Dihydrophencomycin Methyl Ester, a New Phenazine Derivative from a Marine *Streptomycete*†

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The novel 5,10-dihydrophencomycin methyl ester (**4**) and the known microbial metabolites (2-hydroxyphenyl)-acetamide (**1**), menaquinone MK9 (II, III, VIII, IX-H8) (**2**), and phencomycin (**3a**) were isolated from an unidentified marine *Streptomyces* sp. and the structures were elucidated by NMR methods. Compound **4** shows weak antibiotic activity against *Escherichia coli* and *Bacillus subtilis*.

In our screening program for secondary metabolites of marine bacteria, extracts of the streptomycete strain B 8251 inhibited the growth of *Escherichia coli*, *Bacillus subtilis* and *Candida albicans* significantly. We describe here the structures of the main constituents, one of them being the novel dihydrophenazine derivative **4**.

Fermentation of *Streptomyces* sp. B 8251 and Isolation of *N*-(2-Hydroxyphenyl)-acetamide

The streptomycete B 8251 was cultivated on yeast-extract malt-extract agar[1] in artificial sea water for 70~80 hours. The yellow-green ethyl acetate extract of a 20 liters culture was evaporated to dryness, the residue suspended in methanol and extensively extracted with cyclohexane.

Column chromatography of the residue from the methanol layer gave two UV-absorbing fractions, the more polar one yielding 28 mg of colourless needles. The ^{1}H NMR spectrum with ABCD coupling pattern of a 1,2-disubstituted benzene and a methyl singlet at $\delta=2.20$, as well as the formula (HR-MS) of this compound indicated *N*-(2-hydroxyphenyl)-acetamide (**1**), as was proven by comparison with a synthetic sample[2].

Identification of Phencomycin

From a second yellow, less polar zone 46 mg of an antibiotically highly active compound were obtained as yellow-green needles. The physico-chemical properties are summarized in Table 1. The NMR spectrum consisted of only two overlapping ABC signals of adjacent protons in the aromatic region, a methoxy signal and a D_2O exchangable ^{1}H singlet at $\delta=15.25$. The carbon NMR spectrum indicated the presence of 15 carbon atoms, two signals corresponding to acid or ester carbonyl groups. The EI mass spectrum gave only an (M^+-CO_2)-ion at $m/z=238$, but the molecular ion was obtained by FAB-MS. The UV absorption at 368 nm was not changed by treatment with dithionite, thus excluding quinones. Among the known metabolites from microorganisms, only phencomycin[3] (**3a**) or its unnatural 1,9-disubstituted isomer[4] corresponded to these data[5]. As the mixed ethyl-methyl ester did not show an Overhauser effect between methyl and ethyl groups, its structure was confirmed as **3c** and the native precursor was therefore phencomycin (**3a**).

Menaquinone MK9 (II, III, VIII, IX-H8)

Repeated chromatography of the cyclohexane extract on silica gel and Sephadex LH-20 gave two yellow fractions, the less polar one being a yellow oil with a molecular weight of $m/z=792$ (DCI-MS). The NMR spectrum showed two aromatic multiplets, olefin signals at $\delta=5.11$ and 5.01, a doublet of an electron-deficient methylene group at $\delta=3.37$ and overlapping signals in the aliphatic region with an intense singlet of four magnetically equivalent olefin-bonded methyl groups at $\delta=1.58$. With these data, a search in AntiBase[5] pointed to octahydromenaquinone MK9 (II, III, VIII, IX-H8)[6]

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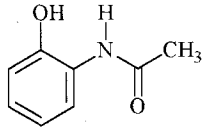
Table 1. NMR data of phencomycin (**3a**) and 5,10-dihydrophencomycin methyl ester (**4**).

C-No.	Phencomycin (**3a**)			Dimethyl 5,10-dihydrophenazine-1,6-dicarboxylate (**4**)		
	δ (C) ppm	δ (H) ppm	J (Hz)	δ (C) ppm	δ (H) ppm	J (Hz)
1	124.8 Cq			108.7 Cq		
2	135.7 CH	8.43 dd	8, 1	122.6 CH	6.94 dd	8, 1
3	130.8 CH	8.12~7.99 m		119.3 CH	6.26 t	8
4	138.2 CH	9.02 dd	8, 1	115.2 CH	6.10 d	8
4a	139.1 Cq			132.7 Cq		
5a	139.8 Cq			138.4 Cq		
6	124.8 Cq			108.7 Cq		
7	131.5 CH	8.35 dd	8, 1	122.6 CH	6.94 dd	8, 1
8	131.8 CH	8.12~7.99 m		119.3 CH	6.26 t	8
9	133.1 CH	8.61 dd	8, 1	115.2 CH	6.10 d	8
9a	141.4 Cq			132.7 Cq		
10a	143.5 Cq			138.4 Cq		
C=O	166.2			168.4		
C=O	165.5			168.4		
NH					8.72 s br	
OMe	53.0 CH_3	4.15 s		51.7 CH_3	3.80 s	
OH		15.25 s br				

Fig. 1. The structures of *N*-(-2-hydroxyphenyl)-acetamide (**1**) and octahydromenaquinone (**2**).



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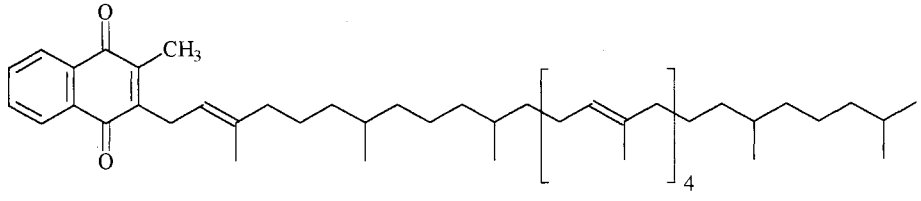

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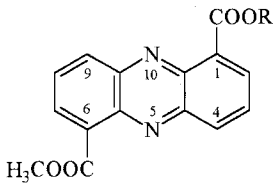
(**2**), a member of the vitamin K group which is already known from various other microorganisms. The structure was confirmed by comparison with reference data.

Structure Elucidation of Dihydrophencomycin Methyl Ester

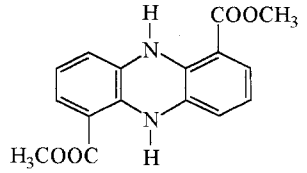
The second yellow, on silica gel greenish fluorescent polar constituent from the cyclohexane phase crystallized as orange-brown needles. The ^{13}C NMR spectrum showed only eight signals, a methoxy carbon, a carbonyl group, and signals of three tertiary and three quaternary aromatic carbons. The HREIMS data pointed to a molecular formula of $C_{16}H_{14}N_2O_4$, thus indicating a symmetrical molecule. The ^1H NMR spectrum showed an NH-proton, a methoxy signal and three adjacent aromatic protons. The IR spectrum confirmed the presence of an NH-proton and a methyl ester.

The substitution pattern of the individual benzene rings is well established by HMBC correlations, and due to the molecular formula both parts must be connected by NH bridges forming a dihydrophenazine system. Because of the symmetry of the molecule, a decision between both annelating directions as in **3a** was not possible. But as phencomycin (**3a**) and its dihydro derivative are produced by the same strain, structure **4** is the more likely one.

Fig. 2. The structures of phencomycin (**3a**) and 5,10-dihydrophencomycin methyl ester (**4**).



3a: R = H
3b: R = Me
3c: R = Et



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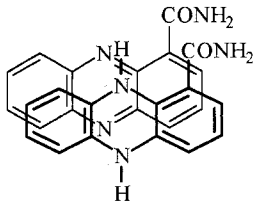

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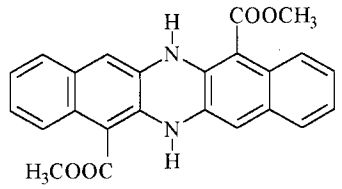

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Table 2. Physico-chemical properties of phencomycin (**3a**) and dimethyl 5,10-dihydrophenazine-1,6-dicarboxylate (**4**).

	3a	**4**
Appearance	Yellow-green needles	Brown needles
mp	263°C	231°C
Molecular formula	$C_{15}H_{10}N_2O_4$	$C_{16}H_{14}N_2O_4$
HR-MS		Found: 298.0953 Calcd: 298.0936
UV (MeOH) λ_{max} nm (log ε)	256 (4.87), 368 (4.20)	216 (4.51), 245 (4.48), 450 (4.04)
Rf value	0.30 ($CHCl_3$) 0.72 ($CHCl_3$/MeOH 95 : 5)	0.92 ($CHCl_3$) 0.64 (C_6H_{12}/EtOAc 9 : 1)

Fig. 3. HMBC NMR ^3J-couplings of 5,10-dihydrophencomycin methyl ester (**4**).

Arrows are directing from C towards H (for ^2J and ^4J couplings see experimental part).

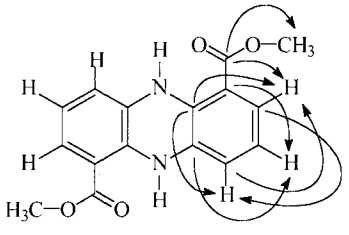

Table 3. Antibiotic activities of phencomycin (**3a**) and dimethyl 5,10-dihydrophenazine-1,6-dicarboxylate (**4**) using agar diffusion tests.

The weights indicate the amounts per paper disk.

Test organism	**3a** [mm i.d./μg]	**4** [mm i.d./μg]
E. coli	15/5, 20/20, 25/30, 30/40	-/10, 12/20, 15/30, 20/40
B. subtilis	15/5, 22/20, 27/30, 30/40	-/20, 9/30, 12/40

Results and Discussion

Compound **4** belongs to a rare class of antibiotics: While phenazines have been isolated quite often in the past[5], only one *N*-unsubstituted simple dihydrophenazine (chlororaphin EDA complex[7,8], **5**) has been obtained from microorganisms so far. Closely related to **4** is caulerpin (**6**), a pigment from green algae[9].

The antibiotic activity of extracts from *Streptomyces* sp. B 8251 was based mainly on the production of phencomycin (**3a**). The dihydrophenazine **4** is less active against *Escherichia coli* and *Bacillus subtilis*. (see Table 3).

Experimental

Material and methods are the same as reported earlier[10].

Isolation and Characterization of Streptomycete B 8251

The strain was isolated from sediment of the Laguna de Terminos at the Gulf of Mexico using cellulose medium containing 50% sea water at 25°C. The pure culture was maintained on yeast-extract malt-extract medium[1]. The vegetative mycelium is brown-yellow, the strain forms yellow aerial mycelium with straight to flexuous (*Rectiflexibiles*) spore chains. The surface of the spores is smooth. Melanin pigment is neither formed on peptone-yeast-extract iron agar as well as on tyrosin agar[11], but a diffusible brown pigment is produced. The temperature optimum is at about 30°C, the temperature maximum at about 45°C. Chitin, starch, casein, gelatin, and esculin are degraded. The strain is catalase positive, nitrate reductase is not formed. The cell wall peptidoglycan of the strain contains major amounts of L-diaminopimelic acid (L-DAP) but no diagnostic sugars (cell wall chemotype I). Due to its chemical and morphological features the strain B 8251 can be assigned to the suprageneric group *Streptomycetes*[††].

Fermentation

The strain B 8251 was cultivated in 1 liter Erlenmeyer flasks containing 160 ml of production medium (1% malt extract, 0.4% yeast extract, 0.4% D-glucose and 50% synthetic sea water, pH=7.8 prior to sterilization) at 28°C for 48 hours. 2 liters of this culture was used as inoculum for a 20-liter fermentor (Fa. Meredos, Göttingen, FRG) with the same medium as used for production; starting pH 7.8 (28°C, aeration of 20 liters/minute with agitation of 250 rpm; the pH remained between 5.9 and 7.5). The fermentation was stopped after 72 hours.

The culture filtrate and mycelium were extracted with ethyl acetate. This afforded 5.34 g of brown crude extract, which was dissolved in 400 ml methanol. The solution was repeatedly extracted with cyclohexane and the extract was subjected to column chromatography on silica gel (cyclohexane/EtOAc, 9 : 1) and Sephadex LH-20 ($CHCl_3$/MeOH 6 : 4) to afford 35 mg of yellow oily **2** and 30 mg of **4**.

The methanolic solution was evaporated to dryness and the residue was separated on silica gel with chloroform to yield 28 mg of *N*-(2-hydroxyphenyl)-acetamide (**1**) and 46 mg of phencomycin (**3a**).

N-(2-Hydroxyphenyl)-acetamide (**1**)

Crystallization (chloroform) gave colorless needles with mp 207°C. Rf=0.15 ($CHCl_3$); 0.5 ($CHCl_3$/MeOH=95 : 5); IR $\tilde{\nu}_{max}$ cm^{-1} KBr 3400 (NH), 3080 (br), 2980, 2750, 2614, 2495, 1680 (CO), 1613, 1595, 1538, 1450, 1392, 1332, 1287, 1252, 1243, 1204, 1109, 1040, 1018, 968, 934, 845, 812, 768, 670, 605; ^{1}H NMR ([d_6]-Aceton, 300 MHz): δ=9.40 (s br, 1H, D_2O exchangable, NH), 9.27 (s br; 1H, OH), 7.39 (dd, ^3J=8 Hz, $^4J\approx$1 Hz; 1H, 6-H), 7.02 (ddd, 2 ^3J=8 Hz, $^4J\approx$1 Hz; 1H, 4-H), 6.89 (dd, ^3J=8 Hz, $^4J\approx$1 Hz; 1H, 3-H), 6.80 (ddd, 2 ^3J=8 Hz, $^4J\approx$1 Hz; 1H, 5-H), 2.20 (s; 3H, CH_3); EI MS (70 eV): *m/z* (%)=151 (18, M^+), 109 (100), 80 (16), 53 (6), 43 (26).

Octahydromenaquinone [MK9 (II, III, VIII, IX-H8), **2**]

Yellow oil, Rf=0.05 (C_6H_{12}); 0.90 (C_6H_{12}/EE=90 : 10); IR $\tilde{\nu}_{max}$ cm^{-1} KBr 2953, 2930, 2859, 1750 (br), 1710 (br), 1663 (CO), 1617, 1598, 1455, 1378, 1331, 1297, 1260, 1228, 1163, 1097, 1028, 973, 952, 894, 840 (br), 787, 717, 695, 610; ^{1}H NMR ($CDCl_3$, 200 MHz): (=8.08 (m; 2 H, 5-H, 8-H), 7.68 (m; 2H, 6-H, 7-H), 5.11 (t, ^3J=6 Hz; 4H, 14′-H, 18′-H, 22′-H, 26′-H), 5.00 (t, ^3J=7 Hz; 1H, 2′-H), 3.47 (d, ^3J=7 Hz; 2H, 1′-CH_2), 2.19 (s; 3H, 3-CH_3), 2.12~1.88 (m; 18H, 4′-CH_2, 13′-CH_2, 16′-CH_2, 17′-CH_2, 20′-CH_2, 21′-CH_2, 24′-CH_2, 25′-CH_2, 28′-CH_2), 1.78 (s; 3H, 3′-CH_3), 1.60 (s; 12H, 15′-CH_3, 19′-CH_3, 23′-CH_3, 27′-CH_3), 1.50~0.84 (m; 26H, 5′-CH_2, 6′-CH_2, 7′-CH, 8′-CH_2, 9′-CH_2, 10′-CH_2, 11′-CH, 12′-CH_2, 29′-CH_2, 30′-CH_2, 31′-CH, 32′-CH_2, 33′-CH_2, 34′-CH_2, 35′-CH), 0.88~0.78 (m; 15H, 7′-CH_3, 11′-CH_3, 31′-CH_3, 2 35′-CH_3); DCI MS: *m/z* (%)=809 (100, $M^+ + NH_3$).

Phencomycin (**3a**)

Greenish-yellow needles from $CHCl_3$/MeOH; IR $\tilde{\nu}_{max}$ cm^{-1} KBr 3440 (br, COOH), 3010, 2960, 2660 (br), 1730 (CO), 1740 (CO), 1615, 1599, 1562, 1533, 1483, 1462, 1427, 1411, 1380, 1350, 1318, 1265, 1228, 1200, 1184, 1145, 1048, 980, 875, 863, 850, 827, 797, 755, 674, 643; NMR spectra see Table 1; EI MS (70 eV): *m/z* (%)=282 (1.6, M^+), 251 (5), 238 (100), 223 (12), 179 (17), 152 (5), 75 (6); FAB MS: *m/z* (%)=282.

†† The strain B 8251 is deposited in the culture collection of marine actinomycetes of the Alfred-Wegener Institute for Polar and Marine Research in Bremerhaven.

Phencomycin Methyl Ester (**3b**)

Phencomycin (**3a**, 5 mg) was dissolved in 0.5 ml etherial diazomethane solution and immediately evaporated to dryness to afford 5 mg **3b** as a greenish-yellow solid with mp 223°C. Rf = 0.35 ($CHCl_3$); 0.80 ($CHCl_3$/MeOH = 95 : 5); IR $\tilde{\nu}_{max}$ cm^{-1} KBr 2958, 2920, 2852, 1718 (CO), 1675, 1615, 1530, 1483, 1460, 1448, 1410, 1381, 1350, 1273, 1219, 1138, 1072, 1045, 856, 828, 810, 763, 745, 672, 643; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 8.48$ (dd, $^3J = 8$ Hz, $^4J \approx 1$ Hz; 2H, 4-H, 9-H), 8.29 (dd, $^3J = 8$ Hz, $^4J \approx 1$ Hz; 2H, 2-H, 7-H), 7.89 (dd, 2 $^3J = 8$ Hz; 2H, 3-H, 8-H), 4.11 (s; 6H, 2 OCH_3).

Phencomycin Ethyl Ester (**3c**)

From **3a** and diazoethane **3c** was obtained as a greenish-yellow solid with mp 228°C. Rf = 0.35 ($CHCl_3$); 0.80 ($CHCl_3$/MeOH = 95 : 5); IR $\tilde{\nu}_{max}$ cm^{-1} KBr 2980, 2960, 2915, 2852, 1723 (CO), 1616, 1533, 1446, 1438, 1410, 1396, 1365, 1347, 1318, 1306, 1268, 1218, 1207, 1139, 1098, 1052, 970, 938, 856, 814, 758, 745, 673, 643; UV (MeOH): λ_{max} nm ($\log \varepsilon$) 214 (4.06), 248 (4.64), 365 (3.97); (MeOH + HCl): λ_{max} nm ($\log \varepsilon$) 214 (4.06), 248 (4.64), 365 nm (3.97); (MeOH + NaOH): λ_{max} ($\log \varepsilon$) = 248 (4.64), 365 (3.97); ^1H NMR ($CDCl_3$, 200 MHz): $\delta = 8.46$ (dd, $^3J = 8$ Hz, $^4J \approx 1$ Hz; 2H, 4-H, 9-H), 8.32 ~ 8.24 (m; 2H, 2-H, 7-H), 7.94 ~ 7.84 (dd, 2 $^3J = 8$ Hz; 2H, 3-H, 8-H), 4.58 (q, $^3J = 7$ Hz; 2H, OCH_2), 4.11 (s; 3H, OCH_3), 1.51 (t, $^3J = 7$ Hz; 3H, CH_3); NOESY ($CDCl_3$, NOESYTP, relax.delay 2.000 sec, F1 300.135 MHz, F2 300.133 MHz): 2-H→3-H, 2-H→4-H, 3-H→4-H, 7-H→8-H, 7-H→8-H, 8-H→9-H, OCH_2→CH_3; EI MS (70 eV): m/z (%) = 310 (47, M^+), 279 (10), 266 (24), 252 (26), 238 (100), 222 (19), 179 (15), 117 (10), 104 (7).

Dimethyl 5,10-Dihydrophenazine-1,6-dicarboxylate (5,10-Dihydrophencomycin Methyl Ester, **4**)

Orange needles from $CHCl_3$/MeOH; IR $\tilde{\nu}_{max}$ cm^{-1} KBr 3322 (NH), 2958, 2930, 2858, 1678 (CO), 1607, 1494, 1442, 1330, 1288, 1271, 1226, 1203, 1155, 1090, 1049, 937, 889, 865, 800, 751, 743, 726, 690, 668, 602; UV (MeOH + HCl): λ_{max} nm ($\log \varepsilon$) 216 (4.50), 244 (4.49), 451 (4.04); (MeOH + NaOH): λ_{max} nm ($\log \varepsilon$) 216 (4.51), 245 (4.48), 451 (4.03); NMR spectra see Table 1; C, H COSY (HETCOR, $CDCl_3$, F1 499.8 MHz, F2 125.7 MHz) (C→H): C-4′→4′-H, C-3′→3′-H, C-2-OCH_3→OCH_3; HMBC NMR (inverse COLOC, $CDCl_3$, INV4NDPT, F1 300.13 MHz, F2 75.48 MHz) (H→C): OCH_3 ^3J→CO; 2-H, 7-H ^3J→CO; 2-H, 7-H ^3J→C-5a, C-10a; 4-H, 9-H ^3J→C-5a, C-10a; NH ^2J→C-4a, C-9a; 3-H, 8-H ^3J→C-4a, C-9a; 2-H, 7-H ^1J→C-2, C-7; 4-H, 9-H ^3J→C-2, C-7; 3-H, 8-H ^1J→C-3, C-8; 4-H, 9-H ^1J→C-4, C-9; 2-H, 7-H ^3J→C-4, C-9; 3-H, 8-H ^3J→C-1, C-6; OCH_3 ^1J→OCH_3.-EI MS (70 eV): m/z (%) = 298.0953 (100, M^+, calcd. 298.0936 for $C_{16}H_{14}N_2O_4$), 266 (29), 238 (91), 206 (76), 178 (20), 149 (12), 117(18), 69 (26); DCI MS: m/z (%) = 298 (100).

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