NI15501A, a Novel Anthranilamide Derivative from a Marine Fungus *Penicillium* sp.

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In the course of our discovery program of new metabolites from marine microorganisms, we isolated a novel anthranilamide derivative named NI15501A from *Penicillium* sp. NI15501. In this report, we describe fermentation, isolation, physico-chemical properties, structure elucidation, and biological activities of the substance.

The producing strain *Penicillium* sp. NI15501, identified according to its morphological characteristics[1], was isolated from the marine sediment which was collected at the depth of 14 m in Tomari port at Tomari village, 110 km west of Sapporo, Hokkaido, Japan. The seed culture was fermented at 25°C for 3 days on a rotary shaker in a yeast-malt extract medium with artificial sea water (1.5%). After inoculation of the seed culture containing the same medium as above, the fermentation for production was carried out at 25°C for 5 days.

The fermentation broth (10 liters) was filtered, and the filtrate was absorbed onto an ODS column. Fractions eluted with aqueous acetone-methanol were combined followed by extraction with butanol. The butanolic extract was purified by repeated ODS column chromatography and gel-filtration to afford 2.2 mg of NI15501A.

NI15501A, $[\alpha]_D^{30}$ $-62.8°$ (c 0.147, MeOH), had a molecular formula of $C_{12}H_{15}N_3O_3$ established by high resolution EI mass spectrum (m/z 249.1091 $\Delta = -2.2$ mmu). The presence of an amide bond was inferred from IR (1661, 1618 cm^{-1}), and ^{13}C NMR spectra. Its ^{13}C NMR spectrum showed 12 signals consisting of 2 methyls, 5 methines and 5 quaternary carbons including 3 carbonyls. A 1,2-substituted benzene, an alanyl moiety and one acetyl group were deduced from ^1H and HH-COSY spectra. The 2D NMR analyses (HMQC, HMBC, NOESY) allowed us to connect these partial structures. The amide proton (δ 12.05) showed correlation between two aromatic carbons (δ 119.7, d and s) in the ring and the amide carbonyl (δ 171.8) in the alanyl moiety which correlated with methyl protons (δ 1.31) and the α-proton (δ 4.20). Another amide proton (δ 8.53) exhibited HMBC correlation between the α-proton in the alanyl moiety and a carbonyl carbon (δ 169.8) in the acetyl group which correlated with singlet methyl protons (δ 1.94). Another substituent to the aromatic ring was a primary amide group. This was inferred from long range coupling of one amide proton (δ 7.76) coupled with aromatic carbon (δ 119.7, s) and methine proton (δ 7.78) to carbonyl carbon (δ 170.7). Based on these observations, the planar structure of NI15501A was established as **1**, *N*-acetylalanylanthranilamide (Fig. 1, Tables 1 and 2).

The absolute stereochemistry was successfully elucidated as *S* by comparison of the optical rotation between the isolated compound and the synthetic standard. The standard sample was prepared by coupling of *N*-acetylalanine with anthranilamide. The optical rotation of NI15501A was consistent with that of (*S*)-enantiomer ($[\alpha]_D^{30}$ $-68.6°$). Thus the structure of NI15501A was established.

Its antimicrobial activities were tested at a concentration of 10 μg/ml against several microorganism as follows: *Mycobacterium ranae*, MRSA, *Klebsiella*

Fig. 1. Structure of NI15501A (**1**).

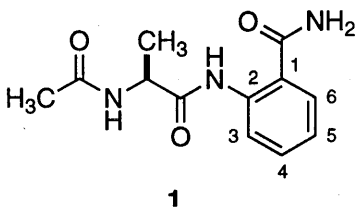

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Table 1. Physico-chemical properties.

Appearance	Colorless solid
$[\alpha]_D^{30}$	$-62.8°$ (c 0.147, MeOH)
Molecular formula	$C_{12}H_{15}N_3O_3$
LR EI-MS m/z (rel. intensity)	249 (9), 231 (28), 188 (82), 163 (67), 146 (52), 136 (100), 119 (50), 87 (67)
HR EI-MS	
Calcd for $C_{12}H_{15}N_3O_3$	249.1091
Found	249.1123
UV λ_{max} (MeOH) nm (ε)	290 (1800), 253 (6100), 216 (12000)
IR ν_{max} (KBr) cm^{-1}	3376, 3267, 3217, 1667, 1618, 1522

Table 2. NMR data of NI15501A in DMSO-d_6.

Position	δ_H[a] (multipulicity, J in Hz)	δ_C[b] (multiplicity)
1-$CONH_2$	8.23, 7.69 (1H each, br)	170.7 s
1		119.7 s
2		139.5 s
3	8.53 (1H, dd, 7.9, 0.8)	119.7 d
4	7.48 (1H, dd, 7.9, 1.3)	132.3 d
5	7.10 (1H, dd, 7.8, 0.8)	122.4 d
6	7.78 (1H, dd, 7.8, 1.3)	128.7 d
Ala CONH	12.05 (1H, s)	171.8 s
Ala α	4.20 (1H, dq, 6.8, 7.3)	50.1 d
Ala β	1.31 (3H, d, 7.3)	17.3 q
Ac CONH	8.47 (1H, d, 6.8)	169.8 s
Ac CH_3	1.94 (3H, s)	22.7 q

[a] Referenced to residual solvent peak at 2.49 ppm.
[b] Referenced to DMSO-d_6 at 39.6 ppm.

pneumoniae, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Trichomonas foetus*, *Candida albicans*, and *Trichophyton mentagrophytes*. At this concentration NI15501A did not exhibit inhibitory activity. No inhibitory activity was shown even at 1 mg/ml against *Bacillus subtilis* and *Escherichia coli*. NI15501A showed no toxicity against mice at 300 mg/kg (po) and at 100 mg/kg (ip) after 3 days.

NI15501A is a new anthranilamide derivative isolated from a marine fungus. It is noteworthy that there have been only a few reports of isolation of anthranilamide derivatives from microorganisms: a fungal metabolite 2-pyruvoylaminobenzamide which was considered to be a precursor of quinazolinone-type alkaloids[2,3]; 2-[(2-hydroxypropionyl)amino]benzamide as a metabolite of *Penicillium chrysogenum*[4]; an antifungal substance 2-acetoamidobenzamide isolated from culture broth of *Streptomyces aurantiogriseus*[5]. Our substance is the first microbial metabolite of an anthranilamide derivative containing an amino acid.

Experimental

Isolation and Fermentation

The producing strain *Penicillium* sp. NI15501 was isolated from the marine sediment which was collected at the depth of 14 m in Tomari port at Tomari village, Hokkaido. The isolation procedure was similar as described in our previous paper[6]. The seed culture was fermented at 25°C for 3 days on a rotary shaker (160 rpm) in a 100-ml medium containing peptone (0.5%), yeast extract (0.3%), glucose (1%), malt extract (0.3%), artificial sea water (1.5%), pH 7 in a 500-ml Erlenmeyer flask. After inoculation of the seed culture into 500-ml Erlenmeyer flasks containing 100 ml of the same medium as above, the fermentation for production was carried out at 25°C for 5 days.

Isolation

The fermentation broth (10 liters) was filtered and the filtrate was absorbed onto an ODS column (Chromatorex ODS 1020TT, 300 g). The column was washed with water, then eluted stepwise with 10, 25, 50, and 75% aqueous MeOH then acetone - MeOH (1 : 1). All the fractions were collected and concentrated to give a residue (8 g) which was further partitioned between BuOH and water. The organic layer was concentrated and extracted with MeOH (20 ml × 3). The methanolic solution was concentrated and applied onto an ODS column (DM1020TT, 100~200 mesh; 30 to 100% aqueous MeOH). 40% - MeOH eluted fraction containing **1** was further purified by Sephadex LH-20 (MeOH) and repeated ODS HPLC (Cosmosil 5C18-AR, 20 × 250 mm, 20 to 30% aqueous CH_3CN). The final purification was carried out by Asahipak GF-310HQ (7.6 × 300 mm, MeOH) to afford 2.2 mg of **1** as a colorless solid.

Synthesis of 1

To a solution of *N*-acetyl-L-alanine (944 mg), *o*-aminobenzamide (816 mg), and 3-hydroxybenzotriazole (1.19 g) in dry DMF (30 ml), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.38 g) was added at 0°C. The mixture was stirred at 0°C for 1 hour and then at room temperature overnight. The mixture was poured into water and extracted with 1-BuOH. The organic layer was concentrated to give a residue, which was chromatographed on SiO_2 ($CHCl_3$ - MeOH 95 : 5 then 90 : 10) to afford 952 mg (53%) of **1** as a colorless needle. An analytical sample was prepared by recrystalization from acetonitrile: MP 203~205°C; $[\alpha]_D^{30}$ −68.6° (*c* 1.00); *Anal.* Calcd for $C_{12}H_{15}N_3O_3$: C 57.82, H 6.07, N 16.86. Found: C 57.77, H 6.12, N 16.90. Other spectroscopic data were in complete agreement with the assigned structure of NI15501A.

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