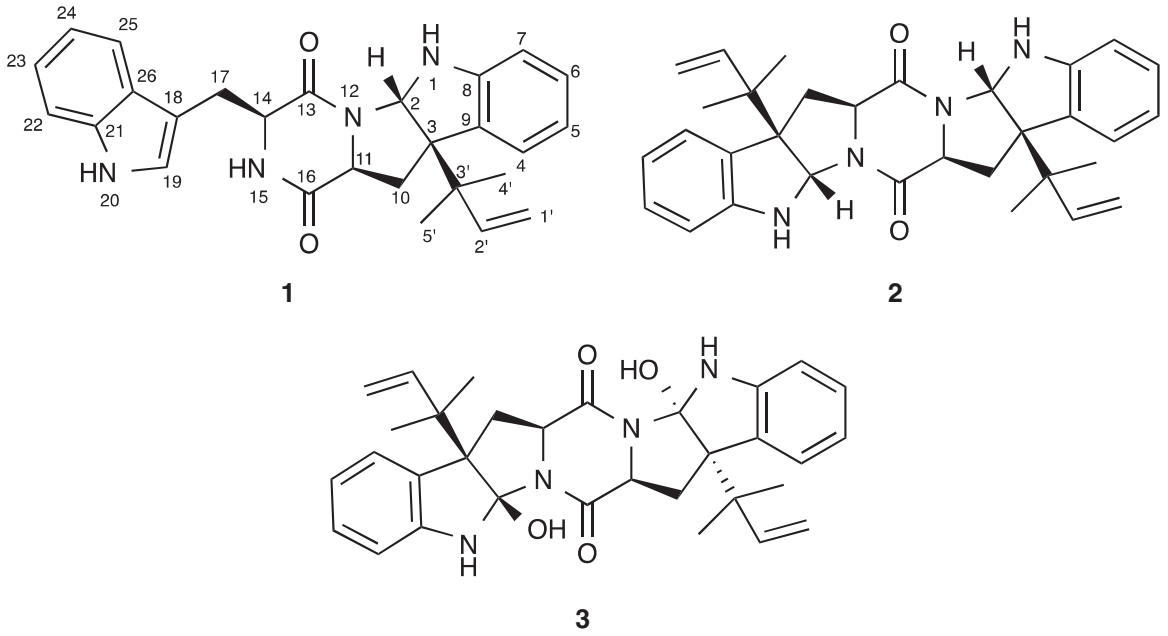

1 **2** **3**

Figure 1 Structures of **1**, amauromine (**2**) and gypsetin (**3**).

Table 1 Physico-chemical properties of 1 and 2

	1	*2*
Appearance	Colorless solid	Colorless solid
$[\alpha]_D^{24.1}$ (c = 0.1, MeOH)	−182.9	−558.7
Molecular weight	440	508
Melecular formula	$C_{27}H_{28}N_4O_2$	$C_{32}H_{36}N_4O_2$
HR-ESI-MS (m/z)		
Calcd	463.2110 $(M + Na)^+$	509.2917 $(M + H)^+$
Found	463.2107 $(M + Na)^+$	509.2913 $(M + H)^+$
UV λ_{max}^{MeOH} (logε)	210 (3.0), 219 (3.0), 244 (2.3) 282 (2.2), 290 (2.2)	208 (3.1), 244 (2.5), 300 (2.1)
IR ν_{max}^{KBr} cm^{-1}	3276, 2970, 1664, 1451, 1317	3386, 2972, 1661, 1462, 1423
CD (MeOH) λ nm (Mol. CD)	220 (3.7), 245 (–5.6), 272 (–1.7), 300 (–2.8)	222 (–0.37), 244 (–4.8), 266 (–0.17), 301 (–1.0)

range couplings of ^2J and ^3J observed in the HMBC experiments gave the following information. (1) The cross-peaks were observed from 1-NH (δ 5.06) to C-3 (δ 61.6) and C-9 (δ 128.9), from 2-H (δ 5.55) to C-8 (δ 149.9), C-9, C-11 (δ 59.0) and C-13 (δ 166.0), from 4-H (δ 7.14) to C-3 and C-8, from 5-H (δ 6.76) to C-9, from 6-H (δ 7.11) to C-8, from 7-H (δ 6.61) to C-9, from 10-H_2 (δ 2.42, 2.51) to C-3, C-9 and C-16 (δ 168.9), from 11-H (δ 3.91) to C-10 and C-16, from 14-H (δ 4.30) to C-13, and from 15-NH (δ 5.69) to C-11, C-13, C-14, C-16 and C-17 (27.0), suggesting the presence of 6-, 5-, 5- and 6-membered ring systems (Part A), including indoline and diketopiperazine rings, which contained the partial structures **I**, **II**, **III** and **IV**. (2) The cross-peaks were observed from 19-H (δ 7.08) to C-18 (δ 109.7), C-21 (δ 136.6) and C-26 (δ 126.6), from NH-20 (δ 8.19) to C-18, C-21 and C-26, from 22-H (δ 7.38) to C-26, from H-23 (δ 7.22) to C-21, from 24-H (δ 7.12) to C-26 and from 25-H (δ 7.55) to C-18, C-21 and C-26, suggesting the presence of the indole ring (Part B) containing the partial structures **IV** and **V**. (3) The cross-peaks were observed from 1′-H_2 (δ 5.08, 5.12) to C-3′ (δ 40.8), from 2′-H (δ 5.97) to C-3, C-4′ (δ 22.8) and C-5′ (δ 22.4), from 4′-H_3 (δ 1.01) to C-2′ (δ 143.5), C-3′ and C-5′ and from 5′-H_3 (δ 1.11) to C-2′, C-3′ and C-4′, suggesting the presence of the prenyl moiety (Part C) containing the partial structure **VII**. Finally, (4) the cross-peaks from 17-H_2 (δ 2.97, 3.74) to C-19 (δ 123.2) and C-26, from 19-H to C-17, from 2-H and 10-H_2 to C-3′ and from 2′-H, 4′-H_3 and 5′-H_3 to C-3 indicated that Part A is attached to Parts B and C, as shown in Figure 2. The structure satisfied the degrees of unsaturation and the molecular formula.

The relative configurations of C-2, C-3, C-11 and C-14 were elucidated by NOE experiments. As shown in Figure 3, the cross-peaks between 2-H and 4′-H_3/5′-H_3 proved the relative configurations, 2*S** and 3*R**. Furthermore, the correlation between H-11 and H-14 proved the relative configurations, 11*S** and 14*R**. As shown in Table 1, the CD spectra of **1** showed positive Cotton effects at 272 and 220 nm and negative Cotton effects at 300 and 248 nm. These data suggested that **1** has the same absolute configurations, 2*S*, 3*R*, 11*S* and 14*R*, as **2**.[13]

SOAT inhibitory activity of **1** and **2** was investigated in the enzyme assay using microsomes prepared from SOAT1- and SOAT2-expressing CHO (hereafter referred to as SOAT1-CHO and SOAT2-CHO, respectively) cells.[14] As summarized in Table 3, **1** and **2** are SOAT inhibitors rather selective toward SOAT2 isozyme in the enzyme assay. Furthermore, the SOAT inhibition was evaluated in a cell-based assay using SOAT1- and SOAT2-CHO cells.[14] As shown in Table 3, **2** inhibited CE synthesis with an IC_{50} value of 0.45 μM in SOAT2-CHO cells, and it became clear that **2** is a SOAT2-selective inhibitor with a selective index (SI) value of 62 in the cell-based assay. However, **1** showed no inhibition of SOAT1 and SOAT2 at 22 μM. It might be that **1** cannot penetrate CHO cells. Shinohara *et al.*[15] reported that structurally related gypsetin (**3**), produced by the fungus *Nannizzia gypsea* var. *incurvata* IFO9228, inhibited rat liver microsomal SOAT activity (IC_{50}, 18 μM) and cholesteryl ester synthesis in macrophage J774 (IC_{50}, 0.65 μM). Now it is known that J774 cells

Table 2 ^{1}H and ^{13}C NMR chemical shifts of 1 (600 MHz for ^{1}H, 150 MHz for ^{13}C)

	1		
Position	*δ_C (p.p.m.)*[a]	*δ_H (p.p.m.)*[b]*, multi, J in Hz*	*HMBC*
1	—	5.06, sd, 1.0	C-3, 9
2	77.7	5.55, s	C-8, 9, 11, 13, 3′
3	61.6	—	—
4	125.1	7.14[c]	C-3, 6, 8
5	118.9	6.76, td, 7.5, 0.8	C-4, 7, 9
6	128.9	7.11[c]	C-4, 8
7	109.2	6.61, d, 7.5	C-5, 9
8	149.9	—	—
9	128.9	—	—
10	36.0	2.42, t, 12.0 2.51, dd, 12.5, 6.0	C-2, 3, 9, 11, 16, 3′
11	59.0	3.91, dd, 11.5, 6.0	C-10, 16
12	—	—	—
13	166.0	—	—
14	54.6	4.30, d, 10.5	C-13, 17, 18
15	—	5.69, s	C-11, 13, 14, 16, 17
16	168.9	—	—
17	27.0	2.97, dd, 10.8, 11.0 3.74, dd, 4.0	C-13, 14, 18, 19, 26
18	109.7	—	—
19	123.2	7.08, sd, 2.0	C-17, 18, 21, 26
20	—	8.19, s	C-18, 19, 21, 26
21	136.6	—	—
22	115.5	7.38, d, 8.5	C-23, 24, 26
23	122.9	7.22, td, 7.5, 1.0	C-22, 24, 25
24	120.1	7.12[c]	C-22, 23, 26
25	118.4	7.55, d, 8.0	C-21, 23, 26
26	126.6	—	—
1′	114.5	5.08, dd, 13.5, 1.2 5.12, dd, 10.5, 1.0	C-2′, 3′
2′	143.5	5.97, dd, 18.0, 10.5	C-3, 1′, 3′, 4′, 5′
3′	40.8	—	—
4′	22.8	1.01, s	C-3, 1′, 2′, 3′, 5′
5′	22.4	1.11, s	C-3, 1′, 2′, 3′, 4′

[a]Chemical shifts are shown with reference to $CDCl_3$ as δ 77.0.
[b]Chemical shifts are shown with reference to $CDCl_3$ as δ 7.26.
[c]Signals are overlapping.

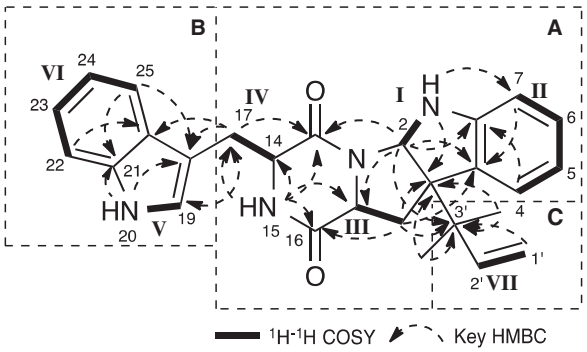

Figure 2 Key cross-peaks observed in ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC experiments of **1**.

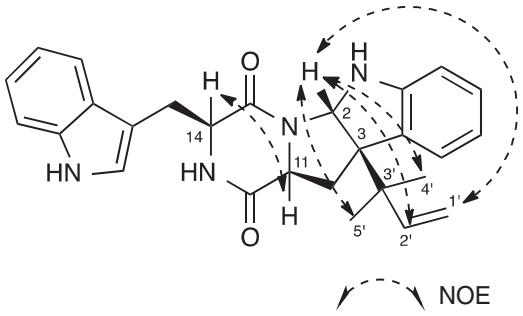

Figure 3 Key NOE experiment of **1**.

Table 3 Effects of 1 and 2 on SOAT isozymes in cell-based and enzyme-based assays

Compound	*Enzyme-based*			*Cell-based*			*Cytotoxicity*[a]
	IC_{50} (μM)			*IC_{50} (μM)*			
	SOAT1	*SOAT2*	*SI*[b]	*SOAT1*	*SOAT2*	*SI*	*IC_{50} (μM)*
1	> 57	21	>2.7	> 22	> 22	1	>22
2	22	1.2	18	> 28	0.45	>62	>28

Abbreviations: SI, selective index; SOAT, sterol *O*-acyltransferase.
[a]Cytotoxicity of the compounds to CHO-K1 cells was measured by the MTT assay.[19]
[b]SI = IC_{50} for SOAT1/IC_{50} for SOAT2.

exclusively express SOAT1 and rat liver mainly expresses SOAT2. Therefore, it will be worth testing **3** in our assay system to make the SOAT selectivity clear.

A number of indoline alkaloid-containing diketopiperazines carry a prenyl moiety at C3. For example, amauromine (**2**), gypsetin (**3**), epiamauromine,[16] roquefortine C[17] and fructigenines A and B[18] were discovered as fungal metabolites, mainly produced by *Penicillium* and *Aspergillus*. In this study, we discovered **1** and **2** from a marine-derived actinomycete, *Nocardiopsis* sp. KM2-16. It will be worth comparing the biosynthetic genes from fungal and actinomycete strains.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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