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Endophenazines A~D, New Phenazine Antibiotics from the Athropod Associated Endosymbiont *Streptomyces anulatus*

II. Structure Elucidation†

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A detailed screening of the secondary metabolite pattern produced by different athropod associated strains of the species *Streptomyces anulatus* resulted in the isolation and structure elucidation of the endophenazines A~D (**2**, **4**~**6**). The structures were assigned by spectroscopic methods and chemical transformations. **4** represents a chromophoric system based on a phenazin-7-one, **5** and **6** are new 5,10-dihydrophenazine derivatives.

In the course of our screening for bioactive compounds from endosymbiontic microorganisms we investigated athropod associated microorganisms by analyzing their metabolite pattern. The extracts of *Streptomyces anulatus* (strains 9663, 9843, 9958 and 10099) were applied to our chemical screening programme[2]. HPTLC analysis resulted in five substances utilising their colour, UV-absorption and colourization by Ehrlich's reagent. The substances were identified as phenazine derivatives.

In the previous paper the taxonomy of *Streptomyces anulatus* as well as the fermentation, isolation and biological activities of the endophenazines have been described[1]. In this part we present the structure elucidation of these antibiotics mainly done by NMR analysis.

Structure Elucidation

Phenazine-1-carboxylic acid (**1**), isolated from the culture filtrate of the strain 9663, was identified by comparison of its spectroscopic data with the data given in the literature[3,4]. The UV-spectrum of endophenazine A (**2**) is very similar to that of **1**, indicating a further phenazine derivative. The ^{1}H NMR spectrum shows six aromatic protons, their coupling pattern points to a 1,6- or 1,9-disubstituted phenazine skeleton. From the molecular formula ($C_{13}H_8N_2O_2$) and the data obtained from the NMR spectra a carboxyl and a C_5-prenyl group can be derived as substituents. Due to a NOE between the acidic proton of the carboxyl and the methine group of the prenyl residue, the regiochemistry of the substituents was unambiguously

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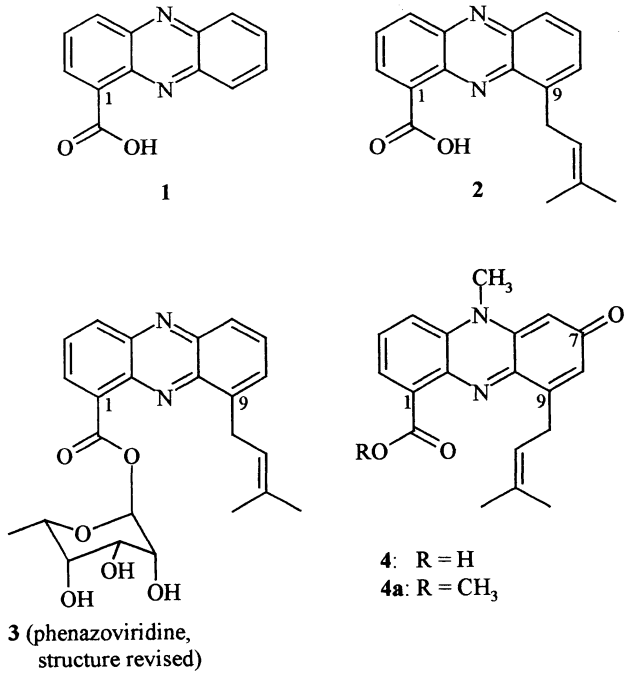
Fig. 1. Structural formulae of and endophenazine A (**2**) and B (**4**) and related compounds.



determined to be [1, 9], which has not been described before. Comparison of the spectroscopic data with the reported [1, 6] isomer[5] shows that the compounds must be identical. That nature in this case prefers the [1,9] regiochemistry must also be assumed for phenazoviridin[6] (**3**).

The striking violet colour of endophenazine B (**4**) indicates a different chromophoric system compared with the yellow-orange coloured compounds **1** and **2**. The ^{13}C NMR spectra of **4** shows signals for three methyl, one methylene and six olefinic/aromatic methine groups in addition to nine quaternary carbonatoms. The signals at δ_C=165.5 and 183.9 indicate a carboxyl group and an unsaturated ketone. From the natural product itself it was not possible to determine the molecular mass *via* EI, DCI or ESI mass spectrometry. Methylation of **4** with diazomethane resulted in the more lipophilic methyl ester **4a**. Its molecular formula $C_{20}H_{20}N_2O_3$ resulted from HREIMS analysis (M^+: *m/z* 336.1473). On the basis of the ^{13}C NMR data of **4** and the molecular formula of **4a** it is possible to determine the molecular formula of **4** as $C_{19}H_{18}N_2O_3$. The ^1H NMR spectrum of **4** indicates the presence of one C_5-prenyl group, one *N*-methyl and five olefinic/aromatic protons. With the remaining signals of the ^{13}C NMR spectrum (including the keto group) and the nitrogen atoms it is possible to predict a phenazine core structure substituted with a prenyl, a methyl and a carboxyl group as substituents. The different elements could be combined by means of 2D NMR spectroscopy using ^1H-^1H COSY and HMBC sequences (Figure 3). The position of the *N*-methyl group was proven by a NOSY experiment, which reveals correlation's between the methyl group and 4-H/6-H. The unusual chromophoric system, a substituted phenazin-7-one, responsible for the violet colour of endophenazine B (**4**) has not been described as part of a natural product so far.

Endophenazine C (**5**) was isolated as a green solid. Its DCI mass spectrum shows the molecular ion at *m/z* 309 $[M+H]^+$. Surprisingly the ^{13}C and ^1H NMR spectra of **5** in DMSO-d_6 exhibit no signals, addition of TFA does not change the situation. Probably **5** is a complex with a paramagnetic metal. Methylation of **5** with diazomethane led to the orange methyl ester **5a**, which gave sufficient NMR spectra. The EI-MS of **5a** shows a molecular ion at *m/z*=322 (M^+, $C_{20}H_{22}N_2O_2$). The ^1H NMR spectrum displays besides the methoxy group the signals for a C_5-prenyl group, one *N*-methyl, six aromatic protons and one exchangeable proton (δ 9.32). Together with the information from the ^{13}C NMR spectrum it is possible to predict a reduced phenazine as core structure substituted with the C_5-prenyl, the *N*-methyl and the methoxycarbonyl group. The positions of the substituents could be determined by a COSY and a HMBC experiment (Figure 3). The position of the *N*-methyl group was additionally confirmed by a NOESY experiment, which reveals contacts to 4-H and 6-H. The structure of **5** is deduced from that of **5a** and from its molecular mass. It seems possible that **5** acts as a chelating agent with a carboxylate and an amino group as binding sites. Which metal is part of the complex is still unknown, from the colour and the behaviour in the NMR spectroscopy we assume that it could be iron.

The colourless endophenazine D (**6**) was isolated from the culture filtrate of strain 9843. The molecular formula of **6** was shown to be $C_{15}H_{12}N_2O_4$ by HREIMS analysis (M^+: *m/z* 284.0797). Its ^1H NMR spectrum displays signals for one methylene and seven aromatic protons between δ_H=6.9 and 7.8, in comparison with the molecular formula three exchangeable protons are left. The APT spectrum shows signals for the carbon atoms carrying protons and additionally five quaternary carbons and two carbonyl groups at δ_C=168.7 and 171.7. These data indicates a reduced phenazine-1-carboxylic acid as core structure, which is esterified with glyoxylic acid resulting in **6**. The assignment of ^{13}C signals (Table 1) was done by HMBC experiments. The esterification results in a downfield shift

Fig. 2. Structural formulae of endophenazine C (**5**), D (**6**) and related compounds.

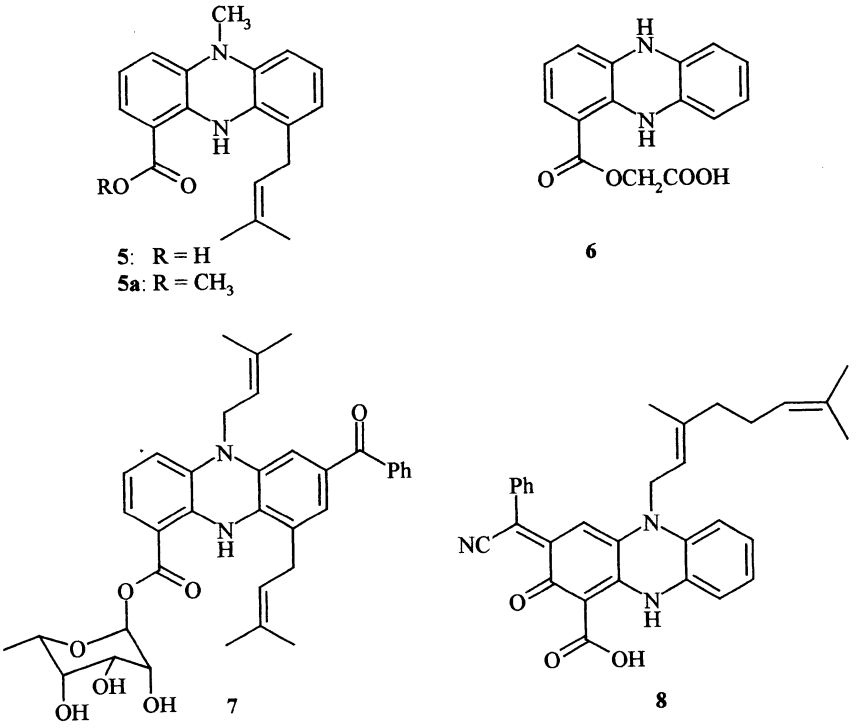
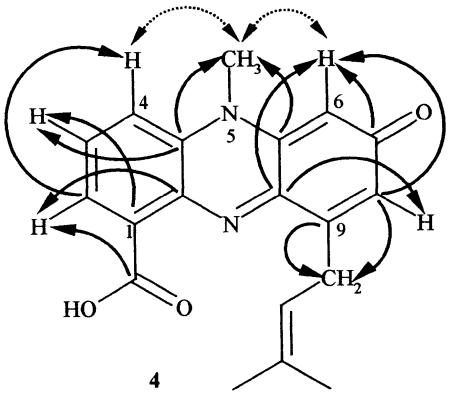


Table 1. ^{13}C NMR data of endophenazine A (**2**), endophenazine B (**4**), endophenazine C methyl ester (**5a**) and endophenazine D (**6**).

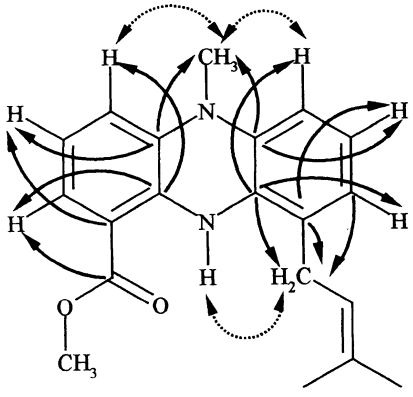
	2[a]	**4**[a]	**5a**[b]	**6**[c]
C-atom	δ	δ	δ	δ
C-1	124.9	126.3	109.2	113.2
C-2	137.0	128.4	121.9	128.7
C-3	130.0	132.6	120.4	119.2
C-4	134.8	118.1	114.3	129.8
C-4a	142.9	132.7	137.9	126.1
C-5a	144.4	138.7	136.3	125.5
C-6	128.0	100.6	110.4	115.7
C-7	131.8	183.9	122.8	127.0
C-8	131.5	135.6	122.8	121.4
C-9	139.14*	142.9	124.7	124.7
C-9a	139.04*	147.7	132.1	138.2
C-10a	139.00*	131.8	142.2	142.8
C-1′	29.8	29.1	30.1	-
C-2′	120.3	118.0	121.7	-
C-3′	135.4	137.0	134.5	-
C-4′	25.7	25.8	25.9	-
C-5′	18.0	18.0	18.0	-
1-COOR	166.0	165.5	169.3	168.7
1-COOCH3	-	-	52.1	-
1-COOCH2COOH	-	-	-	60.0
1-COOCH2COOH	-	-	-	171.7
5-CH3	-	34.7	32.7	-

[a] $CDCl_3$; [b] acetone-d_6; [c] DMSO-d_6
* Assignment may be interchanged

Fig. 3. Important $^{n}J_{C,H}$ long range couplings and NOE's observed in endophenazine B (**4**) and endophenazine C methyl ester (**5a**) by HMBC and NOESY pulse sequences at 500 MHz.



4



5a

^{13}C-^{1}H long-range coupling

NOE

of the methylene group (δ_H=4.16).

Discussion

Phenazines are widespread among microorgansims, up to now about 125 derivatives with different biological activities are known, mainly produced by *Pseudomonas* and *Streptomyces* species[7]. The endophenazines A~D (Figures 1 and 2), named with regard to their biological origin, are new members of this class of natural products. Most of the described phenazines have a fully conjugated tricyclic ring system, dihydrophenazines as endophenazine D (**6**) are rarely found in nature. The only examples are dihydrophencomycin methyl ester from a marine strain of *Streptomyces*[8] and chloraphin, a 3:1 complex of phenazine-1-carboxamide and the corresponding dihydro derivative[9]. *N*-monosubtituted dihydrophenazines like endophenazine C (**5**) are more common in nature, *e.g.* the neuronal cell protecting substance aestivophoenin C[10] (**7**), also possessing the [1, 9] substitution pattern as found for the endophenazines. Most of the published disubstituted phenazines shows the [1, 6] regiochemistry, which in some cases has to be revised.

The striking colour of the violet endophenazine C (**4**) is derived from the new phenazin-7-one chromophore. Such systems have been developed by synthesis[11] and are used as part of a novel concept of organic photovoltaic devices[12]. Benthocyanin C (**8**)[13] also shows a phenazinone skeleton, but the keto group has another position and the chromophoric system is not comparable with that of **4**.

One so far can only speculate about the importance of the different strains of *Streptomyces anulatus* for their host insects. The occurrence of the new endophenazines in the extracts of such strains, which were isolated from different host insects as wood-louse, millipede and leaf-beetle, may be a hint, that these metabolites have a function in improving the living conditions of the host.

Experimental

General

MP's were determined on a Reichert hot-stage microscope and are not corrected. NMR spectra were measured with Bruker AMX 300 (300 MHz) and Varian Inova 500 (500 MHz) instruments. Chemical shifts are expressed in δ values with solvents as internal standards. The mass spectra were taken by Finnigan MAT 95 (EI-MS: 70 eV, high resolution with perfluorkerosine as internal standard; DCI-MS: 200 eV, NH_3 as reactant gas). IR spectra in pressed KBr discs were recorded on a Perkin Elmer FT IR-1600 spectrometer and the UV spectra on a Varian Cary 3E spectrophotometer. TLC was carried out on silica gel 60 F_{254} plates (Merck, 0.25 mm), compounds were viewed under UV lamp at 254 nm and sprayed with anisaldehyde/H_2SO_4 or Ehrlich's reagent followed by heating.

Endophenazine A [9-(3′-Methyl-2′-butenyl)-phenazine-1-carboxylic Acid, **2**]

Yellow solid; Rf 0.63 ($CHCl_3$-MeOH, 9:1); colour reaction with Ehrlich's reagent, grey-green; MP 204°C; UV λ_{max}^{MeOH} nm (log ε) 255 (4.81), 365 (4.06); IR ν_{max} (KBr)

cm^{-1} 3440, 1737, 1532, 1462; ^1H NMR (500 MHz, $CDCl_3$) δ 1.78 (s, 6H, 4′-H_3, 5′-H_3), 4.04 (d, J=7.0 Hz, 2H, 1′-H_2), 5.43 (t, J=7.0 Hz, 1H, 2′-H), 7.78 (dd, J=7.0, 1.5 Hz, 1H, 8-H), 7.86 (dd, J=8.5, 7.0 Hz, 1H, 7-H), 7.99 (dd, J=8.5, 7.5 Hz, 1H, 3-H), 8.13 (dd, J=8.5, 1.5 Hz, 1H, 6-H), 8.46 (dd, J=8.5, 1.5 Hz, 1H, 4-H), 8.92 (dd, J=7.5, 1.5 Hz, 1H, 2-H), 15.60 (br, 1H, 1-COOH); ^{13}C NMR (see Table 1); EI-MS m/z (%) 292.1211 (100) [M^+, calcd. for $C_{18}H_{16}N_2O_2$ and found], 259 (24), 245 (65), 231 (50), 205 (48).

Endophenazine B [1-Carboxy-5-methyl-9-(3′-methyl-2′-butenyl)-phenazin-7-one, **4**]

Violet solid; Rf 0.24 ($CHCl_3$ - MeOH, 9 : 1); UV λ_{max}^{MeOH} nm (log ε) 236 (4.33), 283 (4.44), 374 (3.86), 516 (3.89), 545 (sh) $\lambda_{max}^{MeOH+HCl}$ 272 (4.54), 391 (4.04), 482 (3.73), $\lambda_{max}^{MeOH+NaOH}$ 235 (4.34), 283 (4.44), 373 (3.87), 516 (3.89), 545 (sh); IR ν_{max} (KBr) cm^{-1} 3436, 1735, 1628, 1595, 1548; ^1H NMR (500 MHz, $CDCl_3$) δ 1.68 (s, 3H, 5′-H_3), 1.78 (s, 3H, 4′-H_3), 3.60 (d, J=7.0 Hz, 2H, 1′-H_2), 3.79 (s, 3H, 5-CH_3), 5.30 (t, J=7.0 Hz, 1H, 2′-H), 6.15 (s, 1H, 6-H), 6.97 (s, 1H, 8-H), 7.72 (d, J=8.0 Hz, 1H, 4-H), 7.80 (t, dd, J=8.0, 8.0 Hz, 1H, 3-H), 8.36 (d, J=8.0 Hz, 1H, 2-H); ^{13}C NMR (see Table 1); EI-MS, DCI-MS, ESI-MS no results.

Endophenazine B Methyl Ester [1-Methoxycarbonyl-5-methyl-9-(3′-methyl-2′-butenyl)-phenazin-7-one, **4a**)

To a stirred solution of 0.1 mg **4** in 2 ml MeOH 1 ml of a 0.4 M solution of diazomethane in diethyl ether was added at 0°C. The mixture was stirred for 30 minutes at room temperature and after evaporated to dryness analysed without further purification.

Rf 0.43 ($CHCl_3$ - MeOH, 9 : 1); EI-MS m/z (%) 336.1473 (100) [M^+, calcd. for $C_{20}H_{20}N_2O_3$ and found], 289 (20), 248 (28).

Endophenazine C [5-Methyl-9-(3′-methyl-2′-butenyl)-10*H*-phenazine-1-carboxylic Acid, **5**)

Green solid (as complex), Rf 0.41 (CH_2Cl_2 - MeOH, 19 : 1); UV λ_{max}^{MeOH} nm (log ε) 254 (4.47), 410 (3.77), $\lambda_{max}^{MeOH+HCl}$ 252 (4.44), 426 (3.79), $\lambda_{max}^{MeOH+NaOH}$ 253 (4.53), 387 (3.72); IR ν_{max} (KBr) cm^{-1} 3435, 1651, 1508, 1472, 1254; DCI-MS m/z (%) 309 (100) $[M+H]^+$.

Endophenazine C Methyl Ester [1-Methoxycarbonyl-5-methyl-9-(3′-methyl-2′-butenyl)-10*H*-phenazine, **5a**]

To a solution of 16.7 mg **5** in 5 ml MeOH 2 ml of a 0.4 M solution of diazomethane in diethyl ether was added and stirred at room temperature for 30 minutes. The mixture was evaporated to dryness and the residue was purified by column chromatography (silica gel, CH_2Cl_2 - MeOH, 99 : 1) to yield 4.8 mg **5a** as orange solid.

Rf 0.68 ($CHCl_3$ - MeOH, 9 : 1); UV λ_{max}^{MeOH} nm (log ε) 248 (4.09), 323 (2.60); 432 (3.28); IR ν_{max} (KBr) cm^{-1} 3428, 1678, 1506, 1478, 1260; ^1H NMR (500 MHz, d_6-acetone) δ 1.76 (d, J=1.0 Hz, 3H, 4′-H_3), 1.78 (s, 3H, 5′-H_3), 2.96 (s, 3H, 5-CH_3), 3.14 (d, J=7.0 Hz, 2H, 1′-H_2), 3.87 (s, 3H, 1-COOCH_3), 5.28 (tq, J=7.0, 1 Hz, 1H, 2′-H), 6.33 (d, J=8.0 Hz, 1H, 6-H), 6.41 (d, J=8.0 Hz, 1H, 4-H), 6.50 (dd, J=8.0, 1.0, 1H, 8-H), 6.54 (t, J=8.0 Hz, 1H, 3-H), 6.62 (t, J=8.0 Hz, 1H, 7-H), 7.10 (dd, J=8.0, 1.0 Hz, 1H, 2-H), 9.32 (br, 1H, 10-H); ^{13}C NMR (see Table 1); EI-MS m/z (%) 322.1681 (100) [M^+, calcd. for $C_{20}H_{22}N_2O_2$ and found], 275 (16), 247 (16).

Endophenazine D [1-(Carboxymethylenoxycarbonyl)-5,10-dihydrophenazine, **6**]

Colourless solid, Rf 0.10 ($CHCl_3$ - MeOH, 9 : 1); colour reaction with Ehrlich's reagent, yellow; UV λ_{max}^{MeOH} nm (log ε) 293 (3.82), 351 (3.63); IR ν_{max} (KBr) cm^{-1} 3446, 3344, 1665, 1596, 1488, 1457, 1266, 1094; ^1H NMR (500 MHz, d_6-DMSO) δ 4.16 (s, 2H, CH_2COOH), 6.98 (m, 1H, 3-H), 7.00 (m, 1H, 8-H), 7.13 (m, 1H, 6-H), 7.15 (m, 1H, 7-H), 7.40 (d, J=8.0 Hz, 1H, 9-H), 7.57 (d, J=8.0 Hz, 1H, 4-H), 7.76 (d, J=8.0 Hz, 1H, 2-H), 9.76 (s, 1H, 10-NH), the exchangeable protons 5-NH and 1-COOH are not detectable; ^{13}C NMR (see Table 1); EI-MS m/z (%) 284.0797 (32) [M^+, calcd. for $C_{15}H_{12}N_2O_4$ and found], 225 (62) $[M-CH_2COOH]^+$, 207 (100) $[C_{13}H_7N_2O]^+$, 179 (55).

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