ANDRIJA A. ŠMELCEROVIĆ[1]
SINIŠA M. ĐORĐEVIĆ[2]
RADOSAV M. PALIĆ[3]

[1]Chemical Industry "Nevena", Leskovac, Yugoslavia
[2]Faculty of Technology, Leskovac, Yugoslavia
[3]Faculty of Natural Science and Mathematics, Niš, Yugoslavia

SCIENTIFIC PAPER

UDC 577.121:579.87(26)

A NEW METABOLITE FROM MARINE BACTERIA

The marine actinomycete B 1758 comes from the actinomycete collection of the Alfred Wegener Institute for Polar and Marine Research in Bremerhafen, Germany. 1.079 g of raw extract was obtained by fermentation, from which 3.3 mg of 3,7–dihydroxy–cis,cis–1,8–nonadiene–1,9– dicarboxylic acid diamide was isolated. Since this substance is not included in the Dictionary of Natural Products by Chapman & Hall or in the AntiBase® data bank of natural substances, the metabolite may be considered to be a new one from marine bacteria.

Active substances from marine bacteria

The approach to new metabolites is connected with the choice of micro–organisms. For a long time, only those were investigated that were readily available or easy to cultivate, so that they were within reach in great quantity. As a result, 90% of all the terrestrial bioactive micro–organisms investigated contained only the well known metabolites [1]. In the meantime, a great effort was made to investigate those originating from "exotic" habitats because it was believed that due to specific environmental conditions, these organisms could have developed other, secondary metabolites.

The world seas, that make 2/3 of the earth's surface and 90% of the biosphere, are seen as the new source of natural substances [2,3]. They offer extremely different physical and nutrient conditions: the temperatures range from –1.5°C in the Antarctica to 350°C in hydrothermal deep sea systems, the pressure ranges from 1 to more than 1000 bar and the nutrient conditions vary from euthrophic to oligothrophic. These should have produced exceptional specialisations in groups of organisms, which, in turn, could have produced unusual metabolites.

Marine micro–organisms fulfill two essential conditions for the university research of natural substances. Until now they have not been investigated so frequently and, therefore, there are great possibilities of isolating new substances.

EXPERIMENTAL

Materials and Methods

The ^{1}H–NMR (300.1 MHz) and H–H–COSY (300.1 MHz; H↔H) spectra were recorded on a Bruker WM 300 spectrophotometer. The ^{13}C–NMR (125.7 MHz) and HMBC–NMR (F1 125.7 MHz, F2 499.9 MHz; H→C) spectra were recorderd on a Varian INOVA 500 spectrophotometer. DCI–MS mass spectra were recorded on a Finnigan MAT 95 A instrument; reacting gas NH_3. ESI–MS mass spectra were recorded on a Finigan TSQ 7000 instrument. The column chromatography was accomplished on silica gel 30–60 μm (J.T. Baker). The thin–layer chromatography was carried out on DC–foils Polygram SIL G/UV_{254} (Macherey Nagel & Co). HPLC was carried out on: 1. an analytic apparatus: Jasco Multiwavelength Detector MD–910, two pumps type Jasco Intelligent Prep. Pump PU–987 with mixing chamber; degasser VDS Degasys DG–1310; feed valve: Rheodyne valve with 20 μl samples injection; software: Borwin HPLC Software; analytical column: LiChroCART 4 x 250 mm with 4 x 4 mm pre column. 2. preparation apparatus: Jasco Preparatory flow cuvette, samples 500 μl; stationary phase: Merck Lichrospher 60 RP–select B, 5 μm; preparatory column: Vertex 16 x 250 mm with 16 x 30 mm precolumn; stationary phase: Eurochrom Eurospher RP C18, 100 A, 5 μm. All the elutes were filtered through a membrane filter (water: cellulose acetate, pore size 0.45 μm, Sartorius, Göttingen; acetonitrile: filter RC–felt, pore size 0.45 μm, Sartorius, Göttingen) and ultrasonically degassed. Solvent for HPLC: for the separations acetonitrile/water–azeotrope (83.7% acetonitrile/16.3% water/b.p.: 78.5°C) solvent recovered by fractional distillation was used.

Microorganism, fermentation and isolation

The marine actinomycete B 1758 comes from the actinomycete collection of the Alfred Wegener Institute for Polar and Marine Research in Bremerhafen. The breed B 1758 from soil culture was inoculated to 10 agar plates and incubated for 72 hours at 28°C. For the fermentation, a shake culture was put in 50 Erlenmeyer flasks of 1000 cm^3 each together with 200 cm^3 of nutrient solution and an approx. 1 cm^3 piece of agar and allowed a fermentation period of 72 hours at 28°C. The

Author address: A. Šmelcerović, Hemijska industrija "Nevena", Đorđa Stamenkovića b.b., 16000 Leskovac, Jugoslavija
Paper received: June 20, 2001
Paper accepted: August 20, 2001

culture was mixed with Celite and filtered through a filter press. The filtrate and mycelia were extracted five times by ethyl acetate. The organic phases were put together and evaporated under vacuum at 40°C until dry. The yield was 1.079 g.

The raw extract was added to 300 cm^3 of methanol and degreased by two extractions, each with 200 cm^3 of cyclohexane. The methanol phase was separated by column chromatography on silica gel, with step gradients consisting of chloroform and methanol (1500 cm^3 $CHCl_3$, 1000 cm^3 $CHCl_3$ / 1% CH_3OH, 1000 cm^3 $CHCl_3$ / 2% CH_3OH, 500 cm^3 $CHCl_3$ / 5% CH_3OH, 700 cm^3 $CHCl_3$ / 15% CH_3OH). The fractions were evaluated by fluorescence and coloration with anisaldehyde/sulphuric acid. Seven fractions were separated by thin layer chromatography ($CHCl_3$ / 5% CH_3OH), which still contained the substance mixture (fraction 1, 875 cm^3, 20.3 mg; fraction 2, 375 cm^3, 189.8 mg; fraction 3, 850 cm^3, 100.5 mg; fraction 4, 875 cm^3, 82.7 mg; fraction 5, 950 cm^3, 103.7 mg; fraction 6, 325 cm^3, 71.3 mg; fraction 7, 250 cm^3, 28.8 mg).

Fraction 5 was separated by column chromatography on Sephadex LH 20 with chloroform and methanol (60% $CHCl_3$ / 40% CH_3OH). The fractions were evaluated by fluorescence and coloration with anisaldehyde/sulphuric acid. Three fractions were separated by thin layer chromatography method ($CHCl_3$ / 5% CH_3OH) (fraction 5.1, 10 mg; fraction 5.2, 5.5 mg; fraction 5.3, 65.5 mg). The fraction 5.3 was further separated into nine fractions by HPLC (with CH_3CN–H_2O–azeotrope / H_2O = 10 / 90 at the beginning, increased to CH_3CN–H_2O–azeotrope / H_2O = 100 / 0 in 25 minutes, maintained at this level for 10 minutes, flow rate: 10 ml / min). The fraction 5.3.6 (t_R = 13.45 min, 4.9 mg) was purified HPLC (with CH_3CN–H_2O–azeotrope / H_2O = 50 / 50 at the beginning, increased to CH_3CN–H_2O–azeotrope / H_2O = 100 / 0 in 25 minutes, maintained at this level for 10 minutes, flow rate: 7 cm^3/min). Fraction 5.3.6B (t_R = 7.72 min, 3.9 mg) was further purified by column chromatography on Sephadex LH 20 with chloroform and methanol (60% $CHCl_3$ / 40% CH_3OH). The fraction 5.3.6 B1 yielded 3.3 mg of 3,7–dihydroxy–*cis,cis*–1,8–nonadiene–1,9–dicarboxylic acid diamide as a yellowish substance.

RESULTS AND DISCUSSION

In the ^{1}H–NMR–spectrum of the isolated compound there was a doublet at δ = 7.43 (^3J = 7 Hz) and a double doublet at δ = 6.09 (^3J = 7 Hz, ^3J = 1 Hz) indicating a double bond substituted by an acceptor or donor substituted aromates. The proton signal at δ = 5.03 indicates a double bond or electron attracting substituents in the vicinity (O, N). Several multiplets in the high field range could be perceived, corresponding to alkyl groups. The supposition of a 1,2–di–substituted double bond was confirmed in the ^{13}C–NMR–spectrum with two CH signals at δ = 156.3 and 121.6. Because of the coupling constant, it is most probably a *cis*–substitution. Since there were no more carbon signals in the range between 170 and 90 ppm, an aromate was excluded. Due to the chemical shift of the olefin–carbon atoms and protons, the carbonyl group most probably has a double bond. From the correlation of HMBC–NMR (Figure 3) and H–H–COSY spectra (Figure 4), Fragment I (Figure 1) was obtained. With a chemical shift of δ =83.4, the methine carbon atom in the position near the olefin group should be bound to an oxygen atom. The carbon atom at δ =173.2 belongs to an amide group. Other correlations, such as those shown in Fragment I, could not be found in H, H–COSY and HMBC spectra. The mass spectra (DCI, ESI) indicated a molar mass of 242 Dalton. The structure of 3,7–dihydroxy–*cis,cis*–1,8–nonadiene–1,9–dicarboxylic acid diamide was obtained (Figure 2).

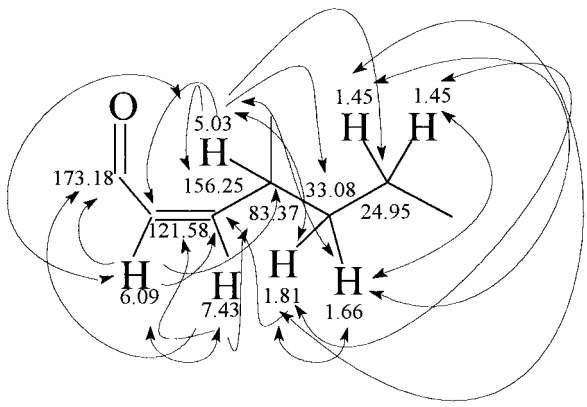

Figure 1. Fragment I obtained by the correlation of H, H–COSY, and HMBC spectra of 3,7–dihydroxy–cis,cis–1,8–nonadiene–1,9–dicarboxylic acid diamide

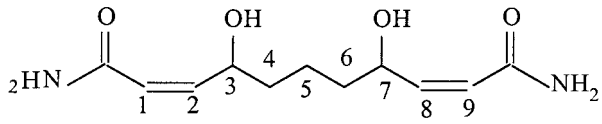

Figure 2. 3,7–dihydroxy–cis,cis–1,8–nonadiene–1,9–dicarboxylic acid diamide

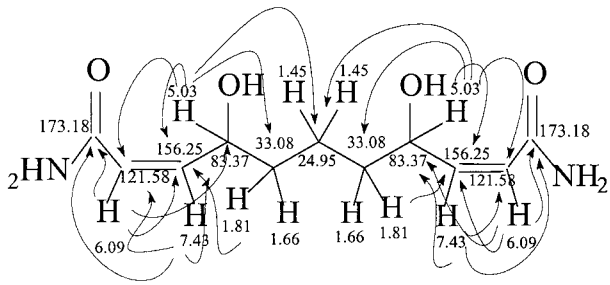

Figure 3. HMBC–NMR of 3,7–dihydroxy–cis,cis–1,8–nonadiene–1,9–dicarboxylic acid diamide

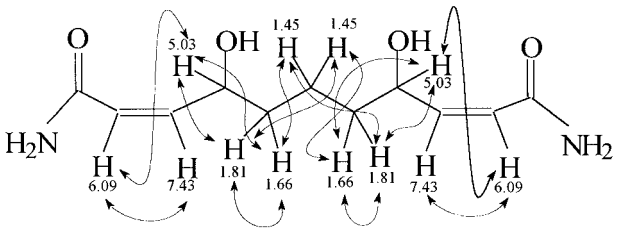

Figure 4. H–H–COSY spectrum of 3,7–dihydroxy–cis,cis–1,8–nonadiene–1,9–dicarboxylic acid diamide

Since 3,7–dihydroxy–*cis,cis*–1,8–nonadiene–1,9–dicarboxylic acid diamide is not included in the Dictionary of Natural Products by Chapman & Hall [4] or in the AntiBase® [5] data bank of natural substances, metabolite can be considered to be a new one from marine bacteria.

3,7–dihydroxy–cis,cis–1,8–nonadiene–1,9–dicarboxylic acid diamide: $C_{11}H_{18}N_2O_4$ (242)

DCI–MS (NH_3): *m/z* (%) = 502.5 $[2M + NH4]^+$ (48), 260.3 $[M + NH_4]^+$ (100)

(+)–ESI–MS: m/z (%) = 507.2 $[2M + Na]^+$ (42), 265.4 $[M + Na]^+$ (100)

^{1}H–NMR ($CDCl_3$, 300 MHz): δ = 7.43 (d, ^3J = 6 Hz, 2 H, 2–H, 8–H), 6.09 (dd., ^3J = 6 Hz, ^3J = 1 Hz, 2 H, 1–H, 9–H), 5.07–4.99 (m, 2 H, 3–H, 7–H), 1.83–1.72 (m, 2 H, 4–H, 6–H), 1.71–1.55 (m, 2 H, 4–H, 6–H), 1.51–1.39 (m, 2 H, 5–CH_2)

^{13}C/APT–NMR ($CDCl_3$, 125 MHz): δ = 173.18 (Cq, C=O), 156.25 (CH), 121.58 (CH), 83.37 (CH), 33.08 (CH_2), 24.95 (CH_2).

ACKNOWLEDGEMENTS

The first author would like to heartily thank Prof. Dr. Hartmut Laatsch of the Institute of Organic Chemistry at the University of Göttingen, Germany, a for generously given work post and the DAAD for a scholarship.

Abbreviations: d = doublet; dd = double doublet; m = multiple.

REFERENCES

[1] H. Laatsch, Personal Communication
[2] W. Fenical, Chem. Rev. 93 (1993) 1673
[3] P. Jensen and W. Fenical, Annu. Rev. Microbiol. 48 (1994) 559
[4] Dictionary of Natural Products on CD–ROM, Chapman & Hall, Chemical Database, Version 7, 1998.
[5] H. Laatsch, Naturstoffdatenbank AntiBase®, Chemical Concept, Weinheim, 1998.

IZVOD

NOVI METABOLIT IZ MORSKIH BAKTERIJA

(Naučni rad)

Andrija A. Smelcerović[1], Siniša M. Đorđević[2], Radosav M. Palić[3]
[1]Hemijska industrija "Nevena", Leskovac, Jugoslavija
[2]Tehnološki fakultet, Leskovac, Jugoslavija
[3]Prirodno–matematički fakultet, Niš, Jugoslavija

Morski aktinomicet B 1758 potiče iz zbirke aktinomiceta za polarna i morska istraživanja instituta Alfred Wegener u Bremerhafenu, Nemačka. Fermentacijom je dobijeno 1,079 g sirovog ekstrakta iz koga je izolovano 3,3 mg 3,7–dihidroksi–*cis,cis*–1,8–nonadien–1,9–diamida dikarboksilne kiseline. Kako ova supstanca nije nađena u bazi prirodnih proizvoda Chapman & Hall, niti u banci podataka o prirodnim supstancama AntiBase®, zaključujemo da je reč o novom metabolitu iz morskih bakterija.

Ključne reči: 3,7–dihidroksi–*cis,cis*–1,8–nonadien–1,9–diamid dikarboksilne kiseline • morska aktinomiceta • izolovanje •
Key words: 3,7–dihydroxy–*cis,cis*–1,8–nonadiene–1,9–dicarboxylic acid diamide • marine actinomycete • isolation •