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

I. Taxonomy, Fermentation, Isolation and Biological Activities[†]

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Four new members of the phenazine family, endophenazines A~D, and the already known phenazine-1-carboxylic acid (tubermycin B) were detected in the culture broth of various endosymbiotic *Streptomyces anulatus* strains by chemical screening in a combination of TLC–staining reagents and HPLC–diode array analysis. The endosymbiotic strains were isolated from four different arthropod hosts at various sites. The new phenazine compounds showed antimicrobial activities against Gram-positive bacteria and some filamentous fungi, and herbicidal activity against *Lemna minor* (duckweed).

In the past, mainly terrestrial habitats were used for the isolation of microorganisms for screening of new secondary metabolites. During the last two decades, marine habitats like sea water and sediments were also empolyed to isolate microbes producing active metabolites[2,3]. A further source for the isolation of microorganisms are arthropods which harbour a great variety of "lodgers"[4~6]. However, the search for bioactive metabolites produced by microorganisms living in symbiosis with arthropods was of minor interest. Only a few reports on bioactive metabolites isolated from symbiotic microorganisms are published so far[7~11].

In a preceding paper, we reported on the results of our screening for bioactive metabolites with endosymbiotic bacilli, which were isolated from various arthropods[12]. In the course of this screening program, we investigated the endosymbiotic actinomycete strains 9663, 9843, 9958 and 10099. They were isolated from the intestine of different arthropods, wood-lice, beetles and millipedes. Based on taxonomic features the actinomycete strains were identified in all four cases as members of *Streptomyces anulatus*. The extracts of the culture broth were analysed by a combination of thin layer chromatography–staining reagents and reversed phase HPLC–diode array monitoring (HPLC-DAD). Five characteristic metabolites were detected in the culture filtrate extracts of these streptomycete strains. They all have nearly the same metabolic pattern. By means of our HPLC-UV-Vis-Database[13] one of the metabolites was identified as phenazine-1-carboxylic acid[14] (tubermycin B), and a second metabolite was predicted to be a phenazine-like compound based on its UV-visible spectrum. The other

[†] Art. No. 26 in "Biosynthetic Capacities of Actinomycetes". Art. No. 25: See ref. 1.

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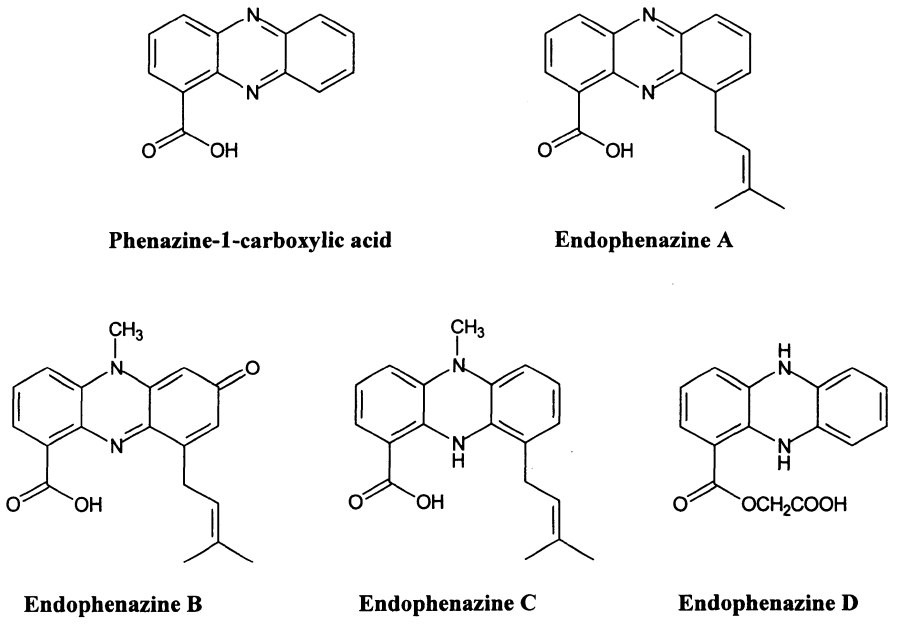
Fig. 1. Phenazine compounds produced by *S. anulatus* strains 9663, 9843, 9958 and 10099.



three metabolites showed no significant similarity to any of the 700 reference compounds stored in our database. Fermentation, isolation and structure elucidation of the metabolites produced by strains *S. anulatus* 9663 and 9843 led to four new phenazine compounds which were named endophenazines A~D, concerning their endosymbiotic origin. The structures of the new phenazine compounds are shown in Fig. 1.

This report deals with the taxonomy of the producing strains, and the fermentation, isolation and biological properties of the new phenazine antibiotics. Details on structure elucidation are reported in the subsequent paper[15].

Materials and Methods

Microorganisms

Streptomycete strain 9663 was isolated from the gut of a wood-louse Gen. spec. (Isopoda), which was collected at Capoliveri, island Elba, Italy. Streptomycete strain 9843 was isolated from the gut of the leaf-beetle *Exosoma lusitanica* (Hexapoda), which was collected in the Cevennes, France. Streptomycete strain 9958 was isolated from the gut of the millipede *Glomeris* spec. (Myriapoda), which was collected at Monte Cetona, Toscana, Italy. Streptomycete strain 10099 was isolated from the gut of the wood-louse *Armadillidium* spec. (Isopoda), which was collected at Monte Cetona, Toscana, Italy. The guts were prepared under the light-microscope and their contents were incubated on humic acid - vitamin agar[16]. The isolated strains are deposited in the culture collection of BASF AG, Ludwigshafen, Germany.

Strains for testing the biological activity spectrum were obtained from DSMZ, ATCC and the strain collection of our laboratory in Tübingen.

Taxonomy

The isolated endosymbiotic strains were characterised by their morphological[17], physiological and chemotaxonomical properties[18~20], as well as by 16S rDNA sequencing[21,22]. The taxonomic investigations were performed by DSMZ, Braunschweig, Germany.

Fermentation

S. anulatus 9663, *S. anulatus* 9958 and *S. anulatus* 10099 were cultivated in 100 ml medium in 300-ml Erlenmeyer flasks with three baffles. The medium was consisted of: glucose 1%; glycerol 1%, starch 1%, corn steep powder 0.25%, casein peptone 0.5%, yeast extract 0.2%, NaCl 0.1%, and $CaCO_3$ 0.3% in tap water (pH 7.0). The fermentation was carried out for 96 hours at 28°C on

a rotary shaker at 180 rpm. The shaking flasks were inoculated with 5 vol-% of a pre-culture grown under the same conditions for 48 hours.

S. anulatus 9843 was cultivated in 100 ml medium in 500-ml Erlenmeyer flasks without baffles. The medium was consisted of: soy peptone 2% and glycerol 2% in tap water (pH 7.5). The fermentation was carried out for 120 hours at 27°C on a rotary shaker at 120 rpm. Shaking flasks were inoculated with 5 vol-% of a pre-culture grown under the same conditions for 48 hours.

Isolation

The culture broth of *S. anulatus* 9663 was centrifuged (4500 rpm, 15 minutes) to separate mycelium and supernatant. The supernatant was adjusted to a Amberlite XAD-2 column, washed with H_2O, and the metabolites were desorbed with MeOH. The eluate was concentrated *in vacuo* to dryness. The residue was dissolved in CH_2Cl_2 and applied on a silica gel column (silica gel 60, 40~63 μm; Merck). Separation was accomplished by a linear gradient elution using CH_2Cl_2 - MeOH starting at 5% MeOH to 10% MeOH. Endophenazines A and C and phenazine-1-carboxylic acid were purified on a silica gel column using cyclohexane - EtOAc (1 : 1) as eluent. Endophenazine B was obtained as a violet powder by silica gel column chromatography using cyclohexane - EtOAc - MeOH (5 : 10 : 1) and CH_2Cl_2 - MeOH (9 : 1) as eluents. Metabolite containing fractions were concentrated and lyophilised.

The culture broth of *S. anulatus* 9843 was adjusted to pH 4 (0.5 N HCl) and extracted three times with an equal volume of EtOAc. The organic layer was concentrated *in vacuo* to dryness. The residue was dissolved in a small volume of MeOH for separation by preparative reversed-phase HPLC using a stainless steel column (VarioPrep ET, 250×21 mm) filled with 25-μm Nucleosil-100 C-18 (Grom). A linear gradient elution with H_2O - MeOH started at 40% MeOH to 90% MeOH within 15 minutes at a flow rate of 20 ml/minute. The preparative system consisted of two high-pressure pumps (Sepapress HPP-200/100; Kronwald), a gradient unit (Sepacon GCU-311), and a Valco preparative injection valve (Mod. 6UW; VICI) with a 5-ml sample loop. The UV absorbance of the eluate was monitored at 260 nm and 280 nm by a Gilson spectrophotometer Mod. 116, equipped with a preperative cell. Endophenazine containing fractions were concentrated *in vacuo* to dryness. The final purification was done on a Sephadex LH-20 column (900×25 mm) using MeOH as eluent. Endophenazine D was obtained as a white powder after lyophilisation.

TLC Analysis

Concentrated extracts (5 μl) were separated on TLC (HPTLC plates silica gel 60; Merck) using $CHCl_3$ - MeOH (9 : 1) and *n*-BuOH - CH_3COOH - H_2O (4 : 1 : 5, upper phase), respectively. The TLC plates were evaluated by UV adsorption at 254 and 366 nm, as well as by colorization with various staining reagents, such as anisaldehyde - H_2SO_4, orcinol reagent, Ehrlich's reagent, and vanillin - H_2SO_4.

HPLC-DAD Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode-array detector, a HP Kayak XM 600 ChemStation and a HPLC software revision A.08.03 (Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360 and 435 nm; the spectrum range was from 200 to 600 nm with a 2-nm step and a sampling rate of 640 milliseconds.

For analysing the culture broth, a 10-ml aliquot was adjusted to pH 4 (0.5 N HCl) and extracted with the same volume of EtOAc. The organic layer was concentrated to dryness. The residue was dissolved in 1 ml MeOH. A 10-μl aliquot of the samples was injected on a HPLC column (125×4.6 mm) fitted with a guard-column (20×4.6 mm) which were filled with 5-μm Nucleosil-100 C-18 (Maisch). The samples were analysed by a linear gradient elution using 0.1% phosphoric acid-acetonitrile at a flow rate of 2 ml/minute, starting at 0% acetonitrile to 100% acetonitrile in 15 minutes with a 1-minute·hold at 100% acetonitrile, followed by a 5-minute post-time under initial conditions.

Biological Assays

To determine the antimicrobial spectrum of endophenazines and tubermycin B, an agar plate diffusion assay was performed. Ten microliters of the samples were applied to filter disks (6 mm diameter). The test plates were incubated for 24~48 hours at a temperature that permitted an optimal growth of the microorganisms.

Herbicidal activity was determined using *Lemna minor* L. (duckweed) as test organism. The assay was performed in 24-well plates as described previously[23]. The herbicidal activity was evaluated by the ratio of bleaching and growth of the *Lemna minor* plants.

Results

Taxonomy

Four endosymbiotic actinomycete strains, 9663, 9843,

9958 and 10099, were isolated from the intestine of a beetle, a millipede and two wood-lice. Chemotaxonomic properties such as the presence of LL-diaminopimelic acid in the peptidoglycan, the absence of mycolic acids, and the typical pattern of saturated *iso*- and *anteiso*-branched fatty acids, assigned the strains to the genus *Streptomyces*. The colour of the aerial mycelium of all strains was yellow-grey. The spore chains were straight-wavy (RF-type) with spores distinguished by a smooth surface. The substrate mycelium of the strains was of a reddish-brown colour as of the soluble pigments. Melanoid pigments were produced on tyrosine agar (ISP 7), but no production was observed on peptone-yeast extract-iron agar (ISP 6). The cultural characteristics and the morphological features of the isolated strains were compared with those of type strains *S. griseus* DSM 40236 and *S. anulatus* DSM 40361. The results are summarised in Table 1. The utilisation pattern of selected carbon sources of the endosymbiotic strains and type strains are shown in Table 2. Additional physiological tests of the endosymbiotic strains[20] indicated a high correlation to the *S. griseus/anulatus* cluster.

The sequences of the 16S rDNA of the endosymbiotic strains were compared to the sequences of other strains belonging to the genus *Streptomyces*. Each of the endosymbiotic strains possessed a sequence similarity value of 100% to the type strain *S. anulatus* DSM 40361.

As indicated by these results the endosymbiotic isolates 9663, 9843, 9958 and 10099 are strains belonging to the species *S. anulatus*. Moreover, the results adduced that the species *S. chrysomallus* ssp. *chrysomallus*, *S. fluorescens*,

Table 1. Cultural and morphological characteristics of endosymbiotic strains 9663, 9843, 9958 and 10099, and type strains *S. griseus* DSM 40236 and *S. anulatus* DSM 40361.

Strain	Aerial spore mass colour	Colour of substrate mycelium	Diffusible pigments	Spore chain morphology	Spore surface
9663	yellow-grey	reddish-brown	reddish-brown	straight-wavy	smooth
9843	yellow-grey	reddish-brown	reddish-brown	straight-wavy	smooth
9958	yellow-grey	reddish-brown	reddish-brown	straight-wavy	smooth
10099	yellow-grey	reddish-brown	reddish-brown	straight-wavy	smooth
S. griseus DSM 40236	yellow-grey	greyish-yellow	none	straight	smooth
S. anulatus DSM 40361	yellow-white	greyish-yellow	none	straight	smooth

Table 2. Carbohydrate utilisation by endosymbiotic strains 9663, 9843, 9958 and 10099, and type-strains *S. griseus* DSM 40236 and *S. anulatus* DSM 40361, according to SHIRLING & GOTTLIEB.[18]

Carbon source	9663	9843	9958	10099	*S. griseus* DSM 40236	*S. anulatus* DSM 40361
D-Glucose	+	+	+	+	+	+
L-Arabinose	+	+	+	+	−	+
D-Xylose	+	+	+	+	+	+
D-Mannitol	+	+	+	+	−	−
D-Fructose	+	+	+	+	+	+
Sucrose	−	−	−	−	−	−
myo-Inositol	+	−	+	+	−	−
Rhamnose	+	+	+	+	−	+
Raffinose	−	−	−	−	−	−

Growth: +, good; −, none

Table 3. Antimicrobal spectrum of endophenazines A~D and phenazine-1-carboxylic acid (tubermycin B), determined by the agar plate diffusion assay at a concentration of 1 mg/ml (inhibition zones in mm).

Organisms	A	B	C	D	Tubermycin B
Arthrobacter aurescens DSM 20116 [a]	32	-	10	-	-
Arthrobacter globiformis DSM 20124 [a]	13	-	14	n.d.	-
Arthrobacter oxydans DSM 6612 [a]	-	-	9	n.d.	-
Arthrobacter pascens DSM 20545 [a]	10	-	12	n.d.	-
Bacillus subtilis DSM 30 [a]	26	-	20	-	-
Bacillus subtilis DSM 30 [b]	28	-	12	-	-
Staphylococcus aureus ATCC 12600 [a]	21	-	12	-	-
Streptomyces viridochromogenes Tü 57 [a]	22	-	18	-	-
Botrytis cinerea Tü 157 [a]	-	-	-	30	36
Mucor hiemalis Tü 179 [a]	15	-	-	-	-
Paecilomyces variotii Tü 137 [a]	12	-	12	n.d.	-

[a] Complex medium

[b] Chemically defined medium (per litre): glucose 5 g, $C_6H_5Na_3O_7 \times 2H_2O$ 0.5 g, KH_2PO_4 3 g, K_2HPO_4 7 g, $MgSO_4 \times 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, Bacto agar 15 g (pH 7.0)

S. baarnensis, *S. citreofluorescens*, *S. fimicarius*, and *S. praecox* are synonyms of the species *S. anulatus*.

Fermentation and Isolation

Fermentations of *S. anulatus* 9663 were carried out in 300 ml Erlenmeyer flasks having three baffles on a rotary shaker and reached a maximal production of endophenazines after 96 hours. The metabolites were purified by Amberlite XAD-2 and silica gel chromatotgraphy. From one litre culture broth endophenazines A, B and C were obtained in amounts of 5.4 mg, 1.1 mg, and 6.3 mg, respectively, and phenazine-1-carboxylic acid yielded in an amount of 12.8 mg.

S. anulatus 9843 was cultivated in 500 ml Erlenmeyer flasks (without baffles) on a rotary shaker. Under such less aerated conditions, only endophenazine D and phenazine-1-carboxylic acid were produced. Maximal endophenazine D concentration in the broth was 42 mg/litre after a 120 hours incubation period. The compound was isolated from the culture broth by extraction and was purified by preparative reversed-phase HPLC and Sephadex LH-20 chromatography. Endophenazine D was obtained as a white powder after lyophilisation.

Biological Properties

The antimicrobal spectra of endophenazines A~D and tubermycin B (phenazine-1-carboxylic acid) were determined by an agar plate diffusion assay (Table 3). The phenazine metabolites showed different biological activities. Endophenazines A and C showed good antimicrobial activity against several Gram-positive bacteria and some fungi, whereas endophenazine D and tubermycin B only inhibited *Botrytis cinerea* Tü 157. No antimicrobial activity was determined for endophenazine B. Gram-negative bacteria, such as *Agrobacterium tumefaciens* DSM 30205, *Escherichia coli* K12, *Proteus mirabilis* ATCC 35501, and *Pseudomonas fluorescens* DSM 50090, and the green algae *Chlorella fusca* were not sensitive against endophenazines.

Testing the herbicidal properties of the isolated metabolites, endophenazine D and tubermycin B revealed a significant effect against *Lemna minor*. Endophenazines A and B showed less herbicidal activity.

Discussion

Widely distributed microorganisms living in symbiosis

with arthropods seem to be an additional source for isolation of new secondary metabolites[8,12], especially in case of actinomycetes, which are continued to be the most potent producers of bioactive secondary metabolites[24]. It is astonishing to find that four members of the species *Streptomyces anulatus* from various arthropod hosts collected at various sites produced the same pattern of phenazine antibiotics. We don't know, if these antibiotically active metabolites are produced during the symbiotic life-cycle and therefore play a role for their hosts. *S. anulatus* is a well known symbiotic microorganism found in the rumen of ruminants and has importance for its hosts by decomposing cellulose[25]. *S. anulatus* strains are further known as producers of epocarbazolines, inhibitors of 5-lipoxygenase[26], the cytotoxic depsipeptide montanastatin[27], telomestatin, a new telomerase inhibitor[28], and dihydroabikoviromycin[29].

Phenazines of the tubermycin B type having a conjugated tricyclic ring system, as in the case of endophenazine A, are abounded secondary metabolites produced by a variety of *Streptomyces* and *Pseudomonas* species. Compared to these wide-spreaded group of antibiotics, 5,10-dihydrophenazines as endophenazines C and D are rarely distributed in nature, such as 5,10-dihydrophencomycin methyl ester from a marine streptomycete[30], and aestivophoenin C from *S. purpeofuscus*[31].

Production of the various endophenazines by *S. anulatus* strains was highly dependent on the fermentation conditions. Under reduced aeration conditions an exclusively production of endophenazine D was observed. Wheras at a normal aeration, as in case of baffled Erlenmeyer flasks and stirred tank fermenters, endophenazine A was the dominant metabolite and endophenazines B and C were minor congeners. Endophenazine D was not produced under such well aerated conditions.

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