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TPU-0031-A and B, New Antibiotics of the Novobiocin Group Produced by *Streptomyces* sp. TP-A0556

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Two novel antibiotics, TPU-0031-A and B, were isolated from the culture broth of an actinomycete strain. The producing strain, TP-A0556, was identified as *Streptomyces* sp. based on the taxonomic study. The new antibiotics were obtained by solvent extraction and chromatographic purification. Spectroscopic analyses showed that TPU-0031-A and B were 7′-demethylnovobiocin and 5″-demethylnovobiocin, respectively. These compounds showed antibiotic activity against Gram-positive and -negative bacteria.

In the course of screening for new antibiotics from actinomycetes associated with plants, we found that new antibiotics TPU-0031-A (**1**) and B (**2**) (Fig. 1) were produced in the fermentation broth of an actinomycete strain TP-A0556. The producing strain was isolated from the plant *Aucuba japonica* Thunb and identified as *Streptomyces* sp. Spectroscopic analyses revealed that these compounds were new analogs of novobiocin (**3**).[1)]

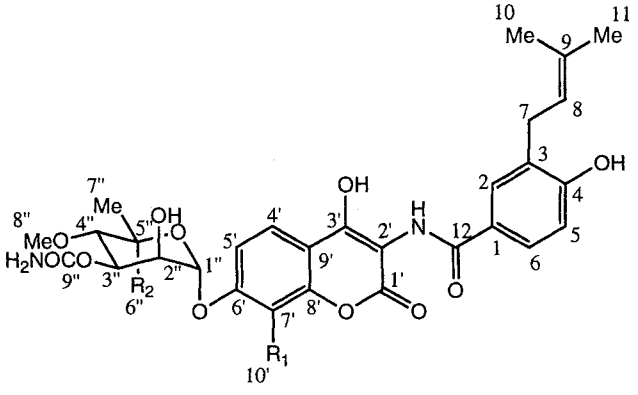
In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structure determination and biological properties of TPU-0031-A and B.

Materials and Methods

Microorganisms

The producing microorganism, strain TP-A0556, was isolated from the wild plant *Aucuba japonica* Thunb collected in Kosugi-machi, Toyama, Japan. The stem of the plant was separated and cut into pieces *ca.* 1 cm in length. They were successively washed with 70% ethanol for 2 minutes and 1% NaClO for 5 minutes. After rinsing with sterilized water, they were placed on an agar plate consisting of agar 1.5%, amphotericin B 0.005% and methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate 0.02% and incubated at 32°C for 30 days. A colony that grew out of a piece of plant was isolated and purified on an agar plate consisting of glucose 0.5%, soluble starch 0.5%, meat

Fig. 1. Structures of TPU-0031-A and B, and novobiocin.



	R_1	R_2
TPU-0031-A	H	CH_3
TPU-0031-B	CH_3	H
novobiocin	CH_3	CH_3

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extract (Kyokuto) 0.1%, yeast extract (Difco Laboratories) 0.1%, NZ-case (Humco Scheffield Chemical Co.) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.5%. A pure culture of strain TP-A0556 was preserved in 20% glycerol at −80°C. It was also maintained at 10°C for laboratory use as a slant on Bennett's agar.

Taxonomy

Taxonomic characteristics of strain TP-A0556 were determined by cultivation on various media described by SHIRLING and GOTTLIEB,[2] WAKSMAN[3] and ARAI.[4] Morphological characteristics were observed after incubation of the culture at 30°C for 14 days on oatmeal agar (ISP. med. 3). Cultural characteristics were determined after growth at 30°C for 14 days. The color names and hue numbers were assigned using the Manual of Color Names (Japan Color Enterprises Co., Ltd., 1987). The carbon utilization was determined by the method of SHIRLING and GOTTLIEB.[2] Cell wall composition was analyzed by the method of LECHEVALIER *et al.*,[5] using thin layer chromatography plates as described by STANECK *et al.*[6]

Instrumental Analysis

Melting points were determined on a Yanagimoto apparatus and are uncorrected. NMR experiments were performed on a JEOL JNM-LA400 NMR spectrometer in CD_3OD at 24°C. The MS spectra were measured on a JEOL JMS-HX110A spectrometer. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were recorded on a Shimadzu FT IR-300 spectrophotometer. Optical rotations were measured on a Horiba SEPA-300 polarimeter.

Biological Assay

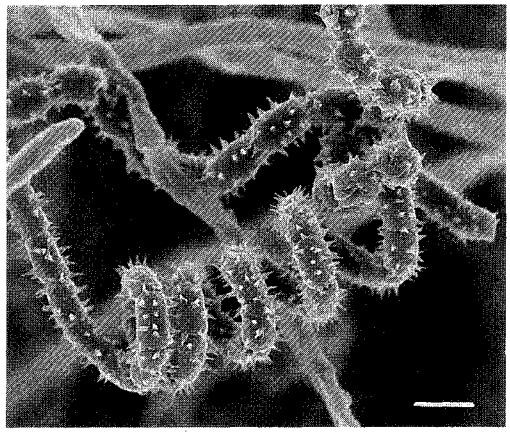
Antibiotic acitivity in fermentation broths and purification samples were monitored by the conventional paper disc assay using *Bacillus subtilis* M-45 as an indicator strain. MIC values were determined by the conventional serial two-fold dilution method against laboratory strains.

Results and Discussion

Taxonomy of the Producing Strain

By scanning electron microscope, the strain TP-A0556 formed spiral type spores and the spores were cylindrical, 0.5~0.7×1.0~1.4 μm in size, having a spiny surface (Fig. 2). The cultural characteristics of TP-A0556 are summarized in Table 1. Color of aerial mycelium was brownish gray and color of reverse side was grayish olive.

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. TP-A0556.



Bar represents 1.0 μm.

Table 1. Cultural characteristics of strain TP-A0556.

Medium	Growth	Aerial mycelium	Reverse side	Diffusible pigment
Yeast extract - malt extract agar (ISP med. 2)	good	Purplish gray (414)	Yellowish brown (99)	None
Oatmeal agar (ISP med. 3)	good	Purplish gray (414)	Grayish olive (169)	None
Inorganic salts - starch agar (ISP med. 4)	moderate	White (388)	Yellowish white (393)	None
Glycerol asparagine agar (ISP med. 5)	poor	White (388)	Yellowish white (393)	None
Tyrosine agar (ISP med. 7)	moderate	Light grayish brown (109)	Dull yellow (152)	None

Diffusible pigments were not formed. The strain TP-A0556 utilized L-arabinose, D-xylose, D-glucose, inositol, D-mannitol, D-fructose, L-rhamnose and raffinose for growth. Analysis of the whole-cell hydrolysates showed the presence of L,L-diaminopimelic acid, glycine, galactose, arabinose and glucose. On the basis of these morphological and chemical characteristics, strain TP-A0556 was assigned to the genus *Streptomyces*.

Fermentation

Streptomyces sp. TP-A0556 cultured on a slant agar medium was inoculated into three 500-ml K-1 flasks each containing 100 ml of the seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case (Humco Scheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The inoculated flasks were shaken on a rotary shaker (200 rpm) at 30°C for 4 days. Three-ml of the seed culture were transferred into fifty 500-ml K-1 flasks each containing 100 ml of the production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2%, pharmamedia (Difco Laboratories) 1.5%, yeast extract (Difco Laboratories) 0.3% and Diaion HP-20 (Mitsubishi Chemical Co.) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were shaken on a rotary shaker (200 rpm) at 30°C for 6 days.

Isolation

The fermented whole broth (5 liters) was centrifuged (8,000 rpm, 15 minutes) to separate the mycelia and the supernatant. The mycelia was extracted with 80% acetone (5 liters) and the extract was concentrated *in vacuo* to an aqueous solution (1 liter). The aqueous solution was combined with the supernatant and applied to a Diaion HP-20 column (1 liter). The column was washed with water (1 liter) and eluted with 40% methanol (1 liter), 80% methanol (2 liters) and acetone (2 liters). The active fraction was concentrated *in vacuo* and extracted twice with ethyl acetate (400 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brown oil (1.97 g). The oily substance was applied to a column of silica gel (Wakogel C-200, 100 g). The column was developed with $CHCl_3$ - MeOH (50 : 1~10 : 1) and the active fractions were combined and concentrated *in vacuo* to give a brown powder (463 mg). This sample was chromatographed on a reversed phase silica gel column (YMC GEL ODS A60; Yamamura Chemical Lab., 0.4 liter) using acetonitrile - 0.15% KH_2PO_4, pH 3.5 (60 : 40), which gave two active fractions containing novobiocin and new analogs. These active fractions were evaporated under reduced pressure to remove acetonitrile and extracted twice with ethyl acetate (20 ml) respectively. Each organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give the mixture of new analogs (24.0 mg) as a brown powder and novobiocin (361.8 mg), respectively. The mixture was further chromatographed on a reversed phase silica gel column (YMC GEL ODS A60, 0.4 liter) using methanol - 0.15% KH_2PO_4, pH 3.5 (30 : 70~80 : 20) to give two active fractions containing TPU-0031-A and B. The fractions were evaporated under reduced pressure to remove methanol and extracted twice with ethyl acetate (20 ml). These organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give TPU-0031-A (2.9 mg) and TPU-0031-B (9.1 mg) as a colorless powder, respectively. No other novobiocin analogs were detected in this culture broth.

Physico-chemical Properties

Physico-chemical properties of TPU-0031-A (**1**) and B (**2**) are summarized in Table 2. These compounds were obtained as colorless powders and are soluble in methanol and ethyl acetate, slightly soluble in acetone and chloroform and insoluble in water. The $[\alpha]_D$ values of **1** and **2** were -116.4 (c=1.00, methanol) and -68.7 (c=0.15, methanol) respectively. The UV spectra of **1** and **2** indicated the existence of the same chromophore in both compounds. The IR spectra suggested the presence of hydroxyl group (3350 cm^{-1}), amide group (1635 cm^{-1}) and lactone carbonyl group (1720 cm^{-1}).

Structure Determination

TPU-0031-A (1)

The structure of TPU-0031-A (**1**) was established on the basis of NMR and MS data. The molecular formula of **1** was determined as $C_{30}H_{34}N_2O_{11}$ based on the HRFAB-MS, which gave a $(M+H)^+$ ion at *m/z* 599.2241 (calcd for $C_{30}H_{35}N_2O_{11}$ 599.2255), and the NMR analyses. The ^1H-NMR spectrum of **1** was very similar to that of novobiocin except for the presence of an aromatic singlet proton at 6.96 ppm instead of the methyl singlet due to C-10′position of novobiocin. In the HMBC spectrum, the aromatic proton was long-range coupled to C-5′, C-6′, C-8′ and C-9′, that confirmed its position at C-7′. Other significant HMBC correlations are shown in Fig. 3. The relative and absolute

Table 2. Physico-chemical properties of TPU-0031-A (**1**) and B (**2**).

	1	**2**
Appearance	Colorless powder	Colorless powder
Mp.	124-126°C	179-180°C
$[\alpha]_D^{28}$ [a]	-116.4 (*c*=1.00, MeOH)	-68.7 (*c*=0.15, MeOH)
HRFAB-MS		
Found:	599.2241 $(M+H)^+$	599.2243 $(M+H)^+$
Calcd:	599.2255 (for $C_{30}H_{35}N_2O_{11}$)	599.2255 (for $C_{30}H_{35}N_2O_{11}$)
Molecular formula	$C_{30}H_{34}N_2O_{11}$	$C_{30}H_{34}N_2O_{11}$
UV λ_{max} nm (log ε)		
in MeOH	209 (4.68), 251 (4.20), 306 (4.13)	214 (4.57), 257 (4.08), 315 (3.97)
in 0.01N HCl-MeOH (1:9)	207 (4.46), 323 (4.22)	213 (4.33), 332 (4.07)
in 0.01N NaOH-MeOH (1:9)	211 (4.71), 251 (4.23), 357 (3.22)	215 (4.50), 257 (4.01), 317 (4.03)
IR ν_{max} (KBr) cm^{-1}	3350, 1720, 1635, 1615	3350, 1720, 1635, 1615
Solubility		
soluble in	methanol, ethyl acetate	methanol, ethyl acetate
slightly soluble in	acetone, chloroform	acetone, chloroform
TLC Rf [b]	0.32	0.32
HPLC Rt (min) [c]	9.0	10.5

[a] novobiocin (lit.[10] –63, *c*=1.00, EtOH)
[b] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=10:1)
[c] HPLC conditions: Cosmosil AR-II (250 x 4.6 mm, i.d.), mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (75:25), flow rate: 0.7 ml/min, detection: UV-254 nm.

configuration of the sugar moiety is determined to be same as that of novobiocin based on the *J* values and $[\alpha]_D$ values. Based on these results, the structure of **1** was determined to be 7′-demethylnovobiocin as shown in Fig. 1.

TPU-0031-B (**2**)

The molecular formula of **2** was determined as $C_{30}H_{34}N_2O_{11}$ based on the HRFAB-MS, which gave a $(M+H)^+$ ion at *m/z* 599.2243 (calcd for $C_{30}H_{35}N_2O_{11}$ 599.2255) and the NMR analyses. In the ^{1}H-NMR spectrum, methyl singlet signals of H-6″ (δ 1.19) and H-7″ (δ 1.39), which were observed in the spectrum of novobiocin, disappeared and a methyl doublet signal (δ 1.25) and methine proton (δ 3.65) were observed. In the ^{13}C-NMR spectrum, one of the methyl signals of C-6″ (δ 23.23) or C-7″ (δ 29.06), which was observed in the spectrum of novobiocin, disappeared. In the homo-decoupling experiment, when the methine proton (δ 3.65) was irradiated, the doublet methyl (δ 1.25) was changed into a singlet signal and the double doublet signal due to H-4″ (δ 3.39) changed into a doublet. Based on this result and the HMBC experiment (Fig. 3), the structure of **2** was determined to be 5″-demethylnovobiocin as shown in Fig. 1. The relative configuration of the sugar moiety was determined as shown in Fig. 1 based on the *J* values. The absolute configuration was tentatively determined in consideration of the biogenesis.

Biological Properties

The antimicrobial activity of TPU-0031-A (**1**) and B (**2**) are shown in Table 4 in comparison with novobiocin (**3**). **1** and **2** exhibited antibacterial activity against Gram-positive and -negative bacteria and no activities against fungi and yeasts. In general, the antimicrobial activities of **1** and **2** were weaker than **3**

The primary molecular target of novobiocin in its antibacterial action is considered to be DNA gyrase.[7] Study on the structure activity relationship demonstrated that novenamine, a substructure consisting of the noviose and the coumarin, was the minimal bioactive structure for the gyrase inhibition.[8] Another report described that the carbamoyl substituent at 3-position of noviose was essential to inhibition activity and chlorobiocin in which the methyl

Table 3. NMR data for TPU-0031-A (**1**) and B (**2**).

Position	**1**		**2**	
	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	124.3		124.3	
2	130.8	7.65 (1H, d, *J*=2.1 Hz)	130.8	7.75 (1H, d, *J*=2.2 Hz)
3	129.9		129.9	
4	160.9		160.9	
5	115.6	6.75 (1H, d, *J*=8.3 Hz)	115.6	6.85 (1H, d, *J*=8.3 Hz)
6	128.5	7.61 (1H, dd, *J*=8.3, 2.4 Hz)	128.5	7.72 (1H, dd, *J*=8.3, 2.4 Hz)
7	29.2	3.24 (1H, d, *J*=7.3 Hz)	29.2	3.33 (2H, m)
8	123.2	5.26 (1H, m)	123.2	5.35 (1H, m)
9	133.9		133.9	
10	17.9	1.65 (3H, s)	17.9	1.74 (3H,s)
11	26.0	1.66 (3H, d, *J*=0.7 Hz)	26.0	1.76 (3H s)
12	169.8		169.8	
1'	163.3*		163.5**	
2'	103.6		103.5	
3'	158.3*		ND**	
4'	126.4	7.79 (1H, d, *J*=9.0 Hz)	123.2	7.80 (1H, d, *J*=9.0 Hz)
5'	115.0	6.97 (1H, dd, *J*=10.0, 2.5 Hz)	112.1	7.23 (1H, d, *J*=9.0 Hz)
6'	161.3		158.3	
7'	103.9	6.96 (1H, s)	115.5	
8'	154.0		151.8	
9'	112.4		112.9	
10'			8.5	2.34 (3H, s)
1''	100.1	5.48 (1H, d, *J*=2.4 Hz)	99.6	5.57 (1H, d, *J*=2.0 Hz)
2''	70.7	4.10 (1H, t, *J*=2.9 Hz)	70.3	4.24 (1H, dd, *J*=3.2, 2.2 Hz)
3''	72.9	5.15 (1H, dd, *J*=9.8, 3.2 Hz)	75.5	5.13 (1H, dd, *J*=9.7, 3.5 Hz)
4''	82.6	3.47 (1H, d, *J*=9.1 Hz)	81.4	3.39 (1H, t, *J*=9.5 Hz)
5''	80.1		70.2	3.65 (1H, dq, *J*=9.5, 6.1 Hz)
6''	23.5	1.07 (3H, s)	18.2	1.25 (3H, d, *J*=6.3 Hz)
7''	28.9	1.26 (3H, s)		
8''	61.9	3.46 (3H, s)	61.0	3.53 (3H, s)
9''	159.1		159.1	

^{1}H and ^{13}C NMR were measured in CD_3OD at 400 MHz and 100 MHz, respectively.

The CD_3OD signals (3.30 ppm for ^{1}H; 49.0 ppm for ^{13}C) were used as a reference.

ND: not detected

*, **: exchangeable

Fig. 3. HMBC analysis of TPU-0031-A and B.

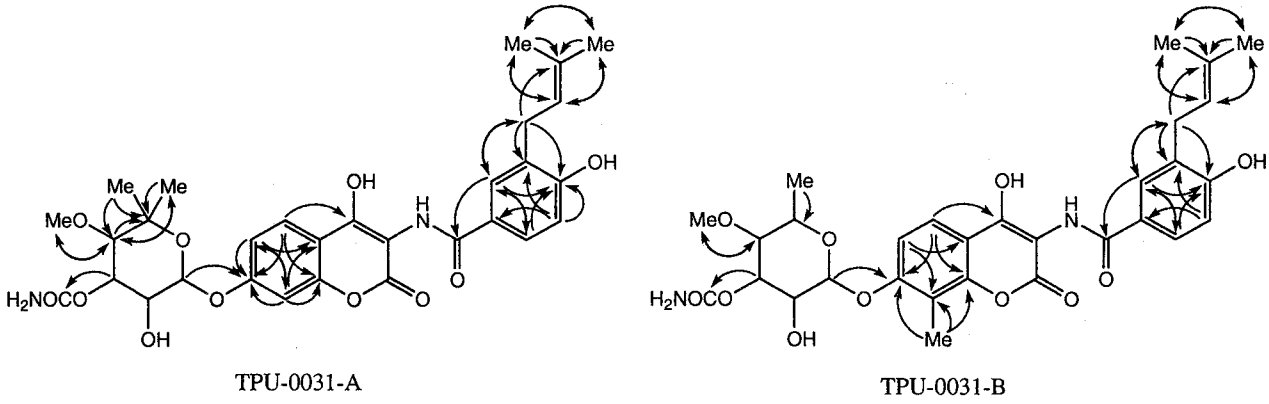


Table 4. *In vitro* antibacterial activities of TPU-0031-A (**1**) and B (**2**), and novobiocin (**3**).

Organism	MIC (μg/ml)		
	1	**2**	**3**
Staphylococcus aureus 209P JC-1	3.1	0.79	<0.20
Staphylococcus aureus F-507 MRSA	12.5	3.1	<0.20
Bacillus subtilis ATCC 6633	50.0	25.0	0.79
Escherichia coli RFM 795	6.3	12.5	<0.20
Escherichia coli NIHJ JC-2	>100	>100	>100
Proteus vulgaris IFO 3851	100	>100	6.3
Proteus mirabilis ATCC 21100	6.3	100	12.5
Pseudomonas aeruginosa A3	>100	>100	>100
Saccharomyces cerevisiae S-100	>100	>100	>100
Candida albicans A9540	>100	>100	>100
Candida tropicalis IFO 1400	>100	>100	>100
Cryptococcus neoformans ATCC 90112	>100	>100	>100
Torulopsis glabrata IFO 0622	>100	>100	>100
Aspergillus fumigatus IFO 8866	>100	>100	>100

group at 7-position of coumarin moiety was substituted with chlorine showed inhibition equivalent to novobiocin.[9] In this study, we showed that loss of either the methyl group at C-10′ or C-6″ lead to the decrease of the activity. This observation is quite interesting in terms of the structure activity relationship of novobiocin and the evolutionary aspect of secondary metabolism.

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