Secondary Metabolites from a *Streptomyces* Strain Isolated from Livingston Island, Antarctica

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Streptomyces sp. 1010, Secondary Metabolites

The producing strain *Streptomyces sp.* 1010 was isolated from a shallow sea sediment from the region of Livingston Island, Antarctica. From the culture broth of this strain naturally active secondary metabolites were isolated identical to phthalic acid diethyl ester ($C_{12}H_{14}O_4$, MW. 222); 1, 3-bis (3-phenoxyphenoxy)benzene ($C_{30}H_{22}O_4$, MW.446); hexanedioic acid dioctyl ester ($C_{22}H_{42}O_4$, MW.370) and the new substance 2-amino- 9, 13 -dimethyl heptadecanoic acid ($C_{19}H_{39}NO_2$, MW.313). These compounds represent diverse classes of chemical structures and provide evidence for the untapped biosynthetic potential of marine bacteria from Antarctica.

Introduction

Livingston Island is the second largest of the South Shetland Islands. Its area is 845 km^2 and 90% of its territory is perpetually covered with ice, the remaining 10% is without ice and snow during the Antarctic summer (Chipev and Velchev, 1996; Lopez-Martinez *et al.*, 1992). The first reports about Antarctic microflora were published at the beginning of this century by (Tsiklinsky, 1908), who had collected data during the French Antarctic Expedition in 1903–1905. Interesting information about the bacterial microflora of Antarctica was also gathered by (Darling and Siple, 1941). They isolated 178 bacterial cultures from ice, water, plant remains and soil.

Based on the preferred temperature of their environment, microorganisms are divided into three groups: psychrophiles (optimal growth temperature 15 °C or lower and minimum temperature for growth at 0 °C. Mesophiles that grow at 5 °C or lower, but show higher (20–28 °C) optimal growth temperatures and thermophiles grow at 45–90 °C (Morita, 1975). Some authors distinguish also a fourth group, the so-called extreme thermophiles, thriving under optimum temperatures of 90–110 °C (Atlas and Bartha, 1992). In microbial ecosystems of Antarctica, psychrophilic and mesophilic organisms play a major role in the biodegradation of organic matter (Chipeva *et al.*, 1996). According to the data reported by (Grossmann, 1994; Grossmann and Dieckmann, 1994), the total quantity of bacteria in water samples from the Antarctic region varies from 1×10^8 to 3×10^8 per litre. The microorganisms isolated from water samples have become important subjects of study in the search for unique, biologically active secondary metabolites. There are no data available in the literature on the isolation and structure elucidation of biologically active secondary metabolites from *Streptomyces* strains, isolated from antarctic water samples (Christova and Naidenova, 1996).

The objective of the present study was to isolate and determine the structure of biologically active secondary metabolites produced by *Streptomyces sp., strain* 1010.

Materials and Methods

Streptomyceal material

Streptomyces strain 1010 was isolated from the shallow sea sediment from the region of Livingston Island, Antarctica and has been classified by morphological and chemotaxonomical methods.

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Sampling had been performed in the course of the Spanish scientific expedition undertaken during the Antarctic summer of 1988/89. A culture of the strain *Streptomyces sp. 1010* was deposited in the collection of Department of Microbiology, Faculty of Pharmacy, University of Barcelona, Spain. Based on the effect of temperature on its growth rate is a mesophil (minimal temperature for growth at 5 °C, but optimal temperature for growth at 20–22 °C).

The cells of *Streptomyces sp.* 1010 were grown in a medium containing 3.0 g meat extract, 10.0 g peptone, 5.0 g NaCl, 4.0 g yeast extract, 0.3 g cysteine and 10.0 g glucose at a temperature of 20–22 °C.

Isolation of the crude product 1010-G and its separation

After 96 hours cultivation of strain 1010 at 20–22 °C, the culture broth (8.00 l) was centrifuged. The mycelium (100 g) was extracted with acetone for 24 h and the acetone extract concentrated to 110 ml aqueous solution which was reextracted with ethyl acetate. The culture filtrate (7.30 l) was extracted with ethyl acetate (1.5:1.0 v/v). The combined ethyl acetate extracts from the mycelium and the filtrate was filtered and evaporated to dryness *in vacuo*, yielding 150 mg crude product (complex 1010-G). The ethyl acetate solution of the crude product was chromatographed on a silica gel 60 (70–230 mesh) the column equilibrated with ethyl acetate and components in the crude product were eluted with ethyl acetate. They were separated and purified by reverse phase high-performance liquid chromatography on a C-18 column with a gradient of 20% to 60% acetonitrile in 0.01 M sodium phosphate buffer. After concentration to dryness *in vacuo*, the main compounds 1010-G1(35.0 mg) and 1010-G2(6.7 mg) were obtained.

Isolation of the crude product 1010-F and its separation

After extraction with acetone as described in the foregoing section, the mycelium of *Streptomyces sp.* 1010 was extracted twice with methanol (700 ml) for 24 h and the culture filtrate extracted with n-butanol (2:1, v/v). The solvent extracts were evaporated to dryness *in vacuo*. The crude products were dissolved in a small amount of methanol (40 ml), combined, filtered and precipitated with ethyl acetate-diethyl ether (1:2, v/v). The precipitate was dissolved in methanol (20 ml) and reprecipitated with acetone-diethyl ether (1:3, v/v) to yield 500 mg crude complex 1010-F after drying *in vacuo*. Final purification and separation of the components of complex 1010-F was achieved by preparative thin-layer chromatography on silica gel plates with chloroform-acetic acid-water (30:35:5 by vol.) as the mobile phase. The compounds were eluted with methanol, the eluates were evaporated and dissolved in methanol. Precipitation with acetone-diethyl ether (1:3, v/v) yielded 18.0 mg of 1010-F1 and 5.0 mg of 1010-F2.

General experimental procedures

Gas chromatography /mass spectrometry (GC/MS) was performed with a Hewlett-Packard Model 5890 gas chromatograph and a HP 5971 A quadropole mass selective detector interfaced with a Hewlett-Packard chemstation using G1034B software for data analysis. An (30m × 25 mm × 2.5 μm) HP-5 with Ph-Me-Silicon 5% capillary column was used in the splitless injection mode. Injector temperature was 250 °C, and oven temperature was 100 °C with a 10 °C/min temperature ramp to a final temperature 240 °C. Helium was used as the carrier gas.

Electrospray mass spectra were recorded on a triple-quadropole instrument Quattro (VG Biotech, Altrincham, England). Electron impact mass spectra (EI-MS) were carried out on a HP-5988A mass spectrometer by 70 eV. ^{1}H and ^{13}C-NMR spectra were measured in $CDCl_3$ and CD_3OD on a Bruker AMX 500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C. The ^{13}C multiplicity data were obtained from DEPT experiments. The chemical shifts are expressed in δ values (ppm). ^{1}H-^{1}H COSY, HSQC and HMBC were obtained by conventional methods.

Results and Discussion

Compound 1010 - G1 was soluble in methanol, ethyl acetate or chloroform but insoluble in water. On silica gel TLC plates it showed the following color reactions with various reagents: vanillin /sulfuric acid (pink), iodine vapour (yellow), anisaldehyde /sulfuric acid (violet). By electrospray mass

spectrometry (ES-MS) 1010-G1 showed a protonated molecular ion at *m/z* 223 $(M+H)^+$ and ions at 240 $(M+NH_4)^+$; 245 $(M+Na)^+$; 261 $(M+K)^+$ and 467 $(2M+Na)^+$. 1010-G1 showed a molecular ion peak in its electron impact mass spectrum (HREI-MS) at *m/z* 222 and series of fragment ions at *m/z* 177 $(M-CO_2)^+$; 149 $(177-2CH_2)^+$; 122 $(149-2CH_2)^+$; 105; 76; 65; 50 (Fig. 1). In the ^{1}H-NMR spectrum for 1010-G1 six methyl protons were observed at δ 1.29–1.38 (two-CH_3 groups); methylene protons at δ 4.30–4.37 (two-CH_2 groups) and four aromatic protons at δ 7.59–7.74, suggesting the presence of a benzene ring in the molecule. The ^{13}C-NMR spectrum of the compound indicated twelve carbons: two methyl carbons at 14.09 ppm (quartets); two methylene carbons at 61.59 ppm (triplets); six aromatic carbons at 128.84, 130.90, 132.26 ppm (4 doublets and 2 singlets) and two COOR groups at 167.60 ppm (singlets). The molecular weight and the elemental composition of 1010-G1 were determined by HREI-MS (M^+: *m/z* 222.1070; calcd. 222.1041 for C_{12} $H_{14}O_4$). The compound 1010-G1 is identical with phthalic acid diethyl ester and possesses an antibacterial action against *Micrococcus luteus* (MIC, 3 μg/ml); *Bacillus subtilis* (MIC,12 μg/ml) and *Staphylococcus aureus* (MIC,25 μg/ml).

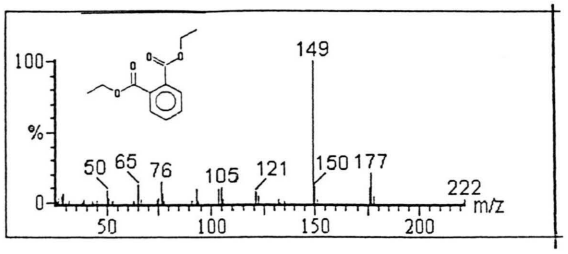

Fig. 1. Electron impact mass spectrum (EI-MS) of phthalic acid diethyl ester.

The other fraction 1010-G2 showed a single major peak by gas chromatography / mass spectrometry (GC/MS) with a M^+ *m/z* = 446, corresponding to a molecular formula C_{30} $H_{22}O_4$ and series of fragment ions at *m/z* 352 $(M - C_6H_5O)^+$; 260 $(352-C_6H_4O)^+$; 168 $(260-C_6H_4O)^+$; 77 $(168-C_6H_4O)^+$; 315 $(M-C_9H_7O)^+$; 223 $(315-C_6H_4O)^+$; 233, 184, 141, 115. Comparison with GC/MS data for a standard sample of 1,3-bis (3-phenoxyphenoxy)benzene indicated that the two compounds were identical.

Substance 1010-F1 was obtained as a white powder, soluble in methanol, ethanol, n-butanol and water. It gave a positive reaction with ninhydrin (red). By electrospray mass spectrometry (ES-MS), the 1010-F1 gave a protonated molecular ion peak at *m/z* 314 $(M+H)^+$; 650 $(2M+Na)^+$ and 665 $(2M+K)^+$. High resolution EI-MS furnished for 1010-F1 *m/z* 313.0094 (M^+; calcd. 313. 0089 for $C_{19}H_{39}NO_2$). [EIMS *m/z* (rel. intensity) obs. $(M)^+$ *m/z* 313 (7); 299 (21); 285 (5); 269 (3); 239 (4); 225 (7); 211 (3); 185 (4); 155 (3); 129 (15); 116 (12); 98 (39); 85 (49) and 57 (100)]. The infrared absorption spectrum of 1010-F1 showed the following: 3426, 2952, 2919, 2853, 1628, 1401, 1347, 1118, 1078, 992, 927, 775 and 535 cm^{-1}. The structure of 1010-F1 was readily settled on the basis of physico-chemical measurements (MS, ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC and HMBC). The assignment of proton and ^{13}C signals is shown in Table I.

A heteronuclear multiple bond correlation (HMBC) experiment showed long rangle coupling from the methylene protons of H-3 at δ 4.42 to a quaternary carbon C-1 (carbonyl) at 174.61 ppm. Coupling was also observed from the methylene protons of H-4 to a C-2 at 71.89 ppm; the methylene protons of H-6 at δ 3.15 and H-5 at δ 3.99 to a C-4 at 63.64 ppm; the methylene protons of H-14 at δ 1.59 to a C-16 at 30.21 ppm; the methyl protons of 9-CH_3 at δ 0.86 to a C-9 at 28.24 ppm

Table I. ^{1}H and ^{13}C NMR chemical shifts for 2-amino-9,13-dimethyl heptadecanoic acid.

Assignment	^{1}H (ppm)	^{13}C (ppm)	Groups
1.		174.611 s	COOH
2.	5.225 (1H, m)	71.892 d	CH
2-NH_2	5.337 (2H, m)		
3.	4.420 (2H, dd, *J*= 3.0,3.3 Hz)	63.646 t	CH_2
4.	4.172 (2H, dd, *J*= 6.6,6.9 Hz)	63.646 t	CH_2
5.	3.990 (2H, t, *J*= 7.2 Hz)	64.949 t	CH_2
6.	3.150 (2H, t, *J*= 5.7 Hz)	41.685 t	CH_2
7.	4.008 (2H, tt, *J*= 4.5,4.5 Hz)	62.934 t	CH_2
8.	1.290 (2H,m)	30.611 t	CH_2
9.	2.000 (1H,m)	28.240 d	CH
9-Me	0.865 (3H, dd, *J*= 2.1 Hz)	11.757 q	CH_3
10.	1.356 (2H,m)	30.834 t	CH_2
11.	2.285 (2H,m)	34.944 t	CH_2
12.	2.332 (2H, dd, *J*= 6.9,7.5 Hz)	37.825 t	CH_2
13.	2.308 (1H,m)	35.118 d	CH
13-Me	0.884 (3H, dd, *J*= 2.7 Hz)	23.053 q	CH_3
14.	1.593 (2H,m)	26.043 t	CH_2
15.	1.346 (2H,m)	30.834 t	CH_2
16.	1.287 (2H,m)	30.211 t	CH_2
17.	0.844 (3H, t, 3.9 Hz)	19.655 q	CH_3

and C-8 at 30.61 ppm; the protons from 13-CH_3 at δ 0.88 to a C-13 at 35.11 and C-12 at 37.82 ppm. A ^1H-^1H chemical shift correlation spectroscopy (COSY) experiment demonstrated coupling from the methine proton of H-2 at δ 5.22 to the methylene protons of H-3 at δ 4.42. Similarly coupling was observed from the terminal methyl at δ 0.84 to the methylene protons at δ 1.28 of H-16.

These data indicated that the structure of the new compound 1010-F1 (Fig. 2) possessed three methine, twelve aliphatic methylene, three methyl residues, one carbonyl carbon (COOH) and one primary amino group. The position of the two methyl groups are established by the signals for C-9 and C-13, and one terminal methyl.

The antimicrobial activity of 2-amino-9, 13-dimethyl heptadecanoic acid was determined by the conventional agar dilution method using Difco heart infusion agar. The substance was active against *Micrococcus luteus* (MIC, 15 μg/ml) and *Bacillus subtilis*(MIC,50 μg/ml) but not against *Candida albicans* and *Escherichia coli.*

Gas chromatography/mass spectrometry (GC/MS) data established also the presence of hexanedioic acid dioctyl ester in the complex. The mass spectrum of 1010 – F2 showed a peak with a M^+ m/z = 370, corresponding to a molecular formula $C_{22}H_{42}O_4$, and series of fragment ions was present at m/z 313 $(M\text{-}C_4H_9)^+$; 284 $(313\text{-}C_2H_5)^+$; 241 $(284\text{-}C_3H_7)^+$; 212 $(241\text{-}C_2H_5)^+$; 199 $(212\text{-}CH)^+$; 160 $(199\text{-}C_3H_3)^+$; 147 $(160\text{-}CH)^+$, 129, 111, 83, 71 and 57. The compound 1010-F2 did not react with ninhydrin. Its identification was confirmed by GC-MS comparison with standard.

The ability of the marine bacteria to produce antibacterial substances was documented more than 50 years ago (Rosenfeld and Zobell, 1947). The diethyl phthalate, 1,3-bis (3-phenoxyphenoxy)-benzene and hexanedioic acid dioctyl ester are known as synthetic products.

The new substance 2-amino-9,13-dimethyl heptadecanoic acid and phthalic acid diethyl ester, 1,3-bis(3-phenoxyphenoxy)benzene, hexanedioic acid dioctyl ester as natural products were found in the culture broth of the strain *Streptomyces sp.* 1010, isolated from water samples in the region Livingston Island, Antarctica.

Acknowledgments

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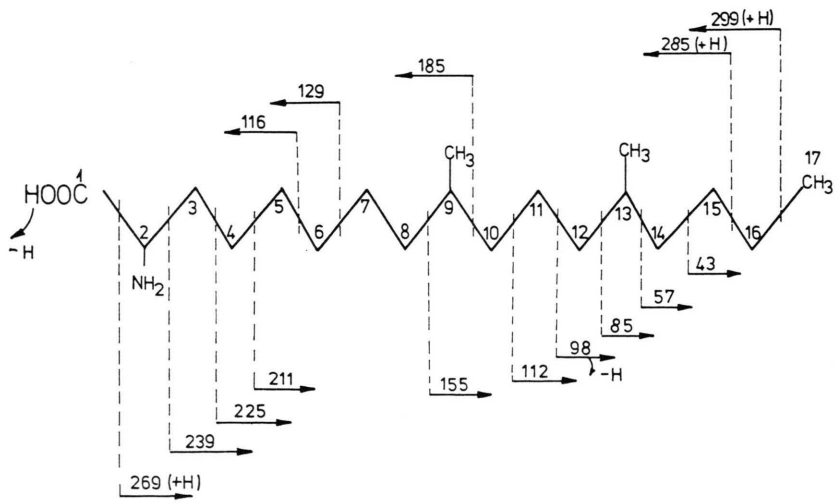

Fig. 2. Chemical structure of 2-amino-9,13-dimethyl heptadecanoic acid.

Atlas K. and Bartha M. (1992), Microbial Ecology. Fundamentals and Applications. 3d. ed., 215 pp.

Chipev N. and Veltchev K. (1996), Livingston island: An environment for Antarctic life. Bulg. Antarct. Research, Life Sci., **1**, 1–6.

Chipeva V., Christova K., Chipev N. and Moncheva P. (1996), Extracellular enzyme activity of *Streptomyces* strains isolated from soils on the Livingston Island, Antarctica. Bulg. Antarct. Research, Life Sci. **1**, 24–30.

Christova K. and Naidenova M. (1996), Isolation and taxonomic investigation of *Actinomyces* strains from Antarctica. Bulg. Antarct. Research, Life Sci. **1**, 19–23.

Darling C. and Siple P. (1941), Bacteria of Antarctica. J. Bacteriol. **42**, 83–98.

Grossmann S. (1994), Bacterial activity in sea ice and open water of the Weddell Sea, Antarctica. A microautoradiographic study. Microbial Ecology, **28**, 1–18.

Grossmann S. and Dieckmann G. (1994), Bacterial standing stock, activity and carbon production during formation and growth of sea ice in the Weddell Sea, Antarctica. Appl. Environ. Microbiol. **60**, 2746–2753.

Lopes-Martínez J., Vilaplana J., Martínez de Pison E., Calvet J., Arche A., Serrat D. and Pallas R. (1992), Geomorphology of selected areas in Livingston Island, South Shetland Islands. In: Geologia de la Antárctida occidental (J. Lopes-Martinez, ed). Simposios **T3**: 271–281.

Morita R. Y. (1975), Psychrophilic bacteria. Bacteriol. Rev. **39**, 144–167.

Rosenfeld W. D. and Zobell C. (1947), Antibiotic production by marine microorganisms. J. Bacteriol. **54**, 393–398.

Tsiklinsky M. (1908), La flore microbienne dans les regions du Pole Sud: Expedition Antarctique française, pp. 1903–1905. Masonet Cie, Paris.