THIOMARINOL, A NEW HYBRID ANTIMICROBIAL ANTIBIOTIC PRODUCED BY A MARINE BACTERIUM

FERMENTATION, ISOLATION, STRUCTURE, AND ANTIMICROBIAL ACTIVITY

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Thiomarinol, an antimicrobial antibiotic, was isolated from the culture broth of a marine bacterium, *Alteromonas rava* sp. nov. SANK 73390. Its structure was deduced as a hybrid composed of a pseudomonic acid analogue and holothin by NMR spectral analysis and chemical degradation. Antimicrobial activity against Gram-positive and Gram-negative bacteria of thiomarinol was stronger than both of pseudomonic acids and pyrrothine antibiotics.

Marine bacteria are very useful microbial resources for finding biologically active substances. For example, bromonitrin[1], marinactin[2], bisucaberin[3], isatin[4], alteramide A[5], *etc.* were isolated from marine bacteria.

In the course of our screening of new antibiotics, a marine bacterium, *Alteromonas rava* sp. nov. SANK 73390 was found to produce a new antibiotic, named thiomarinol, having strong inhibitory activity against Gram-positive and -negative bacteria. Structural characterization of thiomarinol revealed that it was a hybrid of two types of antibiotics; one was pseudomonic acids and the other was pyrrothines. Among pseudomonic acids A[6], B[7], C[8] and D[9], pseudomonic acid C was the most similar to thiomarinol. Pyrrothine antibiotics[10], such as holothin, pyrrothine (*N*-methylholothin), holomycin (acetylholothin), thiolutin (acetylpyrrothine) *etc.*, have a yellowish chromophore containing disulfide.

In this paper we report the fermentation, isolation, structural elucidation and antimicrobial activity of thiomarinol. The producing organism, *Alteromonas rava* sp. nov. SANK 73390 was isolated from seawater by us (K.K.). The taxonomy of this strain will be reported in detail elsewhere[11].

Materials and Methods

Preparation of Mupirocin (Pseudomonic Acid A)

BACTROBAN (15 g of ointment containing 2% of mupirocin, Beecham Lab.) was dissolved in water (150 ml) and applied on a Diaion HP-20 column (50 ml). After washing the column with water and 50% MeOH, mupirocin was eluted with 50% actone. The acetone fraction was concentrated *in vacuo* and lyophilized to give mupirocin (290 mg) as a viscous oily substance.

Fermentation

The strain SANK 73390 was cultured for 1 day at 23°C on marine agar 2216 (Difco). The resulting

culture was suspended in 3 ml of artificial seawater, 0.1 ml of the suspension was taken aseptically and inoculated into a 500-ml Erlenmeyer flask containing 100 ml of marine broth 2216 (Difco). The inoculated flask was incubated for 24 hours at 23°C with shaking at 200 rpm using a rotary shaker. Then, 15 ml aliquots of the culture were transferred to each of four 30-liter jar fermentors each containing 15 liters of the sterile medium described above. The fermentation was carried out at 23°C for 23 hours with aeration of 7.5 liters/minute and agitation rate of 100 rpm.

Isolation

Sixty liters of the culture broth was adjusted to pH 3.0 with HCl, followed by the addition of 60 liters of acetone. The mixture was stirred for 30 minutes and then filtered using Celite 545. The filtrate (110 liters) was extracted once with 60 liters of ethyl acetate, and then twice with 30 liters of ethyl acetate. The combined organic solvent layer was washed with 5% $NaHCO_3$ solution, then with saturated NaCl solution, dried over Na_2SO_4, and concentrated *in vacuo* to give 14 g of a yellowish oily substance. The oily residue was chromatographed on a silica gel column (200 g) in methylene chloride. The column was eluted by elevating the polarity of the developing solvent in the order: CH_2Cl_2 - EtOAc; EtOAc; EtOAc - MeOH. The eluate with EtOAc - MeOH, which contained thiomarinol, was concentrated *in vacuo*, then dissolved in 400 ml of 50% MeOH, and adsorbed on a Diaion HP-20 column (600 ml). After washing with 50% MeOH, thiomarinol was eluted with 90% MeOH, followed by concentration *in vacuo*, and lyophilization. A yellowish powder (1 g) thus obtained was finally purified by a Sephadex LH-20 column, developed with CH_2Cl_2 - EtOAc - MeOH (19 : 19 : 2 v/v). Fractions containing thiomarinol were collected and concentrated *in vacuo* to give thiomarinol (750 mg) as a yellow powder.

Antimicrobial Activity

The MICs were determined by a serial 2-fold plate dilution method with Mueller-Hinton agar (Baltimore Biological Laboratory) on which 1 loopful of 10^6 cfu/ml suspension of test bacteria was streaked, followed by incubation at 37°C for 20 hours.

Results

Physico-chemical Properties

Thiomarinol (**1**) was obtained as orange crystals, mp 106 ~ 110°C (d) by recrystallization from methanol. It was soluble in methanol, ethanol, ethyl acetate, chloroform, and DMSO, but insoluble in water. Based on elementary analysis [Found: C 54.59, H 7.18, N 4.10, S 9.41%. Calcd for $C_{30}H_{44}N_2O_9S_2 \cdot CH_3OH$: C 55.34, H 7.19, N 4.16, S 9.53%] and HR-FAB/MS [$M+H^+$: *m/z* Found, 641.2585; Calcd, 641.2567], the molecular formula was established to be $C_{30}H_{44}N_2O_9S_2$ (MW: 640). It showed $[\alpha]_D^{25}$ +4.3° (*c* 1.0, methanol). The UV spectrum showed maxima at 214 (ε: 26,000), 300 (3,500), and 387 (12,000) nm in methanol or acidic methanol, and at 206 (25,000), 306 (3,200) and 386 (9,600) nm in alkaline methanol. The IR spectrum showed the presence of an ester carbonyl at 1710 and an amide bond at 1680 and 1640 cm^{-1}.

Structural Elucidation

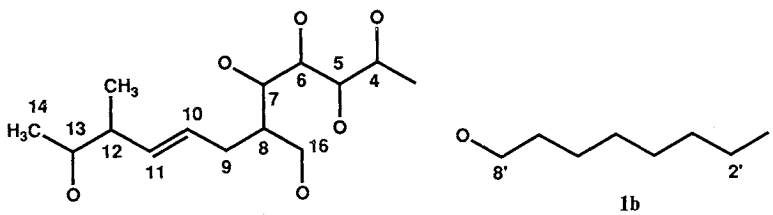
The ^1H and ^{13}C NMR spectral data of thiomarinol are summarized in Table 1. Thirty carbon signals consistent with the molecular formula were classified into 3 × CH_3, 9 × CH_2, 11 × CH and 7 × sp^2 quaternary carbons by DEPT spectra. Six exchangeable protons in addition to the 38 non-exchangeable protons were observed as expected above. Among those, four protons at 4.29, 4.44, 4.62 and 4.88 ppm were observed as doublets, suggesting the presence of secondary alcohols, while the other two protons at 9.78 and 10.67 ppm were observed as singlets. The partial structures **1a** and **1b** of thiomarinol in Scheme 1 were derived from the ^1H-^1H connectivities by DQF-COSY and HOHAHA spectra combined with the ^1H-^{13}C HMQC spectra. The oxygen functions attached to C-4, C-7 and C-13 were directly assigned as hydroxy

Table 1. ^{1}H and ^{13}C NMR chemical shifts of thiomarinol (DMSO).

Carbon No.	^{1}H (δ ppm, J=Hz)	^{13}C (δ ppm)	Carbon No.	^{1}H (δ ppm, J=Hz)	^{13}C (δ ppm)
Monic acid moiety			Fatty acid moiety		
1		166.1 (s)	1′		171.8 (s)
2	5.97 (s)	114.3 (d)	2′	2.33 (t, 7.3)	34.6 (t)
3		160.8 (s)	3′		24.9 (t)
4	4.18 (d, 7.3)	72.4 (d)	4′		28.3 (t)
5	3.54 (m, ov)	76.2 (d)	5′	1.30~1.55 (m)	28.4 (t)
6	3.63 (m, ov)	63.8 (d)	6′		25.3 (t)
7	3.73 (dd)	69.6 (d)	7′		28.2 (t)
8	1.62 (m)	42.2 (d)	8′	4.02 (t, 6.6)	63.0 (t)
9	2.02, 2.17 (m, ov)	31.8 (t)	Chromophore moiety		
10	5.33 (m)	127.7 (d)	1″		167.9 (s)
11	5.41 (m)	134.1 (d)	2″		115.3 (s)
12	2.09 (m, ov)	43.1 (d)	3″		133.9 (s)
13	3.51 (m, ov)	69.2 (d)	4″		133.6 (s)
14	0.95 (d, 5.9)	20.0 (q)	5″	7.04 (s),	110.3 (d)
15	2.03 (s)	15.7 (q)		9.78 (NH)	
16	3.33, 3.64 (m)	64.2 (t)		10.67 (NH)	
17	0.91 (d, 6.8)	15.7 (q)			
	4.29 (d, OH)				
	4.44 (d, OH)				
	4.62 (d, OH)				
	4.88 (d, OH)				

s: singlet, d: doublet, t: triplet, m: multiplet, dd: doublet of doublets, ov: overlap.

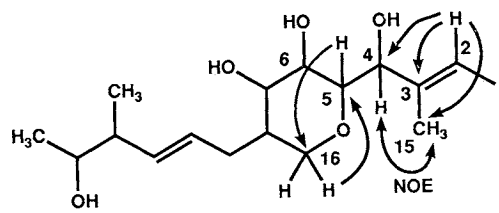
Scheme 1. Partial structures **1a** and **1b**.



1a **1b**

groups due to their coupling patterns which were simplified by addition of a trace amount of D_2O. Since the oxygen functions at C-5 and C-16 could be settled by the formation of a six membered ether ring evidenced by the ^{1}H-^{13}C long range couplings between 5-H and C-16 in the HMBC spectra and *vice versa*, the remaining hydroxy group was assigned to C-6 by elimination. Acetylation of **1** with acetic anhydride in pyridine gave a pentaacetate derivative (**2**), $C_{40}H_{54}N_2O_{14}S_2$ (MW: 850). Additional confirmation of the partial structure **1a** was given by the acetylation shifts of 4-H, 6-H, 7-H and 13-H. In the ^{1}H NMR spectrum of **2**, the corresponding protons were well separated and shifted to low field at 5.27, 4.93, 5.14 and 4.73 ppm, respectively.

Scheme 2. Partial structure **1c**.



1c

The linkage of C-2~C-3~C-4 could be established as shown in Scheme 2, where 15-CH_3 and 2-H were

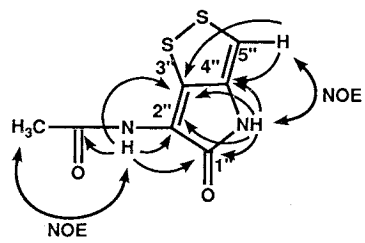
assembled based on their coupling to 4-H with $J=1.5$ and 1.9 Hz, respectively, detected by conventional decoupling experiments processed with a sine bell window to enhance the spectral resolution. These assignments were strengthened with the aid of additional information of the NOE between 4-H and 15-CH_3, and ^1H-^{13}C long range couplings between 2-H and C-3, C-4 and C-15. Thus, the partial structure **1a** was extended to **1c** (Scheme 2).

At this stage, one isolated proton at 7.04 ppm and two exchangable protons at 9.78 and 10.67 ppm were left with six sp^2 quaternary carbons as elements building the unknown part of thiomarinol. Hydrolysis of **1** with a 1:11 mixture of conc HCl-AcOH at 105°C for 20 hours yielded a chromophore moiety (**3**), $C_7H_6N_2O_2S_2$ (MW: 214). The UV spectrum of **3** showing maxima at 250, 310 and 390 nm was very similar to that of **1**. Since the NMR spectra of **3** were interpretable as the assembly of the signals belonging to the unknown part of **1**, the structure of **3** was to be elucidated first. Due to the absence of available ^1H-^1H connectivities, the structural analysis was carried our mainly based on the ^1H-^{13}C long range couplings, where the conventional LSPD experiments were employed to avoid losing the ^1H-^{13}C correlations which might not be detected by the HMBC spectra owing to the small coupling constants. The results were summarized in Scheme 3 along with some NOE data. From the above spectral data, the structure of this compound was deduced as **3**, being found to be identical with holomycin[12]. In **3**, the acetyl group was assumed to be induced from acetic acid of the reaction solvent (Scheme 3).

Finally, the complete structure of thiomarinol was deduced in the following manner. The NH proton at 9.78 ppm was coupled with carbonyl carbon (C-1′) at 171.8 ppm, and also with quaternary carbons at 115.3 (C-2″) and 133.9 (C-3″) ppm in the chromophore moiety, while C-1′ was also coupled with 2′-CH_2 (2.33 ppm) belonging to the partial structure **1b**. In addition, the NOE was observed between this NH proton and 2′-CH_2. Thus, the partial structure **1b** was connected to the chromophore part. The only remaining carbonyl carbon at 166.1 ppm, which was coupled to 8′-CH_2, was connected to the opposite end of the partial structure **1b** to form an ester linkaged which combined the partial structure **1c** to the rest of the molecule. Thus the structure **1d** was established as shown in Scheme 4.

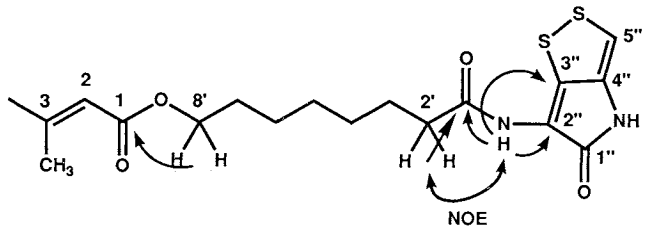
The structure derived mainly from the spectroscopic techniques was further confirmed by chemical degradation. We isolated a monic acid[8] derivative (**4**), its methyl ester (**5**), and acylchromophore (**6**,

Scheme 3. Structure of **3**.



3

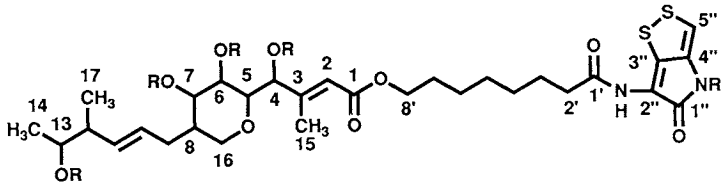
Scheme 4. Partial structure **1d**.



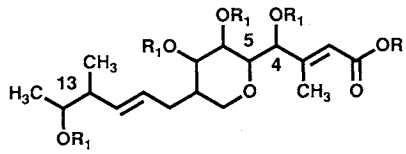
1d

$C_{13}H_{18}N_2O_3S_2$, MW: 314) by hydrolysis of **1** with NaOH in 90% MeOH solution. Reaction of **4** with diazomethane gave a methyl ester (**5**, $C_{18}H_{30}O_7$, MW: 358). Acetylation of **5** gave a tetraacetate (**7**, $C_{26}H_{38}O_{11}$, MW: 526). The ^1H and ^{13}C NMR spectra of **4** were very similar to those of monic acid C[8] except for C-4. Therefore, the structure of **4** was established as 4-hydroxymonic acid C. Moreover, hydrolysis with NaOH in 60% MeOH solution of a 6,7-acetonide of **1**, which was prepared by reaction with 2,2-dimethoxypropane in the presence of *p*-TsOH, yielded a methyl ester derivative (**8**) of the 6,7-acetonide of **4**, and subsequent acetylation with acetic anhydride in pyridine afforded its diacetate (**9**, $C_{25}H_{38}O_9$, MW: 482). In the ^1H NMR spectrum of **9**, 4-H and 13-H shifted downfield to 5.28 and 4.83 ppm and 6-H and 7-H did not change their chemical shifts on acetylation. This comparison in chemical shifts of 4-H, 6-H and 7-H between compounds **8** and **9** suggested the presence of a free hydroxy group at C-4 and an acetonide at C-6 and C-7, which was reported in pseudomonic acid[8]. Therefore, thiomarinol and the

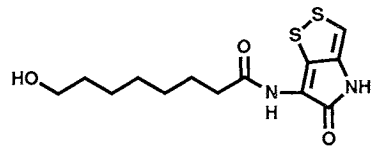
Scheme 5. Structures of **1** to **9**.



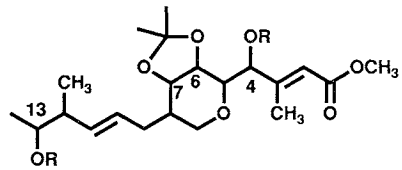
	R
1	H
2	$COCH_3$



	R	R_1
4	H	H
5	CH_3	H
7	CH_3	$COCH_3$



6



	R
8	H
9	$COCH_3$

acylchromophore as described above were firmly established as structures of **1** and **6**, respectively (Scheme 5). In addition, FAB-MS/MS analysis of thiomarinol ($M+H^+$: m/z 641) showed fragment ions at m/z 315 and m/z 172 corresponding to the acylchromophore and chromophore (holothin)[13].

The stereochemistries at C-5, C-6, C-7 and C-8 in the monic acid moiety of thiomarinol were shown to have close structural similarity to those of pseudomonic acid[6] in view of similar coupling constants [$J_{4,5}=2.9$, $J_{5,6}=10.0$, $J_{6,7}=3.1$, $J_{7,8}=3.1$, $J_{8,16a}=0$, $J_{8,16b}=2.4$ Hz in **2**] and acetonide formation. The stereochemistry of hydroxyl at C-4, which does not exist in pseudomonic acids, is now under investigation.

Table 2. Antimicrobial activities of thiomarinol (**1**) and mupirocin.

Test organism	MIC (μg/ml)	
	1	Mupirocin
Staphylococcus aureus 209P JC-1	<0.01	0.2
S. aureus 507 (MRSA)	<0.01	0.2
Enterococcus faecalis NCTC775	<0.01	0.2
Escherichia coli NIHJ JC-2	3.13	>100
Salmonella enteritidis G	3.13	100
Klebsiella pneumoniae IID685	0.78	50
Enterobacter cloacae 963	6.25	>100
Serratia marcescens IAM1184	25	>100
Proteus vulgaris IID874	0.39	100
Morganella morganii IFO3848	12.5	>100
Pseudomonas aeruginosa PA01	0.39	>100

This structure is a hybrid of two antibiotics, a pseudomonic acid analogue and holothin. Very few hybrid antibiotics like **1** have been obtained from natural sources.

Recently new pseudomonic acid derivatives were reported[14]. The structure of one of them was identical with that of thiomarinol except for the chromophore moiety.

Antimicrobial Properties

Thiomarinol showed excellent *in vitro* antimicrobial activity against Gram-positive and Gram-negative bacteria as compared to mupirocin (Table 2). Among the tested bacteria, it was especially active against *Staphylococcus aureus* including MRSA, and it also exhibited antimicrobial activity against Gram-negative bacteria. Its activity was remarkably potent as compared with those of both pseudomonic acids[15] and acylpyrrothines[10].

Discussion

Pseudomonic acids A (mupirocin)[6], B[7], C[8] and D[9] were isolated from *Pseudomonas fluorescens*, and new pseudomonic acid derivatives[14] were recently isolated from a marine bacterium, *Alteromonas* sp.. On the other hand, many pyrrothine antibiotics are produced by *Streptomyces*[10,13] and recently, xenorhabdins[16] have been isolated from *Xenorhabdus* spp.. We have isolated thiomarinol, a hybrid antibiotic of a pseudomonic acid analogue and holothin, from a marine bacterium, *Alteromonas rava* SANK 73390. To our knowledge, only a few compounds having antimicrobial activity have been isolated from marine bacteria[1,4], and hybrid antibiotics have not been reported. Pseudomonic acids are mainly active against staphyococci and streptococci, but are less active against other Gram-positive bacteria and most Gram-negative bacteria[15]. Pyrrothine antibiotics are active against both Gram-positive and Gram-negative bactera[10,16], their activities being dependent on the length of their acyl side chain. Pyrrothine antibiotics having a C4 or longer acyl chain do not show high activity against Gram-negative bacteria[16]. The antimicrobial spectrum of thiomarinol showed characteristics of both pseudomonic acids and short chain pyrrothine antibiotics, its activity being stronger than either of the two types of antibiotics. The mode of action of pseudomonic acid A and thiolutin has been reported. The former strongly inhibited isoleucyl-transfer RNA synthetase in bacteria, but not so strongly the mammalian enzyme[17]. On the other hand, the latter inhibited RNA synthesis[18,19]. Thiomarinol may inhibit both targets Ile-tRNA synthetase and RNA synthesis, as it exhibits broad as well as potent *in vitro* antimicrobial activity.

Experimental

General

NMR spectra were measured on JEOL JNM-GX270, JNM-GX400 or Bruker AMX360 spectrometer. Chemical shifts are given in ppm using TMS as an internal standard. UV spectra were measured on a Shimadzu UV-265FW spectrophotometer. FAB-MS and MS/MS spectra were obtained on a JEOL JMS-HX100 triple analyser tandem mass spectrometer. The samples were dissolved in a 3-nitrobenzyl alcohol matrix on the FAB probe tip and bombarded with 6 keV xenon atoms. FAB MS/MS spectra were obtained by collisional activation. The parent ions were collided with argon gas in the collision cell.

Acetylation of **1**

1 (104 mg) was treated with acetic anhydride (0.5 ml) in pyridine (3 ml) at room temperature overnight. The reaction mixture was evaporated to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2-EtOAc (1:1 v/v, 5 ml) and applied to a Sephadex LH-20 column (200 ml, Pharmacia) using CH_2Cl_2-EtOAc (1:1 v/v) as eluent to give 120 mg of pentaacetate (**2**) as an amorphous solid. FAB-MS: *m/z* 851 ($M+H^+$). ^1H NMR ($CDCl_3$): 0.99 (3H, d, 6.8), 1.13 (3H, d, 6.4), 1.36 (6H, m), 1.6~1.8 (5H, m), 1.97 (3H, s), 2.01 (3H, s), 2.12 (3H, s), 2.16 (3H, s), 2.20 (3H, s), 2.25~2.4 (5H, m), 2.65 (3H, s), 3.57 (1H, d, 11.7), 3.74 (1H, dd, 2.4, 11.7), 3.97 (1H, dd, 2.9, 10.0), 4.08 (2H, t, 6.9), 4.73 (1H, m), 4.93 (1H, dd, 3.1, 10.0), 5.14 (1H, t, 3.1), 5.27 (1H, d, 2.9), 5.43 (2H, m), 5.78 (1H, s), 7.36 (1H, s).

Hydrolysis of **1** with HCl

1 (50 mg) was hydrolyzed with conc HCl-AcOH (1:11 v/v, 5 ml) in a sealed tube at 105°C for 20 hours. The reaction mixture was evaporated to dryness. The residue was chromatographed on a Sephadex LH-20 column (100 ml) using MeOH as eluent to give 8 mg of **3**. EI-MS: *m/z* 214 (M^+), 172 (base peak). ^1H NMR (CD_3OD): 2.10 (3H, s), 7.05 (1H, s), ^{13}C NMR (DMSO-d_6): 22.2 (q), 110.4 (d), 115.3 (s), 133.6 (s), 133.8 (s), 167.8 (s), 168.7 (s).

Alkaline Hydrolysis of **1**

1 (150 mg) was dissolved in MeOH (150 ml) and added with 1 N NaOH (15 ml). After 15 hours at room temperature the reaction mixture was neutralized with 1 N HCl and evaporated. The residue was chromatographed on a Diaion CHP-20P column (100 ml, Mitsubishi Chemical Industrial). The column was developed with 20~90% MeOH by stepwise. 30% MeOH fraction was rechromatographed on a Sephadex LH-20 column (100 ml, eluent: CH_2Cl_2-EtOAc-MeOH; 45:45:10 v/v) to give 5 mg of **4** as an amorphous solid. 70% MeOH fraction containing **5** and 80% MeOH fraction containing **6** were purified by preparative HPLC (Senshu-pak, ODS, H-4251, 10×250 mm, 30% CH_3CN, 5 ml/minute), respectively. 36 mg of **5** and 44 mg of **6** were obtained as an amorphous solid and yellow powder, respectively.

4: FAB-MS: *m/z* 345 ($M+H^+$). ^1H NMR (CD_3OD): 0.98 (3H, d, 6.8), 1.09 (3H, d, 6.3), 1.75 (1H, m), 2.04 (3H, s), 2.1~2.3 (3H, m), 3.5~3.9 (6H, m), 4.27 (1H, s), 5.47 (2H, m), 6.0 (1H, s).

5: FAB-MS: *m/z* 359 ($M+H^+$). ^1H NMR ($CDCl_3$): 1.00 (3H, d, 6.8), 1.16 (3H, d, 6.2), 1.86 (1H, m), 2.16 (3H, s), 2.0~2.2 (3H, m), 3.55 (2H, m), 3.68 (2H, m), 3.70 (3H, s), 3.87 (2H, m), 3.98 (1H, s), 4.30 (1H, s), 5.45 (2H, m), 6.04 (1H, s).

6: EI-MS: *m/z* 314 (M^+), 172 (base peak). ^1H NMR ($CDCl_3$): 1.37 (6H, br s), 1.58 (2H, br s), 1.70 (2H, m), 2.35 (2H, t, 7.3), 3.64 (2H, t, 6.3), 6.7 (1H, s), 7.62 (1H, s, NH), 8.27 (1H, s, NH).

Preparation of **5** from **4**

4 (15 mg) dissolved in a mixture of MeOH (1 ml) and EtOAc (2 ml) was added with diazomethane in ether solution under cooling. After 30 minutes the reaction mixture was evaporated to dryness. The product was identical with **5** by HPLC analysis and NMR spectra data.

Acetylation of **5**

5 (15 mg) was acetylated as described above for **1**. The crude acetate was purified by preparative HPLC (Senshu-pak, ODS, H-4251, 10×250 mm, 30% CH_3CN, 5 ml/minute). **7** (13.4 mg) was obtained as an amorphous solid. EI-MS: *m/z* 526 (M^+). ^1H NMR ($CDCl_3$): 1.00 (3H, d, 6.7), 1.14 (3H, d, 6.3),

1.79 (1H, m), 1.97 (3H, s), 2.01 (3H, s), 2.12 (3H, s), 2.15 (3H, s), 2.21 (3H, s), 2.3~2.4 (3H, m), 3.70 (3H, s), 3.65~3.75 (2H, m), 3.98 (1H, dd, 2.6, 9.8), 4.83 (1H, m), 5.02 (1H, dd, 3.0, 10.0), 5.27 (1H, t, 3.0), 5.31 (1H, s), 5.4~5.5 (2H, m), 5.84 (1H, s).

Preparation of Acetonide of **1**

1 (600 mg) dissolved in 2,2-dimethoxypropane (60 ml) was added with *p*-toluenesulfonic acid (120 mg). After 1 hour at room temperature, water (200 ml) was added followed by extraction with EtOAc (500 ml). The extract was washed with saturated aqueous NaCl and dried over anhydrous $MgSO_4$ - Na_2SO_4 (1 : 1 w/w). The solvent was evaporated and the product was purified by a Sephadex LH-20 column (300 ml) using CH_2Cl_2 - EtOAc (1 : 1) as eluent to give 500 mg of an acetonide of **1** as an amorphous solid. FAB-MS: *m/z* 681 ($M+H^+$). ^1H NMR ($CDCl_3$): 1.36 (3H, s, acetonide-Me), 1.50 (3H, s, acetonide-Me).

Preparation of **8**

Acetonide (100 mg) of **1** dissolved in 60% aqueous MeOH (20 ml) was added with 1 N NaOH (2 ml). After stirring at room temperature for 3 hours, the reaction mixture was neutralized with 1 N HCl and applied on a Diaion HP-20 column (70 ml). The column was washed with 30% MeOH and eluted with 90% MeOH. The fraction containing **8** was evaporated and injected to a preparative HPLC (Senshu-pak, ODS, H-4251, 10×250 mm, 55% CH_3CN, 5 ml/minute). **8** (35 mg) was obtained as an amorphous solid. FAB-MS: *m/z* 399 ($M+H^+$). ^1H NMR ($CDCl_3$): 1.01 (3H, d, 6.9), 1.16 (3H, d, 6.1), 1.36 (3H, s), 1.50 (3H, s), 2.03 (1H, m), 2.15 (3H, s), 2.1~2.3 (3H, m), 3.40 (1H, dd, 2.5, 8.3), 3.58 (1H, m), 3.70 (3H, s), 3.6~3.7 (2H, m), 4.09 (1H, s), 4.17 (2H, m), 5.48, (2H, m), 6.01 (1H, s).

Acetylation of **8**

8 (18 mg) was acetylated as described above for **1**. After preparative HPLC (Senshu-pak, ODS, H-4251, 10×250 mm, 70% CH_3CN, 5 ml/minute), 14.8 mg of product (**9**) were obtained. EI-MS: *m/z* 482 (M^+). ^1H NMR ($CDCl_3$): 0.99 (3H, d, 6.9), 1.14 (3H, d, 6.2), 1.34 (3H, s), 1.50 (3H, s), 1.97 (1H, m), 2.02 (3H, s), 2.17 (3H, s), 2.18 (3H, s), 2.2~2.3 (3H, m), 3.49 (1H, dd, 2.9, 9.2), 3.69 (3H, s), 3.6~3.7 (2H, m), 3.90 (1H, dd, 5.0, 9.2), 4.15 (1H, dd, 2.1, 5.0), 4.83 (1H, m), 5.28 (1H, s), 5.4~5.5 (2H, m), 5.86 (1H, s).

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