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ORIGINAL ARTICLE

Iminimycin A, the new iminium metabolite produced by *Streptomyces griseus* OS-3601

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A new natural product, designated iminimycin A, was isolated from the cultured broth of a streptomycin-producing microbial strain, *Streptomyces griseus* OS-3601, via a physicochemical screening method using HP-20, silica gel and ODS column chromatographies and subsequent preparative HPLC. Iminimycin A is an indolizidine alkaloid, containing of an unusual iminium group and a cyclopropane ring with a triene side chain. The absolute configuration of iminimycin A was elucidated by NMR studies and electronic circular dichroism analysis. Iminimycin A shows anti-bacterial activity against *Bacillus subtilis*, *Kocuria rhizophila* and *Xanthomonas campestris* pv. *orizae*, and cytotoxic activity against HeLa S3 and Jurkat cells with IC_{50} values of 43 and 36 μM, respectively.

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INTRODUCTION

Actinomycetes have long been recognized as prolific producers of useful bioactive metabolites, which have demonstrated a broad spectrum of bioactivity.[1,2] Typically actinomycetes possess over 20 secondary metabolic biosynthesis gene clusters encoding the biosynthesis of a wide range of compounds, including polyketides, peptides and aminoglycosides.[3–5] Many researchers have long been searching for new bioactive compounds from actinomycetes. In our group, many new useful compounds; for example, avermectin,[6] lactacystin,[7] staurosporine,[8] and nanaomycin,[9] as well as known compounds have been discovered as the secondary metabolites of actinomycete strains. These strains, which have the potential of producing novel or previously known bioactive compounds, are routinely preserved via freeze drying for possible future investigation.

New useful compounds have been discovered in the course of various biological screening programs, including anti-bacterial, anti-fungal, anti-viral and other biological activities. In these biological assays, however, it is possible to miss or overlook potential and valuable natural compounds in cases where specific biological assays are unsuitable or nonexistent. Although some natural products were isolated from the cultured broths of these antibiotic-producing strains, it is considered that many products remain unidentified. Therefore, we are examining our preserved actinomycete collection for novel compounds using a physicochemical screening program. Physicochemical screening has led to the discovery of various new compounds, such as mangromicins,[10–12] trehangelins,[13] nanaomycin analogs[14] and actinoallolides.[15] We screened about 330 preserved actinomycete strains that were restored and cultured using four different liquid media. Cultured broths were added to equal volumes of ethanol, and analyzed by LC/UV and LC/MS. Makerview software (AB Sciex, Framingham, MA, USA) was used for data analysis. This software allows the processing of data acquired from nonclassified workflows using principal components analysis. This method compares data across multiple samples, reveals groupings among the data sets and shows the groupings in a scoring plot graphically. The loading plots provide valuable insight into variables leading to sample clustering as well as illustrating different metabolites. Principal component variable grouping helps to discover relationships between peaks and assigns related peaks to specific groups. The *t*-test analysis technique is useful when two or more predetermined classes of samples are present and can help to decide which compounds lead to significant differences between the classes. The MS and UV spectra of each peak selected for new compounds by Makerview software were compared with those of known compounds using existing databases, such as the Dictionary of Natural Products (http://dnp.chemnetbase.com/), as well as in-house databases. This comparison revealed the presence of a unique metabolite, which was predicted to be a new compound, in a cultured broth of *Streptomyces griseus* OS-3601. This strain had been preserved as a streptomycin-producing strain since 1972. As a result of purification guided by LC/MS analysis from the cultured broth of strain OS-3601, a new compound with an iminium cation, designated iminimycin A (**1**), was isolated. Here, we report the fermentation, isolation, structure elucidation and biological activity of **1**.

RESULTS

Isolation of iminimycin A (1)

The procedure for isolation of **1** is summarized in Figure 1. Isolation was guided by physicochemical properties such as molecular formula

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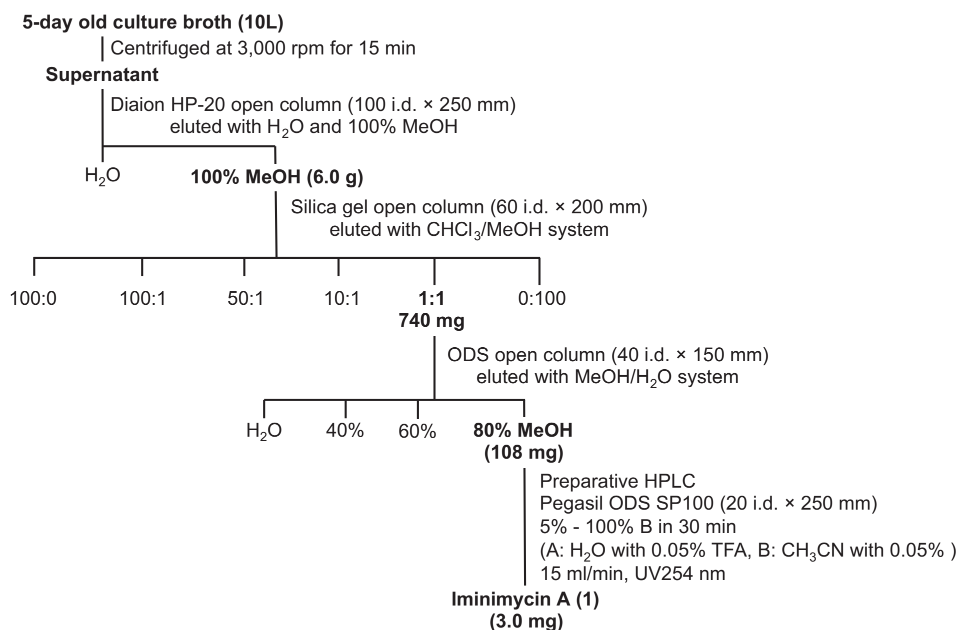

Figure 1 Isolation procedure for iminimycin A.

Table 1 NMR spectroscopic data (CD_3OD) for iminimycin A (1)

Position	δ_C*, mult.*	δ_H *(int., mult., J in Hz)*	δ_H *Calculated*	*HMBC*
1	27.7, CH	2.81 (1H, m)	2.59	
2	22.0, CH	2.33 (1H, m)	2.17	C-3, C-9, C-11
3	76.4, CH	4.70 (1H, dd, 2.4, 9.6)	4.53	C-9, C-11
5a	45.2, CH_2	3.40 (1H, m)	3.04	C-6, C-7, C-9
5b		3.62 (1H, m)	3.72	C-6, C-7
6	20.0, CH_2	1.90 (2H, m)	1.97, 1.45	
7	16.4, CH_2	1.84 (2H, m)	1.95, 1.59	C-6, C-9
8	16.4, CH_2	3.03, 3.39 (2H, m)	3.16, 2.75	
9	190.7, C			
10a	16.2, CH_2	1.00 (1H, ddd, 2.8, 4.8, 4.8)	0.91	C-1, C-2, C-3, C-9
10b		1.63 (1H, ddd, 5.2, 8.0, 8.0)	1.68	C-1, C-2, C-3, C-9
11	123.6, CH	5.63 (1H, dd, 9.6, 14.8)	5.52	C-3
12	139.2, CH	6.63 (1H, dd, 10.8, 14.8)	6.91	C-3, C-13, C-14
13	123.3, CH	6.23 (1H, dd, 10.8, 15.6)	6.42	C-11, C-12, C-15
14	141.8, CH	6.47 (1H, d, 15.6)	6.85	C-12, C-13, C-15, C-16, 15-Me
15	134.3, C			
16	129.6, CH	5.73 (1H, br)	6.22	C-14, 15-Me, 16-Me
17	12.8, CH_3	1.77 (3H, d)	1.93	C-15
15-Me	10.5, CH_3	1.78 (3H, s)	1.82	C-13, C-14, C-15, C-16

($C_{17}H_{24}N^+$) and UV spectrum (λ_{max} 269 and 282 nm), using LC/UV and LC/MS equipment. A strain of *S. griseus* OS-3601 was cultured using a production medium consisting of 2% soluble starch, 0.5% glycerol, 1.0% defatted wheat germ, 0.3% meat extract, 0.3% dry yeast and 0.3% $CaCO_3$. The 5-day-old culture broth (10 l) was centrifuged to separate cells and supernatant. The supernatant was passed through a column of Diaion HP-20 (100 i.d. × 250 mm; Mitsubishi Chemical, Tokyo, Japan) previously equilibrated with water. After washing with water, the fraction containing **1** was eluted with 100% MeOH. The whole eluate was concentrated *in vacuo*. The resultant material (6.0 g) was subjected to column chromatography on silica gel FL100D (60 i.d. × 200 mm; Fuji Silysia Chemical, Aichi, Japan), and eluted stepwise with $CHCl_3$/MeOH (100:0, 100:1, 50:1, 10:1, 1:1 and 0:100 ($v\,v^{-1}$)), to give six fractions. After the 1:1 fraction was concentrated *in vacuo*, the material (740 mg) was subjected on an ODS column (40 i.d. × 150 mm; Senshu Scientific, Tokyo, Japan) previously equilibrated with water. After washing with water, the fraction eluted with 80% MeOH was concentrated *in vacuo* to yield 108 mg. Finally, it was dissolved in a small amount of MeOH, and purified by preparative HPLC on a Pegasil ODS SP100 column (20 i.d. × 250 mm; Senshu Scientific) with elution as a linear gradient from 5 to 100% CH_3CN plus 0.05% trifluoroacetic acid in 30 min at 15.0 ml min^{-1} monitoring at UV 254 nm. The peak at retention time of around 20 min was collected and dried to yield 3.0 mg of **1**.

Physicochemical properties of iminimycin A (1)

Compound **1** is readily soluble in H_2O and MeOH, and was obtained as a white powder ($[\alpha]_D^{23.5}$ 3.4 (c=0.1, MeOH), UV (MeOH) λ_{max} (ε) 269 (2710) and 282 (2680), and IR νmax (KBr) cm^{-1} 1681, 1403, 1203 and 1114).

Structure elucidation of iminimycin A (1)

The HR-ESIMS of **1** produced the M^+ at *m/z* 242.1910 indicating the molecular formula was $C_{17}H_{24}N^+$ (calculated value for $C_{17}H_{24}N^+$, 242.1909). The ^1H and ^{13}C NMR spectra data measured in CD_3OD of **1** are listed in Table 1. The ^1H NMR data indicated the presence of three sp^3 methines, five sp^2 methines, five methylenes and two olefinic methyl groups. The ^{13}C NMR spectrum showed the resonances of 17 carbons, which were classified into six olefinic carbons, three sp^3 methine carbons, five sp^3 methylene carbons, two methyl carbons by HSQC spectra and one iminium carbon. The chemical shifts of H_2-8 at δ_H 3.03 and 3.39 and iminium carbon at δ_c 190.7 were characterized by comparison with ^1H and ^{13}C NMR data of similar substructure.[16]

The ^1H-^1H COSY indicated the presence of four partial structures: (a) C-1/C-2/C-10 to form a cyclopropane ring, (b) C-5/C-6, (c) C-3/C-11/C-12/C-13/C-14 and (d) C-16/C-16-Me, as shown in Figure 2a. Analysis of HMBC data confirmed the presence of a pyrrolidinium ring fused with a cyclopropane, based on the correlations from H_2-10 to C-1, C-2, C-3 and C-9 and from H-2 to C-3 and C-9. An indolizidinium ring was identified, based on HMBC correlations from H_2-5 to C-6, C-7 and C-9, from H_2-7 to C-6 and C-9 and from H-3 to C-9. The presence of an iminium cation was also strongly supported by IR absorption at 1682 cm^{-1} (Supplementary Information S3). Moreover, the correlations from H-13 to C-11, from H-13 and H-14 to C-12, from H-11, H-12 and H-14 to C-13, from H-12, H-16 and H_3-15-Me to C-14, from H-13, H-14, H_3-15-Me and H_3-16-Me to C-15, from H-14 and H_3-15-Me to C-16, from H-14 and H-16 to C-15-Me and from H-16 to C-16-Me confirmed the presence of a 5-methyl-hepta-1,3,5-trienyl unit. All geometries of the triene were determined as *E* based on large coupling constants (Table 1) and ROESY correlations (Supplementary Information S9). Finally, the HMBC correlations from H-2 and H-3 to C-11and from H-11 to C-2 and C-3 revealed that this triene unit was attached to the C-3 position and the structure of **1** was elucidated as shown in Figure 2. The accurate predictions of molecular geometries are confirmed by reliable calculations of magnetic properties. After optimization of **1** in CH_3OH at the B3LYP/6-311+G(2d,p) level, gauge-invariant atomic orbital ^1H NMR chemical shift calculations of **1** have been made by the B3LYP/6-311+G(2d,p) level. The experimental and calculated ^1H NMR chemical shifts in CD_3OD are gathered in Table 1. The calculated assignments corresponded reasonably well with the experimental data (Supplementary Informations S4 and S11).

The relative configuration of **1** was determined by ^1H-^1H coupling constant analysis, differential NOE and ROESY experiments (Figure 2b). ROESY correlations were observed between H-3/H5a and H-3/H10a. These results indicate that the cyclopropane ring and the triene side chain are located on the opposite surface. This was supported by the coupling constant of 2.4 Hz between H-3 and H-2. Therefore, the relative configuration of **1** was determined to be 1*S**, 2*R** and 3*S**, as shown in Figure 2c.

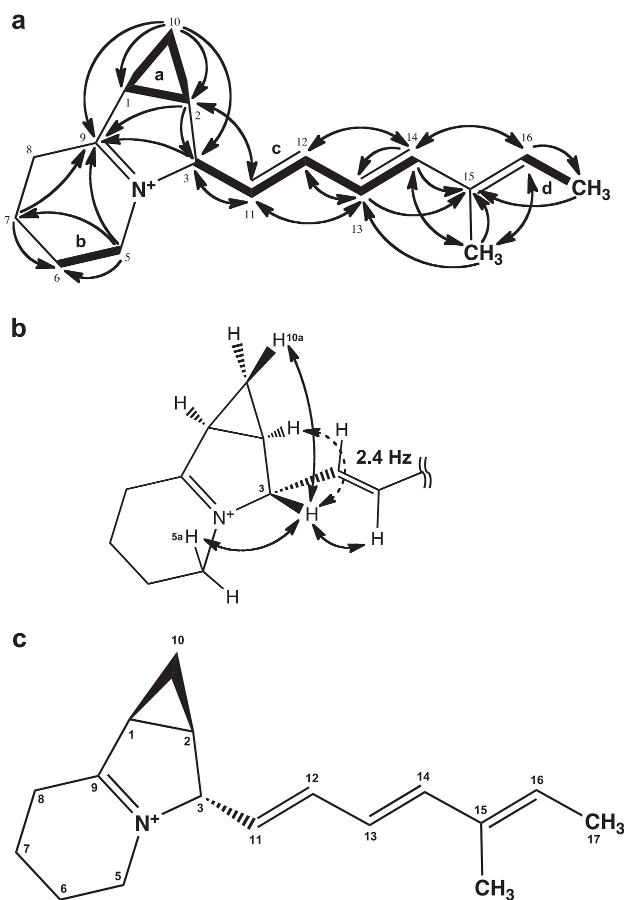

Figure 2 Structure elucidation of iminimycin A. (**a**) ^1H-^1H COSY (bold lines) and selected HMBC correlation (arrows) of iminimycin A (**1**). (**b**) Key ROESY and NOE correlations (bold arrows) and coupling constants (dotted arrows) of iminimycin A (**1**). (**c**) Relative configurations of iminimycin A (**1**).

Absolute configuration of iminimycin A (1)

The absolute configuration of **1** was deduced by electronic circular dichroism (ECD) spectra[17,18] in comparison with their calculated spectra[19] (Figure 3). The conformational analysis of **1** started from MM2 force field structures by ChemDraw 3D, optimized by semi-empirical PM3 calculations in Gaussian 09[20] and then further refined by density functional theory (DFT) at the B3LYP/6-311G level, which yielded additional relevant conformers. Structures of resulting calculated conformers were also supported by our ROESY analyses. Calculated conformers for **1** are shown in the Supplementary Information S1, together with their atomic coordinates and energies. The ECD spectra of **1** were simulated using time-dependent DFT at the same level of theory on the relevant conformers. As a result of the comparison between the experimental and calculated ECD spectra, the absolute configuration of **1** was elucidated to be 1*S*, 2*R* and 3*S*.

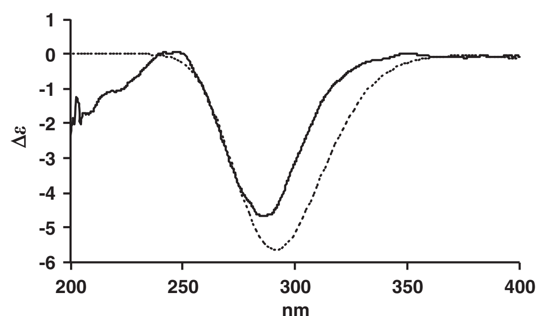

Figure 3 Experimental electronic circular dichroism (ECD) spectrum of iminimycin A (**1**) (line) overlaid with calculated spectrum (dotted line).

Biological activities of iminimycin A (1)

Compound **1** showed weak antimicrobial activities against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341 and *Xanthomonas campestris* pv. *oryzae* KB 88 with inhibition zones of 12, 10 and 10 mm at 100 μg per disk, respectively. No activity was evident against *Escherichia coli* NIHJ, yeast or fungi. Compound **1** displayed toxic activity against HeLa S3 and Jurkat cells with IC_{50} values of 43 and 36 μM, respectively.

DISCUSSION

Natural products with an iminium moiety have previously been isolated mainly from plants[21] and sponges.[22] Although it has been reported that ammosamide C was an iminium cationic compound originating from actinomycetes, it was only detected in ESI-MS analysis and could not be isolated from a culture broth because of very low productivity.[23] To our knowledge, iminimycin A is the first example of an iminium compound isolated from an actinomycete. *S. griseus* was discovered to produce the important aminoglycoside antibiotic, streptomycin, by Waksman and co-workers.[24] Our iminimycin A-producing strain OS-3601 was identified as *S. griseus* by BLAST search based on 16S rRNA gene sequence (Supplementary Information S2).

To date, there has been only two reports of natural compounds with the same structural skeleton as iminimycin A. Indolizomycin was obtained from strain SK2-52 by protoplast fusion treatment between *S. griseus* and *S. tenjimariensis*.[25] Therefore, *S. griseus* may possess the capacity to produce compounds with the same basic skeleton. Compound JBIR-102, containing an octahydroindolizine skeleton, was obtained from *Saccharopolyspora* sp. RL78 isolated from mangrove soil.[26] However, these two compounds have no iminium moiety.

Iminimycin A showed anti-bacterial activity against several bacterial strains, but its activity was very weak in comparison with streptomycin. Therefore, even if iminimycin A was produced by strain OS-3601, it could easily be missed during antibiotic screening because of its minimal antimicrobial activity. Our results showed that we could find new compounds using our unique physicochemical screening system, even from widely studied species such as *S. griseus*.

METHODS

General experimental procedures

All solvents were purchased from Kanto Chemical (Tokyo, Japan). Silica gel and a Pegasil ODS SP100 column were purchased from Fuji Silysia Chemical (Aichi, Japan) and Senshu Scientific (Tokyo, Japan), respectively.

NMR spectra were measured using an Agilent Technologies XL-400 and INOVA 600 (Agilent Technologies, Palo Alto, CA, USA) or JEOL JNM-ECA 500 spectrometer (JEOL, Tokyo, Japan), with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz in CD_3OD. The chemical shifts are expressed in parts per million and are referenced to residual CHD_2OD (3.31 p.p.m.) in the ^{1}H NMR spectra and CD_3OD (49 p.p.m.) in the ^{13}C NMR spectra. LC-ESI-MS spectra were measured using an AB Sciex QSTAR Hybrid LC/MS/MS Systems (AB Sciex). IR spectra (KBr) were taken on a Horiba FT-710 Fourier transform IR spectrometer (Horiba, Kyoto, Japan). UV spectra were measured with a Hitachi U-2810 spectrophotometer (Hitachi, Tokyo, Japan). Optical rotation was measured on a JASCO model DIP-1000 polarimeter (Jasco, Tokyo, Japan).

Fermentation

A strain of *S. griseus* OS-3601, preserved for about 40 years by freeze drying, was used in this study. After growth, the strain was maintained on agar slants, consisting of 1.0% starch, 0.3% NZ amine, 0.1% yeast extract, 0.1% meat extract, 1.2% agar and 0.3% $CaCO_3$. A loop of spores of strain OS-3601 was inoculated into 100 ml of the seed medium, consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% $CaCO_3$ (adjusted to pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask. The flask was incubated on a rotary shaker (210 r.p.m.) at 27 °C for 3 days. A 1-ml portion of the seed culture was transferred to Erlenmeyer flasks (total 100) containing 100 ml of starch medium, consisting of 2% soluble starch, 0.5% glycerol, 1.0% defatted wheat germ, 0.3% meat extract, 0.3% dry yeast and 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization), and fermentation was carried out on a rotary shaker (210 r.p.m.) at 27 °C for 5 days.

Computational methods for conformational analysis

The conformational analysis was performed by the semiempirical PM3 method, as implemented in the program package Gaussian 09[20] on Mac mini Apple (Late 2014), starting from preoptimized geometries generated by the MM2 force field in Chem 3D software (Hulinks Inc., Tokyo, Japan). The corresponding minimum geometries were further optimized by DFT calculations at the B3LYP/6-311G level. The time-dependent DFT -based B3LYP/6-311G method was performed to provide the calculated ECD. In addition, DFT calculations at the B3LYP/6-311 +G(2d,p) level were performed for the prediction of ^{1}H NMR spectra.

Biological activities

Antimicrobial activity of **1** against six microorganisms, *B. subtilis* ATCC 6633, *K. rhizophila* ATCC 9341, *Escherichia coli* NIHJ, *X. campestris pv. oryzae* KB 88, *Candida albicans* KF1 and *Mucor racemosus* IFO4581, was evaluated using the paper disk method (8 mm disk). Media for microorganisms were as follows: nutrient agar (Sanko Junyaku, Tokyo, Japan) for the bacteria, a medium composed of 1.0% glucose, 0.5% yeast extract and 0.8% agar for fungi and yeasts. A paper disk (φ 8 mm) containing a sample (at a final concentration of 100 μg per disk) was placed on an agar plate. Bacteria except *X. oryzae* were incubated at 37 °C for 24 h. Fungi and *X. oryzae* were incubated at 27 °C for 48 h.

Cytotoxic activity of **1** was measured by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) in two cell lines, namely HeLa S3 (human cervical cancer cell line) and Jurkat (human acute lymphocytic leukemia cell line). Briefly, HeLa S3 (5×10^4 cells per well) and Jurkat (3×10^5 cells per well) cell lines were seeded in 96-well plates and cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. After culturing overnight, **1** dissolved in MeOH at appropriate concentrations was added into each well. After 48 h of incubation at 37 °C, WST-8 solution was added to each well and incubated at 37 °C for 4 h. The absorbance at 450 nm of each well was measured using a Corona Grating Microplate Reader SH-9000 (Corona Electric, Ibaraki, Japan).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)