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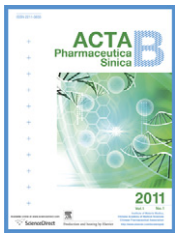

ORIGINAL ARTICLE

New cyslabdans B and C, potentiators of imipenem activity against methicillin-resistant *Staphylococcus aureus* produced by *Streptomyces* sp. K04-0144

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KEY WORDS

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Abstract From a further purification study, two new congeners designated cyslabdans B (**1**) and C (**2**) were isolated along with previously reported cyslabdan (cyslabdan A (**3**) in this study) from the culture broth of *Streptomyces* sp. K04-0144. The structure was elucidated by various spectroscopy including NMR, revealing that **1** and **2** was 18-hydroxy and 1′-methoxy cyslabdan, respectively. Compounds **1** and **2** were found to potentiate imipenem activity against methicillin-resistant *Staphylococcus aureus* by 123 fold and 533 fold, respectively. Comparison with the activity of compound **3** indicated that the introduction of a hydrophilic group at the dimethyl moiety of the decalin ring was unfavorable for its activity.

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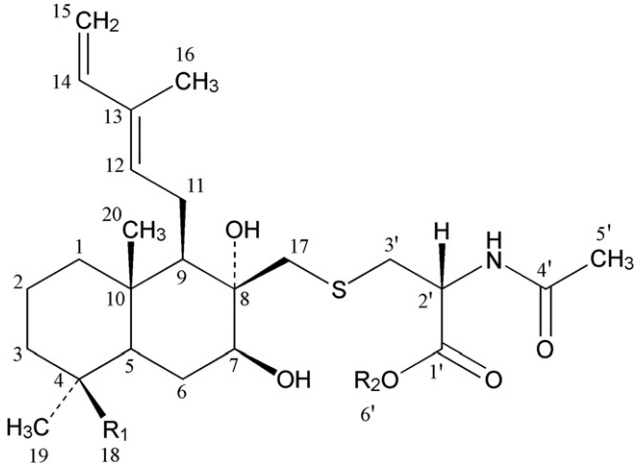
1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), a major and widespread pathogen in hospitals, has developed resistance to many other antibiotics[1]. Moreover, MRSA getting resistant to the last-resort antibiotic, vancomycin, has been reported[2,3]. These facts suggest that MRSA would acquire more resistance to vancomycin in the near future. It is therefore increasingly important and necessary to find new antimicrobial agents and to devise new measures that are effective against MRSA infection.

Based on the new concept of "anti-infective drugs" developed by Omura[4], a screening system was established to search for microbial potentiators of imipenem activity against MRSA[5], and new cyslabdan (cyslabdan A (**3**) in this study) was previously discovered from the culture broth of *Streptomyces* sp. K04-0144[6,7]. From further precise analysis of the culture broth, new congeners cyslabdans B (**1**) and C (**2**) were recently discovered (Fig. 1). In this study, the isolation, structural elucidation, and biological properties of **1** and **2** are described.



	R_1	R_2
Compound **1**	$-CH_2OH$	-H
Compound **2**	$-CH_3$	$-CH_3$
Compound **3**	$-CH_3$	-H

Figure 1 Structures of compounds.

2. Results and discussion

2.1. Structural elucidation of *1* and *2*

The physico-chemical properties of **1** and **2** are summarized in Table 1. Compounds **1** and **2** showed UV absorption at 233 nm. The IR absorption around 3400 cm^{-1} and 1740 cm^{-1} suggested the presence of a hydroxyl residue and/or an amide residue, and a carbonyl residue in their structures, respectively. These physico-chemical properties are very similar to that of **3**.

The structure of **1** was elucidated from various spectral data including NMR experiments. The molecular formula of **1** was determined as $C_{25}H_{41}NO_6S$ on the basis of HR-FAB-MS measurements, indicating that **1** was bigger with one oxygen atom than **3**. The ^{13}C NMR spectrum showed 25 resolved peaks, which were classified into four methyl carbons, nine methylene carbons, four sp^3 methine carbons, two sp^2 methine carbons, three sp^3 quaternary carbons, and three sp^2 quaternary carbons by DEPT spectral analysis. The ^1H NMR spectrum (in CD_3OD) displayed 36 proton signals. The connectivity of the proton and carbon atoms was established from the HMQC spectrum (Table 2). A comparison of the ^1H and ^{13}C NMR spectra of **1** and **3** indicated that they both possessed a labdanic skeleton. However, a methyl proton (H_3-18, δ 0.91) in **3** was absent in **1**, and an oxymethylene (δ 3.33, 3.76) was present in **1** which was absent in cyslabdan A, indicating that **1** is 18-hydroxy cyslabdan A. In fact, cross peaks were observed from H_2-18 (δ 3.33, 3.76) to C-3 (δ 36.5), C-4 (δ 39.6), C-5 (δ 54.4), and C-19 (δ 27.7) in the ^{13}C–^1H HMBC experiments (Fig. 2). The structure satisfied the unsaturation number, UV spectra, and the molecular formula. Taken together, the planar structure of **1** was elucidated as shown in Fig. 1.

The structure of **2** was similarly elucidated. The molecular formula of **2** is $C_{26}H_{43}NO_5S$, indicating that **2** is bigger than **3** with CH_2. Its ^1H NMR spectrum was almost identical to that

Table 1 Physico-chemical properties of **1** and **2**.

Items	Compound **1**	Compound **2**
Apperance	White powder	White powder
Molecular weight	483	481
Molecular formula	$C_{25}H_{41}NO_6S$	$C_{26}H_{43}NO_5S$
HR-FAB-MS (m/z)		
Found	484.2730 $[M+H]^+$	482.2934 $[M+H]^+$
Calcd.	484.2728	482.2927
$[\alpha]_D^{27}$	+22.2 (c 0.1, CH_3OH)	+11.8 (c 0.1, CH_3OH)
UV $\lambda_{max}^{CH_3OH}$ nm($\log\varepsilon$)	233 (4.30)	233 (4.12)
IR ν_{max}^{KBr} cm^{-1}	3427, 2940, 2883, 1741, 1633, 1396, 1132	3419, 2931, 2873, 1738, 1657, 1381, 1135
Solubility		
Soluble	H_2O, CH_3CN, CH_3OH, EtOAc	H_2O, CH_3CN, CH_3OH, EtOAc
Insoluble	$CHCl_3$, n-Hexane	$CHCl_3$, n-Hexane

Table 2 ^{1}H and ^{13}C NMR chemical shifts of **1** and **2** in CD_3OD (^{1}H NMR, 400 MHz).

Compound **1**			Compound **2**		
Position	δ_C	δ_H	Position	δ_C	δ_H
1	41.1	0.92 (1H, m) 1.71 (1H, m)	1	41.1	0.87 (1H, m) 1.67 (1H, m)
2	19.0	1.39 (1H, m) 1.59 (1H, m)	2	19.3	1.39 (1H, m) 1.62 (1H, m)
3	36.5	0.93 (1H, m) 1.84 (1H, m)	3	43.0	1.18 (1H, m) 1.42 (1H, m)
4	39.6		4	34.1	
5	54.4	1.02 (1H, m)	5	53.9	0.87 (1H, m)
6	27.8	1.62 (1H, m) 1.81 (1H, m)	6	28.0	1.59 (1H, m) 1.70 (1H, m)
7	73.1	3.67 (1H, dd, J=4.5, 11.0)	7	73.0	3.69 (1H, dd, J=4.5, 11.0)
8	78.6		8	78.6	
9	55.2	1.34 (1H, m)	9	55.1	1.31 (1H, m)
10	39.8		10	39.9	
11	24.6	2.08 (1H, m) 2.44 (1H, dt, J=6.5, 17.0)	11	24.3	2.07 (1H, m) 2.45 (1H, dt, J=6.5, 17.0)
12	137.4	5.50 (1H, t, J=6.5)	12	137.5	5.49 (1H, t, J=6.5)
13	133.5		13	133.5	
14	143.0	6.35 (1H, dd, J=10.0, 18.0)	14	143.1	6.35 (1H, dd, J=10.0, 18.0)
15	110.4	4.86 (1H, d, J=10.0) 5.05 (1H, d, J=18.0)	15	110.3	4.86 (1H, d, J=10.0) 5.04 (1H, d, J=18.0)
16	12.1	1.76 (3H, br-s)	16	12.1	1.76 (3H, br-s)
17	39.0	2.59 (1H, d, J=12.5) 2.77 (1H, d, J=12.5)	17	39.0	2.57 (1H, d, J=12.5) 2.76 (1H, d, J=12.5)
18	65.0	3.33 (1H, d, J=10.0) 3.76 (1H, d, J=10.0)	18	34.0	0.92 (3H, s)
19	27.7	0.98 (3H, s)	19	22.3	0.86 (3H, s)
20	16.7	0.96 (3H, s)	20	15.9	1.00 (3H, s)
1′	173.9		1′	172.7	
2′	53.8	4.54 (1H, dd, J=4.5, 8.0)	2′	53.9	4.55 (1H, dd, J=4.5, 8.0)
3′	36.4	2.80 (1H, dd, J=8.0, 13.0) 3.02 (1H, dd, J=4.5, 13.0)	3′	36.2	2.77 (1H, dd, J=8.0, 13.0) 2.97 (1H, dd, J=4.5, 13.0)
4′	173.2		4′	173.2	
5′	22.4	1.98 (3H, s)	5′	22.3	1.98 (3H, s)
			6′	52.9	3.72 (3H, s)

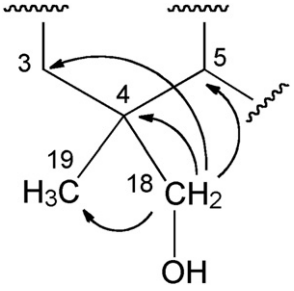


Figure 2 Key HMBC of compound **1**.

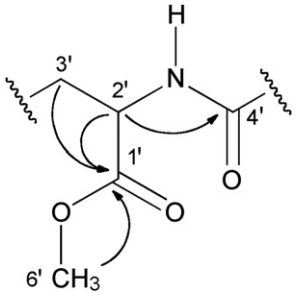


Figure 3 Key HMBC of compound **2**.

of **3** except for the presence of a methoxy proton signal (δ 3.72) in **2**, indicating that one of the hydroxyl groups of **3** was methylated in **2**. The position of the methylation was confirmed by ^{13}C–^{1}H HMBC experiments (Fig. 3): cross peaks were observed from H_3-6′ (δ 3.72), H-2′ (δ 4.55), and H-3′ (δ 2.77, 2.97) to C-1′ (δ 172.7). Thus, compound **2** was elucidated to be 1′-methoxy cyslabdan A (Fig. 1).

Regarding the stereochemistry of **1** and **2**, the data from the ROESY experiments were consistent with those of **3**[6]. The relative configuration of the labdane skeleton in **1** and **2** was shown to be identical to that of **3**; i.e., (12*E*)-labda-12, 14-dien-7β,8α−diol (Fig. 1). The stereochemistry of *N*-acetylcysteine was also defined as *L*[6]. This is reasonable because they share the same biosynthetic pathway.

2.2. Potentiation of imipenem activity by **1** and **2**

The potentiation of imipenem activity against MRSA was investigated by the liquid microdilution method[5,8]. The concentrations of **1** and **2** were set to 10 μg/mL, which had no effect on the growth of MRSA. Compounds **1** and **2** both demonstrated potentiating activity, reducing the MIC value of imipenem from 16 to 0.13 (123-fold potentiation) and 0.03 μg/mL (533-fold potentiation), respectively. Taking the activity of **3** (1070-fold potentiation) into consideration, the introduction of a hydrophilic group at the dimethyl moiety of the decalin ring might have an unfavorable effect on its activity. Further studies of the structure-activity relationship of cyslabdans and their mechanisms of action of **3** are ongoing.

3. Experimental

3.1. Materials

The following materials were purchased from commercial sources: Mueller-Hinton broth (MHB, Difco) and imipenem (Banyu Pharmaceutical).

3.2. General procedures

Streptomyces sp. K04-0144 was used for production of **1** and **2**[6,7]. UV spectra were recorded on a spectrophotometer (8453 model, Agilent). IR spectra were recorded on a Fourier transform infrared spectrometer (FT-710, Horiba). Optical rotations were measured with a digital polarimeter (DIP-370, JASCO). FAB-MS spectra and HR-FAB-MS spectra were recorded on a mass spectrometer (JMS-AX505HA, JEOL). The various NMR spectra were collected with a spectrometer (XL-400, Varian).

3.3. Isolation of compounds **1** and **2**

Five-day old fermentation broth (110 L) of *Streptomyces* sp. K04-0144 was centrifuged to separate the mycelia and supernatant. The supernatant was then applied to a 2.5 L Diaion HP-20 column (Mitsubishi Chemical Co.). After washing with water, the desired substances were eluted with MeOH and concentrated *in vacuo* to dryness to produce brown oily materials (15.8 g), which were then dissolved in MeOH and applied to an ODS column (40 g, Senshu Scientific Co.). The substances were eluted stepwise with 20%, 40%, 60%, 80%, and 100% MeOH (each 1.5 L). The 80% MeOH fraction containing **1**–**3** was concentrated *in vacuo* to dryness to yield brown substances (1.7 g), which were then purified using HPLC (column: PEGASIL ODS, 20 mm × 250 mm (Senshu Scientific Co.); solvent: 55% CH_3CN containing 0.05% TFA; detection: UV at 230 nm; flow: 6.0 mL/min). Under these conditions, the fractions of the peaks eluted at retention times of 10.0 and 58.8 min were repeatedly collected and were concentrated to dryness to give brown oils A (930 mg) and B (46.5 mg), respectively. Brown oil A was rechromatographed using HPLC (under the same conditions as described above except that the solvent was 35% CH_3CN containing 0.05% TFA). The peak with a retention time of 43.0 min was collected and concentrated to give pure **1** as a white powder (18.7 mg). Brown oil B was also rechromatographed using HPLC (column: C30-UG5, 20 mm × 250 mm (Nomura Scientific); solvent: 70% CH_3CN containing 0.05% TFA; detection: UV at 210 nm; flow: 6.0 mL/min), and the fractions of the peaks eluted at 30.5 and 49.6 min were collected to give pure **2** (2.5 mg) and **3** (31.2 mg) as white powders.

3.4. Assay for potentiating activity of imipenem against MRSA

Potentiating activity of imipenem against MRSA in combination with cyslabdans was investigated by the liquid microdilution method as our previously reported[5,8]. Briefly, MHB (85 μL) was added to each well of a 96-well microtiter plate (Corning), sample dissolved in MeOH (5.0 μL) was added to the final concentration of 10 μg/mL, whose concentration has no effect on growth of MRSA. Then, imipenem (5.0 μL) serially diluted dissolved in with distilled water (5.0 μL) were added to each well to make the final concentrations of 0.0005 to 512 μg/mL. After MRSA (5.0 μL) was added at the concentration of 1.0×10^7 CFU/mL, microtiter plates were incubated at 37 °C for 20 h. MIC values were determined in the presence or absence of sample.

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