Chemistry of Natural Compounds, Vol. 42, No. 6, 2006

INDOLIC METABOLITES FROM THE NEW MARINE BACTERIUM *Roseivirga echinicomitans* KMM 6058^T

G. K. Oleinikova,[1*] O. I. Ivchuk,[2] V. A. Denisenko,[1] E. L. Chaikina,[1] N. I. Menzorova,[1] O. I. Nedashkovskaya,[1] and T. A. Kuznetsova[1]

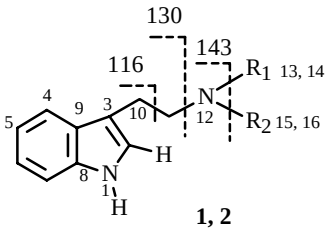
UDC 547.752:577.19: 615.331

*N-Acetyl- (**1**) and N,N-diacetyl- (**2**) tryptamines were isolated from the butanol extract of culture medium of the new marine bacterium* Roseivirga echinicomitans *KMM 6058^T. The structures of the compounds were proved using mass spectrometry, UV, PMR, and ^{13}C NMR spectroscopy and by comparing these data with mass and NMR spectra of synthetic samples of **1** and **2**. Compound **2** was isolated from a natural source and synthesized for the first time. The cytotoxic activity of the compounds was studied using Erlich carcinoma tumor cells, murine erythrocytes, and sperm and egg cells of the sea urchin* Strongylocentrotus intermedius.

Key words: acetyltryptamine, NMR spectroscopy, marine bacterium *Roseivirga echinicomitans*, indolic metabolites, biological activity.

Marine bacteria are good sources for producing physiologically active compounds [1, 2]. During a search of such compounds, we investigated the new marine bacterium *Roseivirga echinicomitans* KMM 6058^T, which is associated with the sea urchin *Strongylocentrotus intermedius*. The bacterium cultivated in liquid medium produced compounds that exhibited cytotoxic activity toward murine erythrocytes.

Fractional extraction followed by column chromatography over silica gel isolated from bacterium culture (2 L) two compounds (0.2 mg each), **1** with R_f 0.2 and **2** with R_f 0.8 using hexane:ethylacetate (1:1). The UV spectra of the compounds had three absorption maxima at 274.4, 281.4, and 290.2 nm, which are characteristic of indole [3]. The PMR spectrum of **1** contained two broad singlets for amide protons at δ_H 8.05 and 5.5 ppm that were assigned to protons on nitrogens of the indole ring and a side chain, respectively [4]. Furthermore, signals of four protons appeared at 7-7.65 ppm as two doublets with SSCC 7.8 and 9.0 Hz and two multiplets. The position of the signals at weak field and their multiplicities were consistent with a disubstituted benzene ring in **1**. A signal at weak field (7.05 ppm, doublet, J = 2.0) was assigned to the C-2 proton in the indole ring [4]. The spectrum also contained two signals for two protons at 3.0 ppm (triplet) and two protons at 3.6 ppm (multiplet), which is characteristic of an $R_3C–CH_2–CH_2–NH–$ system [4]. A 3H singlet at 1.92 ppm was assigned to an acetyl methyl.



1, 2

1: R_1 = H, R_2 = $COCH_3$
2: R_1 = R_2 = $COCH_3$

1) Pacific Institute of Bioorganic Chemistry, Far-Eastern Division, Russian Academy of Sciences, 690022, Vladivostok, pr. 100-Letiya Vladivostoka, 159, fax (4232) 31 40 50, e-mail: oleingk@mail.ru; 2) Far-Eastern State University, 690000, Vladivostok, ul. Sukhanova, 8, Russian Federation. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 581-584, November-December, 2006. Original article submitted October 5, 2006.

0009-3130/06/4206-0713

TABLE 1. PMR and ^{13}C NMR Spectra of **1** and **2** (δ, ppm, J/Hz, $CDCl_3$)

Atom	^1H		^1H-^1H-COSY	HMBC	^{13}C	
	1	**2**			**1**	**2**
NH	8.05 br.s	8.05 br.s			-	-
2	7.05 (d, 2.0)	7.01 (d, 2.8)		3	129.9	122.3
3	-	-			113.1	112.4
4	7.61 (d, 7.8)	7.67 (d, 7.4)	5		118.7	118.7
5	7.14 m	7.15 m	4, 6		119.6	119.7
6	7.22 m	7.22 m	5, 7		122.6	122.3
7	7.39 (d, 9.0)	7.38 (d, 7.9)			111.2	111.3
8	-	-			136.4	136.2
9	-	-			127.4	127.2
10	3.0 (t, 6.6)	3.05 (t, 7.0)		3, 11	25.3	24.8
11	3.8 m	3.95 (t, 8.3)		10, 13	39.8	46.0
12-NH	5.5 br. s	-	11a	11	-	-
13-CO	-	-			170.0	173.4
14-CH_3	1.92 s	2.35 s		13	23.3	26.4
15-CO	-	-			-	173.4
16-CH_3	-	2.35 s		15	-	26.4

The PMR spectrum of **2** was practically identical to that of **1**. However, it contained a signal for an amide proton at 5.5 ppm. Signals for four protons at 3.05 and 3.95 ppm appeared as triplets, which is characteristic of an acyclic $R_3C–CH_2–CH_2–NR_2$ system [4]. A strong singlet corresponding to six protons of two acetyl methyls was shifted to weak field (2.35 ppm compared to 1.92 for **1**). PMR spectra of the indole part of the compounds agreed completely with spectra of indolic metabolites isolated from the bacterium *Xenorhabdus bovienii* A2 [5] and the marine cyanobacterium *Lyngbya majuscula* [6].

Electron-impact mass spectra (EIMS) of **1** and **2** gave molecular weights of 202 and 244 Da, respectively, which differ by 42 mass units ($COCH_3$ group). Both spectra contained the same set of fragment ions with *m*/*z* 116, 130, and 143 (Fig. 1).

The PMR and mass spectra suggested that **1** and **2** were mono- and diacetyltryptamine, respectively.

In order to confirm the proposed structures, **1** and **2** were synthesized by acetylation of tryptamine with acetic anhydride. TLC, UV, mass, and PMR spectra confirmed that the natural and synthetic compounds were identical. ^{13}C NMR spectra were obtained and HMBC, HSQC, COSY, and DEPT experiments (Table 1) were performed for the synthetic compounds and enabled signals for C and H atoms in **1** and **2** to be completely assigned.

The ^{13}C NMR spectrum of **1** confirmed the presence of a carbamide (δ_C 170 ppm). Eight signals in the range 110-140 ppm, three of which were quaternary and five of which were tertiary, were characteristic of indole. The spectrum contained two signals for CH_2 of a side chain at 39.8 and 25.3 ppm and a signal for an acetyl methyl at 23.4 ppm. The C atoms and H atoms corresponding to them were unambiguously assigned using HSQC, COSY, and HMBC correlation spectra. The bonding sequence of C atoms in **1** was also determined (Table 1).

The ^{13}C NMR spectrum of **2** was almost identical to that of **1**. The signals for the indole C atoms coincided completely. However, the signal for the CH_2 bonded to N was shifted to weak field at 46.2 ppm and the singlet for the methyl was twice as strong and also shifted to weak field at 26.4 ppm. The spectrum contained a signal for carbamide C atoms at weak field at 173.6 ppm that was twice as strong, confirming the presence of two equivalent acetyls on the N in the side chain. The C atoms and H atoms corresponding to them were completely assigned using HSQC, COSY, and HMBC correlation spectra. The bonding sequence of the C atoms in **2** was also confirmed (Table 1).

Thus, it was established that the isolated natural compounds **1** and **2** were mono- (**1**) and diacetyltryptamine (**2**). The ability of bacteria to acetylate tryptamine to the diacetyl derivative was observed for the first time. The bacterium *R. echinicomitans* KMM 6058^T is the only natural source of diacetyltryptamine. Tryptamine itself can be generated in bacteria from endogeneous tryptophan [7]. Monoacetylated tryptamine was isolated previously from the actinobacterium *Streptomyces staurospores* AM-2282 [8] and the marine bacterium *Cytophaga* sp. AM 13.1 [9].

The alcohol extract of sea urchin does not contain similar compounds, which confirms that the bacterium produced **1** and **2**.

TABLE 2. Biological Activity of **1** and **2**

Compound	1*		2*	3*	4*
	sperm	egg cells			
1	>50	>50	0	>50	>100
2	7.5	15	100	>6.25<12.5	100

1*, concentration (μg/mL) producing 50% destruction of membrane integrity of sperm and egg cells of sea urchin; 2*, percent inhibition of division of fertilized egg cells of sea urchin at a compound concentration of 50 μg/mL; 3*, compound concentration (μg/mL) causing 100% hemolysis of murine erythrocytes; 4*, compound concentration (μg/mL) causing 50% death of Erlich carcinoma tumor cells.

Table 2 shows that **1** and **2** are weakly cytotoxic toward Erlich carcinoma tumor cells (4^*). The hemolytic activities of **1** and **2** (3^*) showed that **2** is more highly cytotoxic than **1**. Both compounds were checked for the ability to destroy membranes of sperm and egg cells of *S. intermedius*. The percent destruction of membranes was determined from the change of esterase activity using diacetylfluorescein (DAF) as substrate [10]. Compound **2** caused 50% membrane destruction of sperm and egg cells at concentrations of 7.5 and 15 μg/mL, respectively. Compound **1** at 50 μg/mL had no effect on the membrane integrity of these cells. The difference in the cytotoxic activities of these compounds was also noted using fertilized sea-urchin egg cells as the biological model (2^*). At 50 μg/mL, **1** did not inhibit development of embryos whereas **2** caused 100% inhibition of division. Both compounds showed no antimicrobial activity toward gram-positive and gram-negative bacteria and yeast-like fungi.

EXPERIMENTAL

A culture of *R. echinicomitans* KMM 6058^T was isolated from gonads of *S. intermedius* [11], specimens of which were obtained at the Marine Experimental Station of PIBOC FED RAS in Troits Bay (October 2005).

Centrifugation was performed on a MLW K-23 apparatus (Germany) (V = 100 mL) at $v = 4.5\times10^3$ rpm for 30 min with cooling; HPLC, on a Beckman—Altex (USA) chromatograph, 4.6 mm × 25 cm column, Silosorb 600, 12 μm; TLC, on silica-gel plates (CTX-1A, 5-17 μm, 100×50) using hexane:ethylacetate (1:1) with development by anisaldehyde [12] and H_2SO_4.

UV spectra in methanol were obtained on a Specord M 40 UV—Vis spectrophotometer (Germany).

PMR and ^{13}C NMR spectra in $CDCl_3$ were recorded on Bruker DRX-500 (500 and 125 MHz) and Bruker DPX-300 (300 and 75 MHz) instruments with TMS internal standard. Structures of the studied compounds were established and signals in PMR and ^{13}C NMR spectra were assigned based on DEPT, COSY, HSQC, and HMBC experiments.

Electron-impact mass spectra (EIMS) were obtained in an LKB-9000S instrument (Sweden) using direct sample introduction and ionizing potentials 15 and 70 eV.

The cell model for determining membrane integrity were sperm and egg cells of the sea urchin *S. intermedius*. The percent destruction of membranes was determined using esterase activity and diacetylfluorescein (DAF) as substrate [10]. Fluorescence was estimated on a Microplate Fluorescence Reader FL_X 800 (Finland) using E_x/E_m 425/528 nm.

Analysis of Alcohol Extract of *S. intermedius*. Sea urchins (20 specimens) were extracted by ethanol (1 L) at room temperature for 30 d. The resulting extract was concentrated at reduced pressure and analyzed by TLC on silica-gel plates with development by anisaldehyde.

Bacterium Cultivation. Bacterium culture was fermented on rockers in 250-mL Ehrlenmeyer flasks for 7 d at 22°C in standard medium containing peptone (5 g), yeast extract (2.5 g), glucose (1 g), K_2HPO_4 (0.2 g), $MgSO_4$ (0.05 g), tapwater (500 mL), and seawater (500 mL).

Preparation of Total Extracts. Culture (2 L) was centrifuged. The microbial mass was separated. Culture liquid (CL) was successively extracted with hexane, ethylacetate, and butanol. The extracts were concentrated at reduced pressure. The initial extracts were dissolved in a minimum amount of water and reextracted with hexane, ethylacetate, and butanol to produce total hexane, ethylacetate, and butanol extracts of CL.

Isolation of Indolic Compounds from Total Second Butanol CL Extract. The butanol extract was separated by repeated column chromatography over silica gel using hexane:ethylacetate (100:0—0:100) and HPLC using a normal column and hexane:ethylacetate (1:1) to isolate **1** and **2** (0.2 mg each).

Synthesis of Acyltryptamines. Tryptamine (40 mg) was boiled in acetic anhydride (1.5 mL) for 1.5 h at 140°C. The excess of acetic anhydride was distilled in vacuo. The resulting solid was chromatographed over silica gel using hexane:ethylacetate (100:0—0:100) followed by HPLC over a normal column using hexane:ethylacetate (1:1) to isolate two compounds that were identical chromatographically to natural **1** (20 mg) and **2** (10 mg).

***N*-Acyltryptamine (1)**, $C_{12}H_{14}N_2O$, colorless compound. UV spectrum (MeOH, λ_{max}, nm): 274.4, 281.4, 290.2. Mass spectrum (EI, *m*/*z*): 202 $[M]^+$, 144 $[M - NHCOCH_3]^+$, 130 $[M - CH_2NHCOCH_3]^+$, 116 $[C_8H_6N]$. Table 1 gives the PMR and ^{13}C NMR spectra.

***N,N*-Diacyltryptamine (2)**, $C_{14}H_{16}N_2O_2$, colorless compound. UV spectrum (MeOH, λ_{max}, nm): 274.4, 281.4, 290.2. Mass spectrum (EI, *m*/*z*): 244 $[M]^+$, 144 $[M - N(COCH_3)_2]^+$, 130 $[M - CH_2N(COCH_3)_2]^+$, 116 $[C_8H_6N]$. Table 1 gives the PMR and ^{13}C NMR spectra.

Determination of Hemolytic Activity. Hemolytic activity was determined using a suspension of blood erythrocytes from white mongrel mice of optical density 1.0 at 700 nm in 66 mM phosphate buffer at pH 7.4 containing NaCl (120 mM) and KCl (4 mM). The erythrocyte suspension (200 µL) was treated with the studied compound at concentrations of 50, 25, 12.5, and 6.25 µg/mL and incubated at 37°C for 60-180 min. Lysis (100%) was observed visually [13].

Determination of Cytotoxic Activity. Cytotoxic activity was determined using an alcohol solution (0.001 mL) of the studied compound at various concentrations to treat a suspension (0.1 mL) of Erlich carcinoma tumor cells in medium 199 (1×10^6 cells/mL). The vitality of the tumor cells was determined after incubation at 37°C for 2 h using coloration by an isotonic solution of trypan blue (0.17%) and a microscope. The concentration that inhibited tumor-cell vitality by 50% (IC_{50}) was determined [14].

Determination of Membrane Destruction of *S. intermedius* Sperm Cells. A suspension (300 µL) of sperm (2.5×10^7 cells/mL) or egg (3.5×10^3 cells/mL) cells in seawater (2.5×10^7 cells/mL) was treated with the tested compounds at various concentrations. Microplates were held at 22°C for 30 min, treated with DAF (3 µL, 1 mg/mL acetone), and held again for 30 min. The fluorescence was estimated. The control was seawater for which the background fluorescence was determined before adding DAF.

Activities of compounds toward fertilized sea-urchin egg cells were determined using the literature method [15]. **Antimicrobial activity** was determined by a diffusion method in agar in Petri dishes. The test cultures were the bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and the yeast-like fungus *Candida albicans* from the American Type Culture Collection (ATCC) and the Collection of Marine Microorganisms (KMM) of the PIBOC FED RAS.

ACKNOWLEDGMENT

We thank O. S. Radchenko of the PIBOC FED RAS for help with synthesizing the acetyltryptamines. The work was supported by grants of the RFBR No. 06-04-48578 and 05-04-48211; state contracts No. 02.445.11.7263, 02.452.11.7041, and 02.445.11.7280 of the Federal Agency for Science and Innovation of the RF Ministry of Education and Science; and a grant of the FED RAS Presidium No. 06-III-A-05-121.

REFERENCES

1. J. W. Blunt, B. R. Copp, M. G. Munro, P. T. Northcote, and M. R. Prinsep, *Nat. Prod. Rep.*, **22**, 15 (2005).
2. W. Gul and M. T. Hamann, *Life Sci.*, 422 (2005).
3. J. A. Joule, G. Smith, and K. Mills, *Heterocyclic Chemistry*, 3rd Ed., Chapman and Hall, New York (1994).
4. B. Riemer, O. Hofer, and H. Greger, *Phytochemistry*, **45**, 337 (1997).
5. J. Li, G. Chen, and J. M. Webster, *J. Nat. Prod.*, **58**, 1081 (1995).
6. L. T. Tan, T. Okino, and W. H. Gerwick, *J. Nat. Prod.*, **67**, 952 (2000).

7. T. A. Dvornikova, *Microbiological Synthesis and Transformation of Indolic Compounds* [in Russian], Shtiintsa, Kishinev (1980).
8. Y. Shu-Wei and G. A. Cordell, *J. Nat. Prod.*, **60**, 44 (1997).
9. M. Shaaban, R. P. Maskey, I. Wagner-Dobler, and H. Laatsch, *J. Nat. Prod.*, **65**, 1660 (2002).
10. B. Rotman and B. W. Papermaster, *Proc. Nat. Acad. Sci. USA*, **55**, 134 (1966).
11. O. I. Nedashkovskaya, S. Bum Kim, A. M. Lysenko, M. S. Park, V. V. Mikhailov, K. S. Bac, and H. Y. Park, *Int. J. Syst. Evol. Microbiol.*, **55**, 1797 (2005).
12. A. A. Lur′e, *Chromatographic Materials (Handbook)* [in Russian], Khimiya, Moscow (1978).
13. N. G. Prokof′eva, E. B. Shentsova, S. I. Stekhova, et al., *Izv. Akad. Nauk, Ser. Biol.*, 493 (1999).
14. N. G. Prokof′eva, N. K. Utkina, E. L. Chaikina, and A. E. Makarchenko, *Comp. Biochem. Physiol. B: Biochem, Mol. Biol.*, **139**, 169 (2004).
15. N. G. Prokof′eva, M. M. Anisimov, N. M. Rebachuk, M. M. Kisileva, and N. D. Pokhilo, *Izv. Akad. Nauk, Ser. Biol.*, 645 (2002).