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Cyslabdan, a New Potentiator of Imipenem Activity against Methicillin-resistant *Staphylococcus aureus*, Produced by *Streptomyces* sp. K04-0144

I. Taxonomy, Fermentation, Isolation and Structural Elucidation

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Abstract Cyslabdan, a new potentiator of imipenem activity against methicillin-resistant *Staphylococcus aureus*, was isolated from the culture broth of *Streptomyces* sp. K04-0144 by Diaion HP-20 and ODS column chromatographies and preparative HPLC. The structure of cyslabdan was elucidated by spectroscopic analyses including NMR. The compound has a labdane-type diterpene skeleton connecting with an *N*-acetylcysteine *via* thioether linkage.

Keywords cyslabdan, imipenem potentiator, methicillin-resistant *Staphylococcus aureus*, MRSA, structural elucidation

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is known as a major nosocomial pathogen which has also 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 Ōmura [4], a screening system was established to search for microbial potentiators of imipenem activity against MRSA, and new stemphones were recently discovered from the culture broth of *Aspergillus* sp. FKI-2136 [5]. During our continuous screening program, an actinomycete strain K04-0144 was found to produce a strong potentiator of imipenem activity against MRSA. Activity-guided purification lead to the discovery of a novel compound designated cyslabdan (Fig. 1). From the structural elucidation, the fundamental

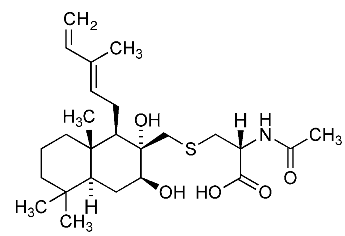

Fig. 1 Structure of cyslabdan.

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skeleton of cyslabdan is similar to that of totarol previously presented as a β-lactam potentiator [6]. As described in detail in the accompanying paper [7], cyslabdan showed almost no anti-MRSA activity by itself (MIC, 64 μg/ml), but the compound (10 μg/ml, no activity against MRSA) enhanced the imipenem activity against MRSA over 1,000 folds, indicating that clinically resistant MRSA strains become clinically susceptible in the presence of cyslabdan.

In this study, the taxonomy of the producing strain, fermentation, isolation, and structural elucidation of cyslabdan are described.

Materials and Methods

General Experimental Procedures

Strain K04-0144 was isolated from a soil sample collected at Ishigakijima Island, Okinawa, Japan, and was used for production of cyslabdan. For determination of the amount of cyslabdan in culture broths, the samples dissolved in MeOH were analyzed by HP1100 system (Hewlett-Packard) under the following conditions: column, Symmetry (2.1×150 mm, Waters Inc.); flow rate, 0.2 ml/minute; mobile phase, a 20-minute linear gradient from 5.0% CH_3CN/0.05% H_3PO_4 to 100% CH_3CN/0.05% H_3PO_4; detection, UV at 210 nm. The peak of cyslabdan was eluted with a retention time of 17.4 minutes.

UV spectra were recorded on a spectrophotometer (DU640, Beckman). 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).

Taxonomic Studies

The International *Streptomyces* Project (ISP) media recommended by Shirling and Gottlieb [8] and media recommended by Waksman [9] were used to investigate the cultural and physiological characteristics. Cultures were routinely observed after the incubation for two weeks at 27°C. Color names and hue numbers were determined according to the Color Harmony Manual [10]. The utilization of carbon sources was tested by growth on Pridham and Gottlieb's medium containing 1.0% carbon at 27°C [11]. The morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL). The type of diaminopimelic acid (DAP) isomers was determined by the method of Becker *et al.* [12]. Menaquinones were extracted and purified by the method of Collins *et al.* [13], then were analyzed by HPLC (Gulliver System, JASCO) equipped with a Capcell Pak C18 column (4.6×250 mm, Shiseido) [15].

Absolute Sterochemistry of Amino Acid Residue

To detemine the configuration of *N*-acetylcysteine, cyslabdan was cleaved to labdane skeleton and *N*-acetylalanine, by hydrogenation with Raney nickel as a catalyst [16~19]. The absolute configuration of *N*-acetylalanine was determined by direct comparison of the retention times of the authentic *N*-acetyl-L-alanine and *N*-acetyl-D-alanine by HPLC (column, Sumichiral OA-5000 (4.6×250 mm); solvent, 2.0 mM $CuSO_4$ in 5.0% CH_3CN; flow rate, 1.0 ml/minute; detection, 254 nm).

Results

Taxonomy of the Producing Strain

The vegetative mycelia grew abundantly on yeast extract-malt extract agar, inorganic salts-starch agar and others, and did not show fragmentation into coccoid forms or bacillary elements. The color of vegetative mycelia showed pale yellow. The aerial mycelia grew abundantly on yeast extract-malt extract and glycerol-asparagine agar and the aerial mass color showed white to gray. From observation of the scanning electron micrograph of the strain (Fig. 2), the spore chains were spiral and each had more than 20 spores per chain. The spores were cylindrical in shape, 1.0×1.0~1.2 μm in size and had a smooth surface. Whirls, sclerotic granules, sporangia and flagellate spores were not observed. Growth temperature range was 12°C to 42°C. D-Glucose, D-mannitol, melibiose and L-rhamnose were used well, and L-arabinose, D-fructose, *myo*-inositol, raffinose, sucrose and D-xylose were used slightly as sole carbon. Melanoid pigment and other soluble pigments were not produced. Hydrolysis of starch was positive, and

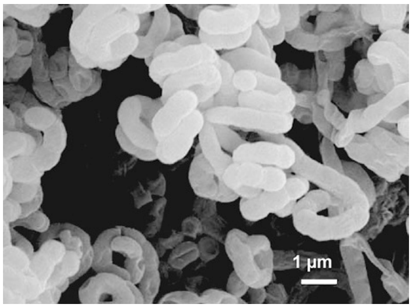

Fig. 2 Scanning electron micrograph of *Streptomyces* sp. K04-0144.

Bar represents 1 μm.

coagulation and peptonization of milk were positive.

The isomer of DAP in whole-cell hydrolysates of strain K04-0144 was determined to be LL-form. Major menaquinones were MK-9 (H_6) and MK-9 (H_8).

Based on the taxonomic properties described above, strain K04-0144 (NITE BP-107) is considered to belong to the genus *Streptomyces* [14].

Fermentation

A slant culture of the strain K04-0144 grown on the slant medium (starch 1.0%, N-Z-amine 0.3%, yeast extract 0.02%, meat extract 0.1%, $CaCO_3$ 0.3%, agar 1.2%, pH 7.0) was used to inoculate five 500-ml Erlenmeyer flasks each containing 100 ml of the seed medium (starch 2.4%, glucose 0.1%, peptone 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4%, pH 6.0). The flasks were shaken on a rotary shaker at 27°C for 3 days. The seed culture (500 ml) was transferred into a 90-liter jar fermenter containing 50 liters of the production medium (glucose 0.5%, corn steep powder 0.5%, oatmeal 1.0%, Pharmamedia 1.0%, K_2HPO_4 0.5%, $MgSO_4 \cdot 7H_2O$ 0.5%, trace salts solution (containing $FeSO_4 \cdot 7H_2O$ 0.1%, $MnCl_2 \cdot 4H_2O$ 0.1%, $ZnSO_4 \cdot 7H_2O$ 0.1% $CuSO_4 \cdot 5H_2O$ 0.1%, and $CoCl_2 \cdot 6H_2O$ 0.1%) 0.1%, pH 7.0). The fermentation was carried out at 27°C for 6 days with an aeration of 5.0 liters/minute and an agitation of 200 rpm. A typical time course of the fermentation is shown in Fig. 3. The production of cyslabdan was observed at day 1 after inoculation, and reached a maximum (2.3 μg/ml) at day 5.

Isolation

The 6-day old culture broth (50 liters) was centrifuged to separate the mycelium and supernatant. The supernatant was applied on a Diaion HP-20 column (100×200 mm, Mitsubishi Chemical. Co.). After washing with H_2O, active principles were eluted with MeOH and concentrated *in vacuo* to dryness to yield brown oily materials (32 g). The materials were dissolved in H_2O, and undesired materials were removed by extraction with EtOAc. The H_2O layer was concentrated to yield brown materials (27 g). A half of the materials were dissolved in a small volume of H_2O and applied on an ODS column (50×280 mm). Materials were eluted stepwise with 40, 60, 80 and 100% MeOH (4.0 liters each). The 80% MeOH fraction showing the activity was concentrated to give brown materials (134 mg). The materials were finally purified by HPLC (column, PEGASIL ODS (Senshu Scientific. Co.) 20×250 mm; mobile phase, 60% CH_3CN containing 0.05% H_3PO_4; flow rate, 8.0 ml/minute; detection, UV at 210 nm). Under these conditions, cyslabdan was eluted as a peak with a retention time of 32 minutes. The fraction was applied on a Diaion HP-20 column to remove H_3PO_4. After washing with H_2O, cyslabdan was eluted with MeOH and concentrated to yield a white powder (17 mg).

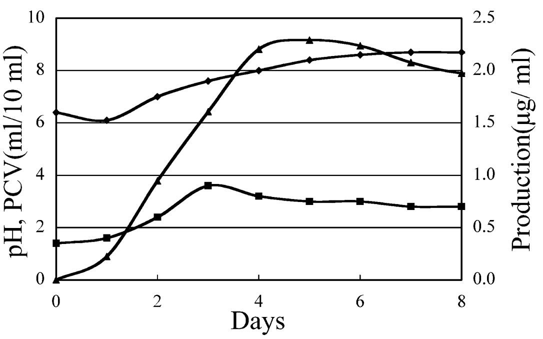

Fig. 3 A time course of cyslabdan production by *Streptomyces* sp. K04-0144.

At the indicated times, whole culture broth (10 ml) was removed from the flask, and centrifuged at 3,000 rpm. The pH of the supernatant and packed cell volume (PCV, ml) were measured. The amount of cyslabdan was determined by HPLC as described in Materials and Methods. ◆; pH, ■; PCV, ▲; production.

Structural Elucidation

Physico-chemical properties of cyslabdan are summarized in Table 1. The IR absorption at 3427 cm^{-1} suggested the presence of a hydroxy residue in the structure. The molecular formula was determined as $C_{25}H_{41}NO_5S$ on the basis of HRFAB-MS measurement. The ^{13}C-NMR spectrum showed 25 resolved peaks, which are classified into five methyl carbons, eight methylene carbons, four sp^3

Table 1 Physico-chemical properties of cyslabdan

Appearance	: White powder
Melting point	: 138°C
Molecular formula	: $C_{25}H_{41}NO_5S$
Molecular weight	: 467
Pos. FAB-MS (*m/z*)	: 512 $[M-H+2Na]^+$
HR Pos. FAB-MS (*m/z*)	
Obsd.	: 512.2416
Calcd.	: 512.2423 ($C_{25}H_{40}NO_5SNa_2$)
$[\alpha]_D^{25}$	: +26.8 (*c* 0.1, CH_3OH)
UV $\lambda_{max}^{CH_3OH}$ nm (log ε)	: 232 (4.19)
IR ν_{max}^{KBr} cm^{-1}	: 3427, 2927, 2864, 1641, 1604, 1398, 1124
Solubility	
Soluble	: H_2O, CH_3CN, CH_3OH
Insoluble	: EtOAc, $CHCl_3$, *n*-Hexane
Color reaction	
Positive	: Molybdatophosphoric acid
Negative	: H_2SO_4, ninhydrin reagent

Table 2 ^{1}H- and ^{13}C-NMR data of cyslabdan

No.	δ_C (M)	δ_H
1	41.1 (t)	0.84 (2H, m)
2	19.3 (t)	1.35 (1H, m)
		1.63 (1H, m)
3	43.0 (t)	1.14 (1H, m)
		1.63 (1H, m)
4	34.1 (s)	
5	53.7 (d)	0.91 (1H, m)
6	27.8 (t)	1.63 (2H, m)
7	72.3 (d)	3.82 (1H, dd, *J*=4.5, 11.0)
8	78.8 (s)	
9	55.2 (d)	1.35 (1H, m)
10	39.8 (s)	
11	24.4 (t)	2.13 (1H, m)
		2.42 (1H, dt, *J*=6.5, 17.0)
12	137.6 (d)	5.55 (1H, t, *J*=6.5)
13	133.2 (s)	
14	143.1 (d)	6.35 (1H, dd, *J*=10.0, 18.0)
15	110.1 (t)	4.86 (1H, d, *J*=10.0)
		5.03 (1H, dd *J*=18.0)
16	12.0 (q)	1.75 (3H, bs)
17	39.2 (t)	2.63 (1H, d, *J*=12.5)
		2.77 (1H, d, *J*=12.5)
18	34.0 (q)	0.91 (3H, s)
19	22.3 (q)	0.84 (3H, s)
20	16.0 (q)	1.00 (3H, s)
1′	177.4 (s)	
2′	55.8 (d)	4.41 (1H, dd, *J*=4.5, 8.0)
3′	37.7 (t)	2.82 (1H, dd, *J*=8.0, 13.0)
		3.02 (1H, dd, *J*=4.5, 13.0)
4′	172.5 (s)	
5′	22.8 (q)	1.98 (3H, s)

^{1}H-NMR: 400 MHz in CD_3OD (ref. 3.31 ppm, *J* value in Hz).
^{13}C-NMR: 100 MHz in CD_3OD (ref. 49.0 ppm), M: multiplicity.

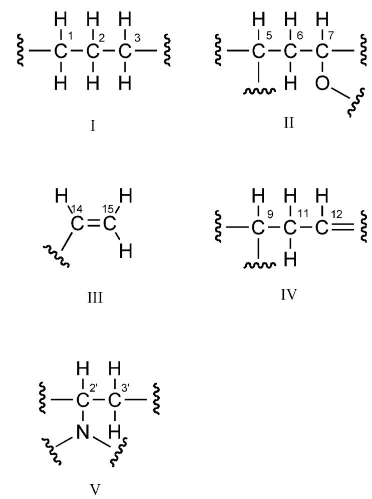

Fig. 4 Partial structures of cyslabdan.

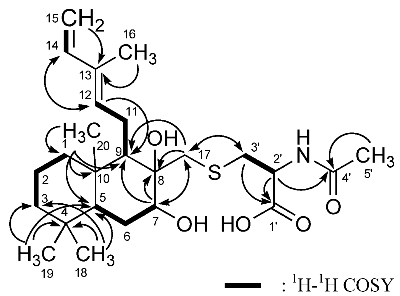

Fig. 5 ^{1}H-^{1}H COSY and ^{13}C-^{1}H HMBC experiments of cyslabdan.

methine carbons, two sp^2 methine carbons, three sp^3 quaternary carbons and three sp^2 quaternary carbons by analysis of the DEPT spectra. The ^{1}H-NMR spectrum (in CD_3OD) displayed 37 proton signals. The connectivity of proton and carbon atoms (Table 2) was established by the HMQC spectrum. Analayses of the ^{1}H-^{1}H COSY revealed the presence of the five partial structures I to V as shown in Fig. 4. Furthermore, ^{13}C-^{1}H long-range couplings of ^2J and ^3J observed in the ^{13}C-^{1}H HMBC spectrum (Fig. 5) gave the following linkages: 1) The cross peaks from H_3-19 (δ 0.84) to C-3 (δ 43.0), C-4 (δ 34.1) and C-5 (δ 53.7) and from H_3-18 (δ 0.91) to C-3, C-4 and C-5 showed the connection between the partial structures I and II. 2) The cross peaks from H-7 (δ 3.82) to C-8 (δ 78.8), C-9 (δ 55.2) and C-17 (δ 39.2), from H_2-17 (δ 2.63, 2.77) to C-7 (δ 72.3), C-8 and C-9, and from H_3-20 (δ 1.00) to C-1 (δ 41.1), C-5, C-9 and C-10 (δ 39.8), showed the connection among the partial structures I, II and III, indicating the presence of a labdane skeleton. 3) The cross peaks from H_2-15 (δ 4.86, 5.03) to C-13 (δ 133.2), from H_3-16 (δ 1.75) to C-13 and from H-14 (δ 6.35) to C-12 (δ 137.6) showed the connection between the partial structures III and IV. 4) The cross peaks from H_2-3′ (δ 2.82, 3.02) to C-17 and chemical shifts of C-3′ suggested connection between the partial structure V and the labdanic skeleton *via* thioether linkage. 5) The cross peaks from H_2-3′ to C-1′ (δ 177.4), from H-2′ (δ 4.41) to C-1′ and C-4′ (δ 172.5) and from H_3-5′ (δ 1.98) to C-4′ and chemical shifts of C-1′ and C-4′ suggested the presence of *N*-acetylcysteine. Taken together, the structure of cyslabdan was elucidated as shown in Fig. 5.

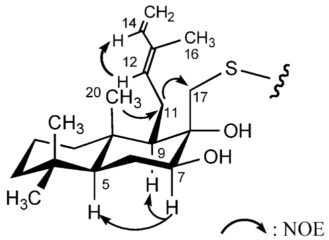

Fig. 6 Key NOE correlations of cyslabdan.

Configuration

NOE experiments suggested the relative configuration of the diterpene part (Fig. 6). Irradiation at H-12 (δ 5.55) produced NOE enhancement of the signals of H-14 (δ 6.35), but not of the signal of H_3-16 (δ 1.75), suggesting 12*E*-configuration. The *trans*-decalin configuration was indicated by the absence of NOE enhancement at H-5 (δ 0.91) when H_3-20 (δ 1.00) was irradiated. Irradiation at H-7 (δ 3.82) (axial proton geminal to the hydroxyl substituent) produced NOE enhancements of the signals of H-5 (δ 0.91) and H-9 (δ 1.35), but not of the signal of H_2-11 (δ 2.13, 2.42) and H_2-17 (δ 2.63, 2.77). Therefore, H-5, H-7 and H-9 were suggested to be on the same side of decalin plane. Furthermore, NOEs betweeen H_3-20 (δ 1.00)/H_2-11 (δ 2.13, 2.42) and H_2-11 (δ 2.13, 2.42)/ H_2-17 (δ 2.63, 2.77) indicated the configuration of C-8 and C-9. Thus, the relative configuration of the labdane skeleton was shown to be (12*E*)-labda-12,14-dien-7β,8α-diol.

To elucidate the configuration of *N*-acetylcysteine, cyslabdan was degraded as described in Materials and Methods. Under the HPLC conditions, the retention times of the authentic *N*-acetyl-L-alanine and *N*-acetyl-D-alanine were 9.25 and 10.57 minutes, respectively. Since the obtained *N*-acetylalanine was eluted with the retention time of 9.25 minutes, it was identified as *N*-acetyl-L-alanine. Therefore, the absolute configuration of *N*-acetylcysteine part of cyslabdan was elucidated as L-form.

Discussion

In this study, cyslabdan was isolated from the streptomycete metabolites, and the structure was elucidated to be a labdane-type diterpene skeleton connecting with an *N*-acetylcysteine *via* thioether likage. Furthermore the relative configuration of the labdane part and the absolute configuration of the *N*-acetylcysteine part were demonstrated. By comparison with the structure of plant labdane diterpenoids [20∼23], the total absolute configuration of cyslabdan was suggested as shown in Fig. 1.

However, there are two different parts of the stereochemistry between actinomycete cyslabdan and plant labdanes; 1) regarding the double bond at C-12, cyslabdan has 12*E*-configuration, while some plant labadanes have 12*Z*-configuration [20], and 2) regarding the hydroxy moiety at C-7, cyslabdan and nidorellol from plants *Nidorella* spp. show 7*S* configuration, while the labdane isolated from liverwort *Porella perrottetiana* shows 7*R* [22, 23]. Labdane diterpenoids had been isolated only from plant metabolites. Therefore, cyslabdane is the first actinomycete metabolite having a labdane-type diterpene structure.

Moreover, cyslabdan has a thioether-linked *N*-acetyl-L-cysteine moiety. Several compounds having an *N*-acetylcysteine of actinomycete orgin have been reported, and their configurations are all L-form as well as cyslabdan [24, 25].

Cyslabdan was found to strongly enhance the β-lactam imipenem activity against MRSA. The potentiation indicates that clinically resistant MRSA strains become clinically susceptible in the presence of cyslabdan. The biological activity of cyslabdan will be described in detail in the accompanying study.

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