Bioactive metabolites of the marine actinobacterium *Streptomyces* sp. KMM 7210

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New 3-(4-hydroxybenzyl)piperazine-2,5-dione, together with the known *N*-[2-(4-hydroxyphenyl)ethyl]acetamide (*N*-acetyltyramine), was isolated for the first time from the marine actinobacterium *Streptomyces* sp. The chemical structures of these compounds were determined by NMR spectroscopy and mass spectrometry. The cytotoxic activities of the compounds were estimated from their effects on sperm and eggs of the sea urchin *Strongylocentrotus intermedius*.

Key words: marine actinobacteria *Streptomyces* sp., piperazine-2,5-diones, *N*-acetyltyramine, biologically active substances.

Actinobacteria isolated from marine materials are of interest as producers of biologically active compounds.[1—5] By screening biologically active compounds in ethanol—chloroform extracts of marine actinobacteria, we found that *Streptomyces* KMM 7210 produces substances with an antimicrobial effect on Gram-positive bacteria and a cytotoxic effect on the gametes of the sea urchin *S. intermedius*.

The combined extracts of the cultural broth and the biomass were extracted successively with hexane, ethyl acetate, and butanol. The ethyl acetate extract that exhibited the cytotoxic activity was separated into two fractions 1 and 2 on Sephadex LH-20 in methanol.

3-(4-Hydroxybenzyl)piperazine-2,5-dione (**1**) (1.3 mg) was isolated from fraction 1 by HPLC. The molecular mass of compound **1** (220 Da) was determined from the value of the quasimolecular ion $[M - H]^-$ (*m/z* 219) by chemical ionization mass spectrometry (CIMS). The ^{1}H NMR spectrum of compound **1** contains two doublets for four protons; their chemical shifts and multiplicity indicate the presence of the *para*-substituted aromatic ring (Table 1). The ^{13}C NMR spectrum of compound **1** shows, apart from the signals for the C atoms of the hydroxyphenyl fragment, signals for a methine and a methylene C atoms (δ_C 57.6 and 40.4); a signal at δ_C 171.1 can be assigned to the amide C atom. Our spectroscopic data are close to the literature data for the tyrosine residue in some peptides.[6,7] The signals for two protons at δ_H 2.6 and 3.5 in the ^{1}H NMR spectrum of compound **1** (see Table 1) were assigned to the α-methylene group of the glycine fragment.[8] The glycine residue was also identified from the presence of signals for another amide C atom and another methylene C atom (δ_C 169.9 and 44.7). Complete acid hydrolysis of compound **1** gave two amino acids identified as glycine and

Table 1. ^{1}H and ^{13}C NMR spectra of compound **1**[a] (500 MHz, CD_3OD)

Atom	δ_C	δ_H, *J*/Hz
	Tyr	
C(2)	171.2 [172.8]	—
α-CH	57.6 [54.0]	4.28 m [4.53 m]
β-CH_2 (H_a)	40.2 [37.0]	2.93 (dd, *J* = 4.6, *J* = 14.0) [2.97 m]
(H_b)		3.16 (dd, *J* = 3.5, *J* = 14.0) [3.42 m]
C(1′)	127.4 [127.1]	—
C(2′), C(6′)	133.2 [131.5]	7.04 (d, 2 H, *J* = 8.5) [6.76 d]
C(3′), C(5′)	117.0 [115.7]	6.79 (d, 2 H, *J* = 8.5) [6.59]
C(4′)	157.3 [157.3]	—
	Gly	
C(5)	169.9 [168.4][b]	—
α-CH_2 (H_a)	44.8 [42.6][b]	2.6 (dd, *J* = 1.2, *J* = 18.1) [3.37 m][b]
α-CH_2 (H_b)		3.5 (dd, *J* = 0.9, *J* = 18.1) [3.99 m][b]

[a] The literature data in brackets refer to the tyrosine and glycine fragments.[6—8]

[b] The spectra were recorded in DMSO-d_6.

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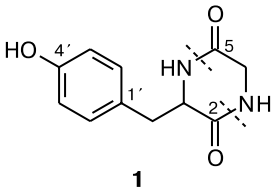
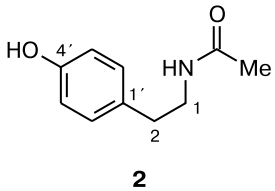
L-tyrosine (HPLC data for their derivatives).[9] The mass and NMR spectra suggested that compound **1** is cyclic dipeptide (*cyclo*-(Gly-L-Tyr)). Although piperazine-2,5-diones (products of cyclocondensation of two amino acids) are widely known as biologically active metabolites of fungi[10] and bacteria,[11—15] *cyclo*-(Gly-L-Tyr) is a novel compound isolated for the first time from *Streptomyces* sp. Compound **1** is weakly cytotoxic against the sea urchin sperm and eggs. The percentage of the damaged cells was determined from the level of esterase activity with diacetylfluorescein as a substrate.[16] At a concentration of 50 μg mL^{-1}, the egg membranes remained intact and only 25% of the sperm membranes was damaged. However, the fertilized sea urchin eggs were more sensitive to compound **1**. So, at a concentration of 25 μg mL^{-1}, compound **1** stopped the development of embryos in the step of 2—4 blastomeres.



1



2

Column chromatography of fraction 2 on silica gel in hexane—ethyl acetate (100 : 0 → 0 : 100) gave *N*-acetyltyramine (**2**) (13 mg). Its molecular mass (179 Da) was determined from the value of the quasimolecular ion $[M + H]^+$ (*m/z* 180) by CIMS. The ^{1}H and ^{13}C NMR spectra contain signals characteristic of the *para*-substituted aromatic ring (Table 2). In addition, the ^{13}C NMR spectrum shows signals for an amide and two methylene C atoms (δ_C 173.8, 36.2, and 42.9). The presence of the CH_2CH_2 fragment between the aryl and *N*-acetylamide groups was confirmed by ^{1}H—^{1}H COSY data. Thus, compound **2** was identified as *N*-acetyltyramine.[17] It should be noted that compound **2** has been earlier isolated from the marine fungus *Fusarium* sp. and the actinomycete *Streptomyces griseus*.[17,18] At a concentration of 25 μg mL^{-1}, compound **2** stopped the development of sea urchin embryos in the step of 2—4 blastomeres. At a concentration of 50 μg mL^{-1}, compound **2** did not damage the egg membranes and only 15% of the sperm membranes of the sea urchin were lysated.

Table 2. ^{1}H and ^{13}C NMR spectra of compound **2** (500 MHz, CD_3OD)

Atom	δ_C	δ_H* (*J*/Hz)
C(1′)	131.9	—
C(2′), C(6′)	131.3	7.02 (d, 2 H, *J* = 8) [7.02]
C(3′), C(5′)	116.8	6.70 (d, 2 H, *J* = 8) [6.73]
C(4′)	157.5	—
C(2)H_2	36.3	2.67 (t, 2 H, *J* = 7.3) [2.67]
C(1)H_2	43.0	3.32 (t, 2 H, *J* = 7.3) [3.37]
CO	173.8	—
Me	23.1	1.89 (s, 3 H) [1.88]

* The ^{1}H NMR data for *N*-acetyltyramine[18] are given in brackets.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-500 and Bruker DPX-300 spectrometers in CD_3OD with Me_4Si as the internal standard. Mass spectra were recorded on Varian MAT 371 (70 eV) and Varian 311 A spectrometers (70 eV). UV spectra were recorded on UV-1601PC spectrophotometers (Shimadzu, Japan). TLC was carried out on Sorbfil plates (Silica gel STKh-1A, ZAO Sorbpolimer) in chloroform—methanol (90 : 10) and chloroform—methanol (80 : 20). Spots were visualized with 4-methoxybenzaldehyde prepared according to a known procedure.[19] Marfey´s reagent *N*-α-(5-fluoro-2,4-dinitrophenyl)-L-alanylamide and D- and D,L-tyrosines were purchased from Sigma. The antibacterial activity and effects of the compounds on fertilized eggs of the sea urchin were studied as described earlier.[20]

Cultivation of the bacterium *Streptomyces* sp. and isolation of compounds 1 and 2. The bacterium was isolated from bottom sediments in the Sea of Japan (Troitsa Bay, Posiette Bay, August, 2001) and cultivated in the medium of the following composition: edible potato starch (10 g L^{-1}), peptone (2 g L^{-1}), a yeast extract (2 g L^{-1}), $CaCO_3$ (1 g L^{-1}), pH 7.7, distilled water—sea water (1 : 1). Fermentation was carried out under continuous aeration at ~20 °C for 6 days. The total volume of the cultural broth was 10 L. The cells were separated from the cultural broth by filtration. The bacterial cell walls were broken by ultrasound. The cultural broth was extracted with butanol and the cells were extracted with ethanol and acetone. The extracts were evaporated to dryness and combined. The total weight of the dry residue was 300 mg.

The dry residue was dissolved in water—ethanol (4 : 1) (800 mL) and the products were extracted successively with hexane (3×200 mL), ethyl acetate (3×250 mL), and butanol (3×250 mL). The ethyl acetate extract was concentrated and the residue was dissolved in methanol and chromatographed on Sephadex LH-20 in methanol. Two fractions were obtained. Fraction 1 was separated by HPLC (column 250×4 mm, Diaspher-110-C_{18} (Biokhimmak)), a Waters-510 chromatograph with an LKB Bromma 2151 detector, λ_{det} = 235 nm, flow rate of water—acetonitrile (89 : 11) 0.4 mL min^{-1}). The retention time of compound **1** was 13 min. The yield of compound **1** with respect to the weight of the combined extract was 0.43%. Fraction 2 was chromatographed on silica gel in hexane—ethyl acetate with an increase in the polarity of the system. Compound **2** (13 mg) was eluted from the silica gel with ethyl acetate. The yield with respect to the dry residue was 4.3%.

3-(4-Hydroxybenzyl)piperazine-2,5-dione (1), $C_{11}H_{12}N_2O_3$, is an amorphous powder well soluble in MeOH. The powder turns blue upon a reaction with 4-methoxybenzaldehyde. CIMS, *m/z*: 219 ($[M - H]^-$). UV (MeOH), λ_{max}/nm: 227.8 (ε 1.748), 276.4 (ε 0.358). The ^{1}H and ^{13}C NMR spectra are given in Table 1.

***N*-[2-(4-Hydroxyphenyl)ethyl]acetamide (2)**, $C_{10}H_{13}NO_2$, is an amorphous powder. CIMS, *m/z*: 180 ($[M + H]^+$).

Table 3. Retention times τ of *N*-α-(5-fluoro-2,4-dinitrophenyl)-L-alanylamide derivatives of amino acids during HPLC on Diasfer-110-C_{18}

Amino acid derivatives	τ/min
L-Tyrosine	19.2
D-Tyrosine	23.4
Glycine	16.5
Hydrolyzate of **1**	19.4, 16.2

UV (MeOH), λ_{max}/nm: 200.4 (ε 1.507), 224.4 (ε 0.492), 277.4 (ε 0.113). The ^{1}H and ^{13}C NMR spectra are given in Table 2.

Acid hydrolysis of compound 1 and the synthesis of Marfey´s derivatives. Complete acid hydrolysis of compound **1** was carried out according to a known procedure.[21] The resulting mixture of amino acids (1 mg) was dissolved in distilled water (0.5 mL). Then 1 *M* $NaHCO_3$ (2 mL) and a 1% solution of Marfey´s reagent in acetone (1 mL) were added. The reaction mixture was kept at 37 °C for 75 min and then 2 *M* HCl (0.2 mL) was added. The resulting solution was analyzed by HPLC.

Marfey´s derivatives were separated by HPLC on a Diaspher-110-C_{18} column (250×4 mm, Biokhimmak; Agilent 1100 chromatograph). Detection was performed at λ = 340 nm (flow rate 0.7 mL min^{-1}, water—acetonitrile, concentration gradient of acetonitrile from 1 to 30%, 30 min, 25 °C). The retention times of Marfey´s derivatives for the amino acids in compound **1** were compared with those for derivatives of standard amino acids (Table 3).

Degree of damage of the sea urchin sperm or egg membranes was determined from the level of the esterase activity with diacetylfluorescein (DAF) as a substrate.[16] Test compounds in concentrations of 25, 50, 75, and 100 μg mL^{-1} were added to a suspension of sperm cells ($2.5 \cdot 10^7$ cells mL^{-1}) or eggs ($3.5 \cdot 10^3$ cells mL^{-1}) in sea water (300 μL). Microplates were kept at 22 °C for 30 min and then 3 μL of DAF (1 mg mL^{-1} in acetone) was added. After 30 min, fluorescence was measured on a Microplate Fluorescence Reader FL_X 800 (Finland) at a E_x/E_m ratio of 425/528 nm. The blank sample contained the same amounts of the test compound and DAF in sea water without sperm or eggs. Before addition of DAF, we measured the background fluorescence intensity at the above wavelengths.

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