ORIGINAL ARTICLE

Panowamycins A and B, new antitrypanosomal isochromans produced by *Streptomyces* sp. K07-0010

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Two new isochromans, panowamycins A and B, were purified by solvent extraction, silica gel and octadecylsilyl silica gel (ODS) column chromatography followed by preparative HPLC, from a culture broth of *Streptomyces* sp. K07-0010, together with the known compounds NFAT-133, conglobatin, piericidin C series and dinactin. Structures of panowamycins were elucidated as new analogs of NFAT-133 by spectroscopic studies including various NMR experiments. Panowamycins A and B showed moderate antitrypanosomal activity, with IC₅₀ values of 0.40 and 3.30 µg ml⁻¹, respectively.

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INTRODUCTION

Human African Trypanosomiasis, also known as sleeping sickness, is caused by two subspecies of the parasitic protozoan, *Trypanosoma brucei*. Unique to Africa, Human African Trypanosomiasis has, in past epidemics, caused significant and widespread mortality and morbidity. The World Health Organization estimated that Human African Trypanosomiasis caused 54 000 deaths in 2008, projecting a continuing fall to a total of 36 000 in 2015.¹ Even though cases are in steep decline and the disease may possibly be eliminated in the near future, significant problems remain with the four parenteral drugs commonly used for treatment (suramin, pentamidine, eflornithine, melarsoprol), as well as the newly introduced nifurtimox. All are toxic, expensive, difficult to administer, have difficulty in crossing the blood/brain barrier and parasite resistance to them is increasing. However, only one molecule (fexinidazole) is currently in clinical development for treatment of Human African Trypanosomiasis.² Therefore, there is an urgent need for new antitrypanosomal drugs that are more effective, safer, affordable, easier to use and that, ideally, have a novel mode of action and can thus cross the blood/brain barrier safely to treat the more deadly late-stage diseases.

Our research group has focused on the screening of antitrypanosomal agents from microbial metabolites.^{3–5} Our ongoing studies have led to the discovery of two novel NFAT-133 analogs, panowamycins A (1) and B (2) isolated from a culture broth of *Streptomyces* sp. K07-0010, along with NFAT-133 (3),⁶ conglobatin (4),⁷ piericidins C₁–C₄ (5–8)⁸ and dinactin (11)⁹ (Figure 1). In this paper, the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and structure elucidation of 1 and 2, and antitrypanosomal activities of isolated compounds are described.

RESULTS

Taxonomy of the producing strain K07-0010

Strain K07-0010 was isolated from mangrove soil collected at Kasturi Ghat, Cox's Bazar (other name 'Panowa'), Bangladesh. The vegetative mycelia developed well on yeast extract–malt extract agar, oatmeal agar and others, and did not show fragmentation into coccoid forms or bacillary elements. The color of vegetative mycelia was yellow to brown. Aerial mycelia were produced poorly on inorganic salts–starch agar, glycerol–asparagine agar and others, and the aerial mass color was white to gray. The mature spore chains were spiral and each had more than 20 spores per chain. The spores were cylindrical in shape, 1.0–1.1 × 0.5–0.7 µm in size and had a hairy surface (Figure 2). No soluble pigment was produced. The isomer of diaminopimelic acid in whole-cell hydrolysates of strain K07-0010 was determined to be LL-form. An almost complete 16S rDNA sequence (1450 nucleotides) was determined. The result of the identification analysis by EzTaxon.org server version 2.1¹⁰ showed the strain belongs to the genus *Streptomyces* and relates closely to *Streptomyces kasugaensis* M338-M1^T (AB024441, 98.4%). Based on the morphological and cultural properties and 16S rDNA sequence similarity, the strain K07-0010 was identified as a *Streptomyces* species¹¹ and designated *Streptomyces* sp. K07-0010.

Isolation

The procedure for isolation of 1 and 2 is summarized in Scheme 1. All compounds were obtained by bioassay-guided purification. The 6-day-old culture broth (31) was added to an equal amount of ethanol and then filtered. The filtrate was concentrated under reduced pressure to remove ethanol and the aqueous solution was extracted three times

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Figure 1 Structures of panowamycins A (1) and B (2) and known compounds isolated from a culture broth of K07-0010.

Figure 2 Scanning electron micrograph of *Streptomyces* sp. K07-0010.

with ethyl acetate (31). The organic layer was dried over Na₂SO₄ and concentrated to dryness *in vacuo* to afford a crude material (1.75 g). The ethyl acetate extract (1.69 g) was applied to silica gel column chromatography, which was eluted with each 550 ml of a mixture of CHCl₃–MeOH (100:0, 100:1, 50:1, 10:1, 1:1, 0:100) in this order. The CHCl₃–MeOH (100:1) fraction (272 mg of 359 mg) was applied to an octadecylsilyl silica gel (ODS) column chromatography. It was eluted with 20, 40, 50, 60, 70, 80, 90 and 100% CH₃CN (each 7 ml×3 fractions). The third 40% aq. CH₃CN eluate and the first and second 50% CH₃CN eluate (10.2 mg) were dissolved in a small amount of MeOH and applied to a preparative HPLC (Pegasil ODS, 20 i.d.×250 mm) with 60% MeOH (flow rate, 5.0 ml min^{−1}; detection, UV 210 nm). The peaks with retention times of 58 and 64 min were collected and concentrated *in vacuo* to dryness to afford 1 (1.7 mg) and 2 (2.1 mg), respectively.

The second 60% CH₃CN (6.6 mg) of the ODS column consists of pure 4. The combined crude mixture of 70, 80, 90 and 100% CH₃CN (161.7 mg) was separated by HPLC (Pegasil ODS, 20 i.d.×250 mm) with 90% MeOH (flow rate, 5.0 ml min^{−1}; detection, UV 210 nm), giving piericidins C₁ (5, r.t.=17 min, 11.4 mg), C₂ (6, r.t.=19 min, 16.0 mg), C₃ (7, r.t.=22 min, 39.2 mg) and C₄ (8, r.t.=24 min, 38.0 mg) as yellow powder. Compound 3 (9.0 mg) was isolated as a white powder from the eluate of CHCl₃–MeOH (50:1) fraction (173.4 mg) of the silica gel column by a preparative HPLC (Pegasil ODS, 20 i.d.×250 mm) with 40% CH₃CN. A member of the macrotetrolide polynactins, 11, was isolated from the CHCl₃–MeOH (1:1) fraction (255 mg) followed by LH-20 gel filtration column chromatography and trituration with MeOH.

Physico-chemical properties

The physico-chemical properties of 1 and 2 are summarized in Table 1. They are readily soluble in CHCl₃ and MeOH. They showed absorption maxima at 218 and 276 nm in UV spectra. The broad IR absorption at 3400 cm^{−1} of 1 and 2 suggested the presence of hydroxyl groups, and the sharp IR absorption at 1700 cm^{−1} of 1 suggested the presence of ketone groups. The similarity in physico-chemical properties strongly suggested that they are structurally related to 3.

Structure elucidation of 2

The molecular formula of 2 was elucidated by HR-FAB-MS to be C₁₇H₂₆O₃, requiring five degrees of unsaturation. The ¹H and ¹³C NMR spectra data (CDCl₃) of 2 are listed in Table 2. The ¹³C NMR and HSQC spectra indicated 17 carbons, which were classified into three *sp*² quaternary carbons, three *sp*² methine carbons, three oxygenated *sp*³ methine carbons, two *sp*³ methine carbons, one oxygenated *sp*³ methylene carbon, one *sp*³ methylene carbon and four methyl carbons. The ¹H and ¹³C NMR spectra suggested the presence of a 1,2,4-trisubstituted benzene ring by the coupling pattern

Scheme 1 Purification of panowamycins A (1) and B (2).**Table 1** Physico-chemical properties of panowamycins A (1) and B (2)

	Panowamycin A (1)	Panowamycin B (2)
Appearance	Light yellow amorphous solid	Light yellow amorphous solid
$[\alpha]_D^{25}$ (MeOH)	-49.4° (c 0.1)	-61.6° (c 0.1)
Molecular formula	$C_{17}H_{24}O_3$	$C_{17}H_{26}O_3$
HR-ESI-MS (m/z)	Found: 277.1803 $[M+H]^+$ Calcd: 277.1804 for $C_{17}H_{25}O_3$	Found: 279.1959 $[M+H]^+$ Calcd: 279.1960 for $C_{17}H_{27}O_3$
UV λ_{max} (MeOH) nm (ϵ)	217 (7,728), 276 (855)	218 (9,535), 277 (1,084)
IR ν_{max} (KBr) cm^{-1}	3429, 2927, 2341, 1707, 1630, 1090, 1057	3427, 2972, 2881, 2364, 1622, 1109, 1057, 1018

of proton signals at H-5 (δ_H 6.83), H-7 (δ_H 7.00) and H-8 (δ_H 7.01). The 1H - 1H COSY of **2** indicated the alignments from C-1 to C-3 and from 10-CH₃ to C-14 (Figure 3). The connection of these partial structures was deduced from the observation of the HMBC correlations as shown in Figure 3. The correlations from H-3 (δ_H 4.97) to C-4 (δ_C 136.5) and C-9 (δ_C 138.3), from H-10 (δ_H 2.90) to C-4, C-8 (δ_C 129.0) and C-9, and from 10-CH₃ (δ_H 1.18) to C-9 revealed the connection between C-3 and C-4, and between C-9 and C-10. The correlations from 6-CH₃ (δ_H 2.29) to C-5 (δ_C 124.5), C-6 (δ_C 135.8) and C-7 (δ_C 127.5) indicated 6-CH₃ is attached to C-6. The correlations from H-11 (δ_H 3.60) to C-3 (δ_C 78.6) indicated the linkage of

C-3 and C-11 through an oxygen atom to form a tetrahydro-2H-pyran ring. Thus, the planar structure of **2** was elucidated as shown in Figure 3, and it was designated as panowamycin B.

The relative configuration of **2** was elucidated by 1H - 1H coupling constant and ROESY experiments (Figure 4). The ROESY correlations were observed for H-3/H-11, H-10/H-11 and 10-CH₃/H-12. Moreover, a large vicinal coupling constant ($J=9.0$ Hz) and a small coupling constant ($J=2.4$ Hz) were observed between H-11 and H-12 and between H-10 and H-11, respectively. These results suggested that **2** has the relative configuration of 3S*, 10R*, 11S* (Figure 1).

Table 2 ^1H and ^{13}C NMR spectral data of panowamycins A (1) and B (2) in CDCl_3

Position	Panowamycin A (1)				Panowamycin B (2)			
	δ_{H}		Hz	δ_{C}	δ_{H}		Hz	δ_{C}
1	3.83	ddd	7.2, 9.6, 16.8	60.4	3.82	ddd	14.4, 7.2, 3.6	61.3
	3.90	ddd	16.2, 9.0, 7.2		3.87		14.4, 7.2, 3.6	
2	1.88	m		38.0	2.02	m		37.9
	2.11	m			2.30	m		
3	4.99	dd	16.8, 3.6	73.0	4.97	dd	8.4, 3.6	78.6
4				136.3				136.5
5	6.77	d	3.0	125.7	6.83	br.s		124.5
6				137.1				135.8
6-CH ₃	2.23	s		21.1	2.29	s		21.2
7	6.92	dd	8.0, 3.0	128.7	7.00	dd	9.0, 2.2	127.5
8	6.88	d	8.0	127.6	7.01	d	9.0	129.0
9				135.7				138.3
10	2.67	qd	6.8, 2.4	34.0	2.90	qd	6.0, 2.4	34.5
10-CH ₃	1.04	d	6.8	17.1	1.18	d	6.0	17.4
11	3.88	dd	10.1, 2.4	71.7	3.60	dd	9.0, 2.4	79.2
12	2.81	dd	10.1, 6.8	49.2	1.71	ddd	9.0, 6.0, 2.4	40.3
12-CH ₃	1.23	d	6.8	15.3	1.06	d	6.0	8.9
13				210.9	4.08	qd	6.0, 2.4	67.2
14	2.17	s		29.2	1.23	d	6.0	21.5

Figure 3 COSY and HMBC correlations of panowamycins A (1) and B (2).

Structure elucidation of 1

The molecular formula of **1** was elucidated by HR-FAB-MS to be $\text{C}_{17}\text{H}_{24}\text{O}_3$, indicating that **1** had lost two hydrogen atoms from **2**. The ^1H and ^{13}C NMR spectra data of **1** are listed in Table 2, and they were similar to those of **2**. However, one ketone carbonyl carbon at δ_{C} 210.9 was observed in **1** instead of the oxygenated sp^3 methine carbon of **2**. The ^{13}C – ^1H long-range couplings of 2J and 3J in the HMBC experiments are shown in Figure 3, giving the following results. The cross-peaks from 14- H_3 (δ_{H} 2.17) and 12- CH_3 (δ_{H} 1.23) to carbonyl carbon (δ_{C} 210.9) indicated that **1** has a ketone group instead of the hydroxyl group of **2** at C-13. Therefore, the structure of **1** was elucidated as shown in Figure 3, and designated as panowamycin A.

The relative configuration of **1** was elucidated by ^1H – ^1H coupling constant and ROESY experiments (Figure 4). The ROESY correlations were observed for H-3/H-11, H-10/H-11 and 10- CH_3 /H-12. Moreover, a large vicinal coupling constant ($J=15.6\text{ Hz}$) and a small coupling constant ($J=2.4\text{ Hz}$) were observed between H-11 and H-12, and between H-10 and H-11, respectively. Therefore, the relative configuration of **1** was determined to be $3S^*,10R^*,11S^*$, which was the same as that of **2**.

Biological activities

Antitrypanosomal activity of the isolated compounds was evaluated using *Trypanosoma brucei brucei* GUTat 3.1 strain (Table 3). We have also evaluated the antitrypanosomal activity of other polynactins, nonactin (**9**), monactin (**10**), trinactin (**12**) and tetranactin (**13**), isolated from a culture broth of *Streptomyces* sp. A00582 and together with dinactin (**11**). As a result, these compounds were classified into three groups as follows: (i) Panowamycins A (**1**) and B (**2**), NFAT-133 (**3**), conglobatin (**4**), piericidin C_1 (**5**) and **9** showed antitrypanosomal activity with IC_{50} values of 0.40 – $5.38\text{ }\mu\text{g mL}^{-1}$, which was in a similar range to that of the commonly-used therapeutic drugs, suramin and eflornithine. These compounds, except **9**, exhibited weak cytotoxicity against MRC-5 cells, with IC_{50} values of 2.95 – $21\text{ }\mu\text{g mL}^{-1}$. A Selectivity Index (SI) was created to allow direct comparisons of effectiveness and cytotoxicity. Compounds **1**–**5** showed SIs of 3.8 – 8.7 . (ii) Compounds **10**–**13** showed potent antitrypanosomal activity, with IC_{50} values of 0.021 – $0.42\text{ }\mu\text{g mL}^{-1}$. However, they also showed potent or moderate cytotoxicity, with IC_{50} values of 0.18 – $0.26\text{ }\mu\text{g mL}^{-1}$. Among them, **10** had moderate selectivity (SI of 12). (iii) Piericidins C_2 (**6**), C_3 (**7**) and C_4 (**8**) showed potent antitrypanosomal activity, with IC_{50} values of 0.34 – $0.48\text{ }\mu\text{g mL}^{-1}$ and low cytotoxicity. They demonstrated high selectivity (SIs of 71 – 88), in excess of those of the commonly used drugs and 10 – 20 -fold stronger than panowamycins.

DISCUSSION

Streptomyces sp. K07-0010 produced at least nine compounds, which were observed to fit into four groups based on their molecular skeletons. It is interesting that a single microorganism simultaneously produces metabolites with a wide variety of different skeletal structures.

Among them, we found **1** and **2**. Their structures are similar to that of **3**, which was reported to inhibit NFAT-dependent transcription.⁶ Panowamycins might be formed from **3** by an intramolecular cyclization between C-3 of olefine and the hydroxyl group at C-11. Though **1** was listed in the CAS Registry file (Registry No. 1217652-79-3), there are no published data or no reports on its stereochemistry.

Figure 4 Relative configuration of panowamycins A (1) and B (2).

Table 3 *In vitro* antitrypanosomal activity against *Trypanosoma brucei* GUTat 3.1 and cytotoxicity in MRC-5 cells of compounds isolated from a culture broth of *Streptomyces* sp. K07-0010

Compound	<i>IC</i> ₅₀ (μg ml ⁻¹)		
	Antitrypanosomal activity <i>T. b. b.</i> GUTat 3.1	Cytotoxicity MRC-5	Selectivity index MRC-5/GUTat 3.1
Panowamycin A (1)	0.40	2.95	7.4
Panowamycin B (2)	3.30	13	3.8
NFAT-133 (3)	2.78	21	7.5
Conglobatin (4)	2.46	13	5.2
Piericidin C ₁ (5)	0.44	3.81	8.7
Piericidin C ₂ (6)	0.48	42	88
Piericidin C ₃ (7)	0.34	24	71
Piericidin C ₄ (8)	0.37	28	76
Nonactin (9)	5.38	0.15	0.03
Monactin (10)	0.021	0.26	12
Dinactin (11)	0.065	0.21	3.2
Trinactin (12)	0.27	0.20	0.7
Tetranactin (13)	0.42	0.18	0.4

Eflornithine ^a	2.27	> 100	> 44
Suramin ^a	1.58	> 100	> 63

^aClinically used antitrypanosomal drugs.

Regarding the antitrypanosomal activity, as shown in Table 3, piericidins C₂, C₃ and C₄ had the more potent activity and best SI. It was previously reported that piericidins are inhibitors of NADH dehydrogenase in the mitochondrial respiratory chain.¹² We reported that some inhibitors of the mitochondrial respiratory chain, such as leucinostatin,¹³ trichosporins⁴ and citreoviridin,⁵ also showed potent antitrypanosomal activity. Therefore, it is likely that all these compounds possess a similar mode of action.

Compounds 1–3 showed moderate activity. Compound 1 showed more potent antitrypanosomal activity and better SI than 2, and therefore the ketone group at C-13 of 1 is probably essential for the activity.

METHODS

General experiments

All solvents (EtOAc, CHCl₃, MeOH and CH₃CN) and Na₂SO₄ were purchased from Kanto Chemical (Tokyo, Japan). Silica gel was purchased from Merck (Darmstadt, Germany). Pegasil ODS was obtained from Senshu Scientific Co. (Tokyo, Japan). LH-20 gel was purchased from GE Healthcare (Buckinghamshire, England, UK).

NMR spectra were measured using a Varian XL-400 spectrometer or an Inova 600 spectrometer (Agilent Technologies, Santa Clara, CA, USA), with ¹H NMR at 400 or 600 MHz and ¹³C NMR at 100 or 150 MHz in CDCl₃. The

chemical shifts are expressed in p.p.m. and are referred to CHCl₃ (7.26 p.p.m.) in the ¹H NMR spectra and to CDCl₃ (77.0 p.p.m.) in the ¹³C NMR spectra. FAB-MS spectra were measured on a JMS AX-505 HA mass spectrometer (JEOL, Akishima, Japan). IR spectra (KBr) were taken on a FT-210 Fourier transform infrared spectrometer (Horiba, Kyoto, Japan). UV spectra were measured with a DU640 spectrophotometer (Beckman Coulter, Brea, CA, USA). Optical rotation was measured on a model DIP-181 polarimeter (JASCO, Hachioji, Japan).

Taxonomic studies

The International Streptomyces Project media recommended by Shirling and Gottlieb¹⁴ and media recommended by Waksman¹⁵ were used to investigate the cultural characteristics. Cultures were observed after incubation for 2 weeks at 27 °C. Morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL). Isomers of diaminopimelic acid in whole-cell hydrolysates were determined by TLC following the standard methods of Becker *et al.*¹⁶ and Hasegawa *et al.*¹⁷ 16S rDNA was amplified by PCR and sequenced directly on an ABI model 377A automatic DNA sequencer using a PRISM Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA).

Fermentation

Strain K07-0010 was grown and maintained on agar slants consisting of 1.0% starch (Wako Pure Chemical Industries, Osaka, Japan), 0.3% NZ amine (Wako Pure Chemical Industries), 0.1% yeast extract (Oriental Yeast, Tokyo, Japan), 0.1% meat extract (Kyokuto Pharmaceutical Industrial, Tokyo, Japan), 1.2% agar (SSK Sales, Shizuoka, Japan) and 0.3% CaCO₃ (Kanto Chemical). A loop of spores of strain K07-0010 was inoculated into 100 ml of seed medium, consisting of 2.4% starch, 0.1% glucose (Wako Pure Chemical Industries), 0.3% peptone (Kyokuto Pharmaceutical Industrial), 0.3% meat extract, 0.5% yeast extract and 0.4% CaCO₃ (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 500-ml Erlenmeyer flasks (total 32), each containing 100 ml of production medium, consisting of 2.0% glycerol (Kanto Chemical), 1.0% glucose, 0.5% corn steep powder, 1.0% soybean, 0.2% meat extract, 0.01% MgSO₄·7H₂O (Wako Pure Chemical Industries) and 0.2% CaCO₃ (adjusted to pH 7.0 before sterilization) and fermentation was carried out on a rotary shaker (210 r.p.m.) at 27 °C for 6 days.

Antitrypanosomal activity *in vitro*

In vitro antitrypanosomal activities against *T. b. b.* strain GUTat 3.1 were measured, using the method described previously.¹⁸ This strain, donated by Dr Y Yabu (Nagoya City University, Japan), is a clone derivative of a stock EVE (Edinburgh Veterinary Expedition) 10 that was originally isolated in 1966 from a naturally infected bovine in Uganda.

Cytotoxic activity *in vitro*

Measurement of cytotoxic activity against human fetal lung fibroblast MRC-5 cells was carried out as described previously.¹⁹

Isolation of macrotetrolides from the culture broth of *Streptomyces* sp. A00582

Macrotetrolides, including **11**, were isolated from the culture broth of *Streptomyces* sp. A00582 supplied by the Marine Biotechnology Institute, Kitasato University. They were extracted by ethyl acetate and successively washed by *n*-butanol, methanol and *n*-hexane. The insoluble material was then purified by silica gel and LH-20 column chromatography and HPLC. Compounds **9–13** were identified by 2D NMR and MS analysis.

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