Isolation and Structure Determination of Phenazostatin D, a New Phenazine from a Marine Actinomycete Isolate *Pseudonocardia* sp. B6273*

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A new phenazine derivative, phenazostatin D (**1a**), was isolated from an extract of the actinomycete isolate *Pseudonocardia* sp. B6273 *via* a TLC-guided chemical screening. The structure of the compound was assigned by spectroscopic methods and found to be the *meso*-form of phenazostatin B (**1b**). Phenazostatin D was inactive against the tested bacteria and fungi. The strain also produced the known phenazine antibiotic methyl saphenate (**2**).

Key words: Phenazostatin, Marine Actinomycetes, *Pseudonocardia*

Marine microorganisms are a potent and prolific source of secondary metabolites. In particular actinomycetes from the marine environment have been shown to be highly productive and may even surpass their terrestrial counterparts. Therefore, our efforts in this field have focussed upon antimicrobial and chemical screening of marine actinomycetes. In the course of this work, we became interested in the extract of the *Pseudonocardia* sp. B6273 which was isolated from a marine sediment because it exhibited two nonpolar light yellow TLC spots that turned orange upon spraying with anisaldehyde reagent and dark red with concentrated sulphuric acid. This and the missing colour reaction with sodium hydroxide pointed to the phenazine skeleton.

A TLC-guided fractionation of the extract afforded a new phenazine antibiotic, phenazostatin D (**1a**), and the known metabolite saphenic acid methyl ester (**2**) [1]. This paper deals with the taxonomy of the producing organism, the isolation and structure assignment of compound **1a**.

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Results and Discussion

Eighty 1-l Erlenmeyer flasks each containing 250 ml of malt extract/yeast extract/glucose in 50% artificial sea water were inoculated with agar cultures of strain B6273 and incubated for 3 days at 28 °C with 110 rpm. The ethyl acetate extract of the entire culture was subjected to vacuum flash chromatography on silica gel. The fraction with UV absorbing zones was rechromatographed by silica gel vacuum flash chromatography. Sub-fractionating by TLC delivered two faintly yellow compounds. The ^{1}H NMR spectrum of the more polar metabolite displayed two deep-field shifted doublets at δ 8.94 and 8.48 typical for phenazine derivatives. As the mass was *m/z* 268, this compound was easily identified as methyl saphenate (**2**) and confirmed by comparison with reference data [1, 2].

The aromatic pattern and the methoxy signal at δ 4.10 in the ^{1}H NMR spectrum of the less polar compound B were similar to those of saphenic acid methyl ester (**2**), which indicated also a 1,6-disubstituted phenazine derivative. The molecular mass was determined by EI, DCI and ESI mass spectroscopy to be 530 Dalton, and the molecular formula was $C_{32}H_{26}N_4O_4$ according to the high resolution (530.1953). The ^{13}C NMR spectrum, however, indicated only 16 carbon signals, one of them at δ 167 was probably due to an ester carbonyl. The ^{1}H and ^{13}C NMR data and the molecular formula pointed

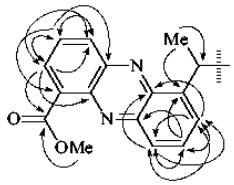
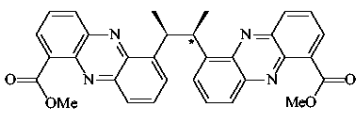


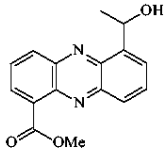
Fig. 1. H,H COSY (↔) and HMBC couplings (H → C) in Phenazostatin D (**2a**).

therefore to a symmetrical dimer. A search in AntiBase [2] led to phenazostatin B (**1a**) [3 – 5] which on direct comparison indeed showed very similar yet not identical NMR data (Table 1) and also had a different R_f value.

By H,H COSY, HMQC and HMBC measurements (Fig. 1) in combination with the molecular formula, the same connectivity as in **1b** was assigned for compound B, which means that both phenazines are diastereomers.



1a
1b: configuration reverse at *



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Phenazostatin B (**1b**) was described first by Yoo *et al.* [3] and recently re-isolated in our group from a marine *Streptomyces* isolate B7693. Our **1b** sample showed a positive CD signal at λ = 268 nm whereas compound B did not give any CD effects. Additionally, our phenazostatin B possessed a positive optical rotation at 365 nm whereas the new isomer was optically inactive. This leads unambiguously to the conclusion that compound B is the *meso*-form **1a** of phenazostatin B (**1b**). We suggest to name it phenazostatin D.

In contrast to our sample, the phenazostatin B described in the literature was optically inactive as well. As the reported shift values were superimposable with the data of our dextrarotatory phenazostatin B (**1b**), Yoo *et al.* [3] might have had a racemic (±)-phenazostatin B in hand.

More than 85 natural phenazine antibiotics are known, and for most of them antimicrobial [2], enzyme inhibitory or neuroprotective effects [6] were reported. According to the literature, **1b** inhibits the toxicity of glutamate in neurons [3, 6]. A direct comparison with the diastereomer **1a** should be therefore of some interest. In our agar diffusion tests against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* (ATCC 6051), *Streptomyces viridochromogenes* (TÜ 57), *Mucor miehei* (TÜ 284), *Candida albicans*, *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus*, both isomeric phenazostatins B (**1b**) and D (**1a**) were inactive in a concentration of about 25 – 30 μg per 6 mm paper disc.

Table 1. ^{1}H and ^{13}C NMR data of phenostatin B and D in $CDCl_3$.

	Phenazostatin D (**1a**)		Phenazostatin B (**1b**)	
C Atom	^{1}H	^{13}C	^{1}H	^{13}C
1	–	131.2	-	131.2
2	8.22	131.9	8.20	131.7
3	7.78	128.3	7.82	128.3
4	8.01	133.9	8.39	133.9
4a	–	141.7	–	141.7
5a	–	142.6	–	141.7
6	–	145.5	–	146.3
7	7.66	128.0	7.61	127.9
8	7.70	130.7	7.36	130.7
9	8.03	127.6	7.82	127.7
9a	–	143.7	–	143.6
10a	–	140.4	–	140.4
1’	5.06	38.0	5.06	37.5
2”	1.32	19.1	1.67	21.2
CO	–	167.3	–	167.3
OMe	4.10	52.6	4.05	52.6

Experimental Section

FT-IR spectra: Perkin Elmer, model 297 (KBr). UV spectra: Perkin-Elmer Lambda 15 UV/vis spectrometer. – ^{1}H and ^{13}C NMR spectra: Varian Inova 500 (TMS as internal standard). – Mass spectra: Finnigan MAT 95 (70 eV, high resolution with perfluorokerosene as reference); HPLC/MS: Finnigan LCQ. – Thin layer chromatography (TLC): TLC sheets Polygram SIL G/UV$_{254}$ 4 × 8 cm (silica gel, Macherey-Nagel & Co, Düren, Germany). Vacuum flash chromatography: Silica gel 60 (0.05 – 0.2 mm and 0.04 – 0.063 mm / 230 – 400 mesh ASTM; Macherey-Nagel & CO). – Assay discs: ∅ 6 and 9 mm, Schleicher & Schuell, Dassel, Germany.

Taxonomic studies on the producing strain

The actinomycete isolate B6273 was derived from a littoral sample of Mauritius (Indian Ocean) and was isolated on chitin agar [7] containing 50% natural seawater. The almost complete 16S rRNA sequence of the strain B6273 shows a relatively low similarity of 97% to the strains *Pseudonocardia hydrocarbonoxydans*, *P. halophobica*, *P. sulfidoxydans.*

The strain B6273 forms a yellow substrate mycelium and a white aerial mycelium with zig-zag shaped spore chains. Spores are long cylindrical and smooth. Melanin pigment is neither produced on peptone-yeast extract-iron agar [8] nor on tyrosine agar [8]. Optimum growth temperature is at about

30 °C. The strain grows neither at 10 °C nor at 45 °C. No growth in media with 7% or higher seawater salinity. Starch, casein, esculin, chitin, and cellulose are not hydrolized. The strain is catalase positive and does neither produce nitrate reductase nor H_2S.

The use of carbon sources was tested with SFN2-Biolog (Hayward, CA, USA) using BMS-N without agar as basal medium [9]. The following organic compounds can be utilized for growth: dextrin, tween 40, tween 80, D-arabitol, D-fructose, D-galactose, D-glucose, m-inositol, maltose, D-mannitol, D-mannose, sucrose, xylitol, methylpyruvate, mono-methylsuccinate, D-gluconic acid, α-hydroxy butyric acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, α-keto butyric acid, α-ketovaleric acid, quinic acid, D-saccharic acid, succinic acid, bromo succinic acid, succinamic acid, alaninamide, L-alanine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-leucine, threonine, inosine, uridine, phenyl ethylamine, putrescine, glycerol.

The reference culture of *Pseudonocardia* sp. B6273 is maintained on yeast extract-malt extract agar[7] in the Collection of Marine Actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research in Bremerhaven.

Fermentation of the strain B6273

The strain was subcultured from its soil culture on agar plates with yeast extract-malt extract-glucose [7] in 50% artificial sea water and incubated 72 h at 28 °C. Eighty 1-l Erlenmeyer flasks each containing 250 ml of the same medium were inoculated with well grown agar cultures of strain B6273 and incubated for 3 days at 28 °C with 110 rpm.

The entire culture broth was mixed with diatom earth (*ca.* 1 kg) and pressed through a pressure filter, and the culture filtrate and mycelium were extracted separately each three times with 6 l of ethyl acetate. Evaporation of the combined organic layers to dryness gave 2.7 g of crude extract. The latter was subjected to vacuum flash chromatography on silica gel (100 g) in a sintered glass funnel and successively eluted with cyclohexane (200 ml) and a CH_2Cl_2/MeOH gradient (each 100 ml, stepwise increasing from 1 to 10%). The fraction III eluted with CH_2Cl_2/2% MeOH was rechromatographed by vacuum flash chromatography on silica gel (60 g) and eluted successively with 500 ml cyclohexane, 100 ml cyclohexane/1% CH_2Cl_2, cyclohexane/2% CH_2Cl_2, and cyclohexane/4% CH_2Cl_2. The sub-fraction IIIb eluted with cyclohexan/2% CH_2Cl_2 was finally purified by PTLC (20×20 cm, CH_2Cl_2/5% MeOH) and delivered 1.5 mg **1a** (R_f = 0.10, CH_2Cl_2; 0.50, CH_2Cl_2/5% MeOH) and 3 mg **2** (R_f = 0.05, CH_2Cl_2; 0.24, CH_2Cl_2/2% MeOH).

*Phenazostatin D (**1a**)*

Yellow solid, R_f = 0.10 (CH_2Cl_2), 0.50 (CH_2Cl_2/2% MeOH). IR (KBr) ν = 2958, 2924, 2854, 1720, 1650, 1634, 1531, 1457, 1433, 1382, 1288, 1263, 1239, 1197, 1170, 1152, 1097, 1046, 1014, 856, 815, 797, 748, 709 cm^{-1}. UV-vis ($CHCl_3$): λ_{max} (log ε) = 385 (sh 2.92), 366 (3.34), 350 (sh 3.16), 256 (3.95). $[\alpha]_D^{25} = 0°$ (c = 0.163, $CHCl_3$). DCI-MS (NH_3): *m/z* = 531 $[M + H]^+$. (+)-ESI-MS: *m/z* = 553 ($[M + Na]^+$, 35), 531 ($[M + H]^+$, 100). EI-MS (70 eV): *m/z* = 530 ($[M]^+$, 30), 313 (26), 265 (100), 239 (22), 206 (24), 57 (18), 43 (16). HREIMS (*m/z*) 530.1950 (M^+ calcd. for $C_{32}H_{26}N_4O_4$ 530.1948). NMR see Table 1.

Acknowledgements

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