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Arylomycins A and B, New Biaryl-bridged Lipopeptide Antibiotics Produced by *Streptomyces* sp. Tü 6075

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

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New lipopeptide antibiotics, colourless arylomycins A series and yellow arylomycins B series were detected in the culture filtrate and mycelium extracts of *Streptomyces* sp. Tü 6075 by HPLC-diode-array and HPLC-electrospray-mass-spectrometry screening. Arylomycins are a family of lipohexapeptide antibiotics, which represent the first examples of biaryl-bridged lipopeptides. They show antibiotic activities against Gram-positive bacteria.

In the course of ongoing research efforts aimed at exploring the biosynthetical potential of freshly isolated actinomycetes, the secondary metabolite profile of streptomycete strain Tü 6075 was subjected to a closer scrutiny. HPLC-diode-array analysis (HPLC-DAD) coupled with HPLC-electrospray-mass-spectrometry (HPLC-ESI-MS) revealed a pattern of metabolites in the extracts from the culture filtrate and mycelium that could not be identified by means of our HPLC-UV-Vis-Database[2]. The database contains about 700 reference compounds, most of which are antibiotics. Two series of homologous metabolites with retention times between 9.0 and 11.4 minutes were produced by strain Tü 6075, having end-absorption in the UV range and a side-maximum at 290 and 295 nm, respectively. The spectra showed a high conformity with reference compounds of the peptide sub-library stored in the HPLC-UV-Vis-Database. Analysis by HPLC-ESI-MS resulted in molecular masses of 838 and 883 Da for two major components of arylomycins A and B series, respectively.

This report deals with the taxonomy of the producing strain, fermentation, isolation and biological activities of arylomycins. Investigations on their chemical structures are reported in the subsequent paper[3]. These results indicated that colourless arylomycins A series are lipopeptide antibiotics with a unique biaryl bridge between *N*-methyl-4-hydroxyphenylglycine[5] (MeHpg[5]) and tyrosine[7] in their hexapeptide backbone. In the cases of yellow arylomycins B series, tyrosine[7] in A series is replaced by 3-nitro-tyrosine[7]. Their structures are shown in Fig. 1.

Materials and Methods

Microorganisms

Strain Tü 6075 was isolated from a soil sample collected in the tropical rain forest at Cape Coast, Ghana, using yeast extract - malt extract agar with addition of cycloheximide (50 mg/liter) and nalidixic acid (20 mg/liter)[4]. The strain is deposited in the culture collection of our laboratory under

[†] Art. No. 24 in 'Biosynthetic Capacities of Actinomycetes'. Art. No. 23: See ref. 1.

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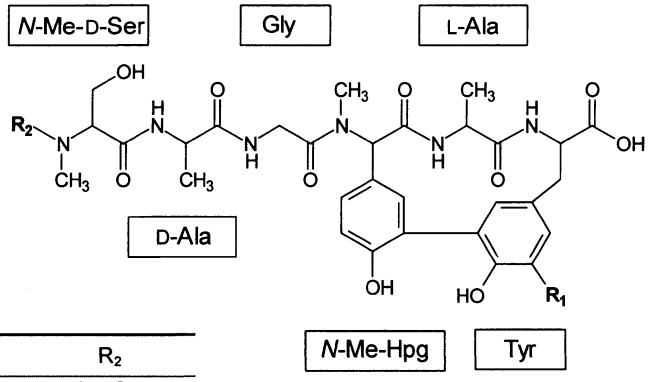
Fig. 1. Structures of arylomycins.



Arylomycin	R_1	R_2
A_1	H	*iso*-C_{11}
A_2	H	*iso*-C_{12}
A_3	H	*n*-C_{12}
A_4	H	*anteiso*-C_{13}
A_5	H	*iso*-C_{14}
B_1	NO_2	*iso*-C_{11}
B_2	NO_2	*iso*-C_{12}
B_3	NO_2	*n*-C_{12}
B_4	NO_2	*anteiso*-C_{13}
B_5	NO_2	*iso*-C_{13}
B_6	NO_2	*iso*-C_{14}
B_7	NO_2	*anteiso*-C_{15}

the accession number Tü 6075.

Strains for testing the minimal inhibition concentrations were obtained from DSMZ, ATCC and the culture collection of our laboratory in Tübingen.

Taxonomy

Strain Tü 6075 was characterised by morphological[5] and chemotaxonomic methods[6] as a member of the genus *Streptomyces*. For molecular characterization fresh bacteria cells were ground using a sterile micro pestle (Eppendorf) to obtain a uniform suspension. Preparation of genomic DNA from the pure culture was performed according to the protocol given by PUKALL *et al.*[7]. The amplification of nearly complete 16S rDNA was done as described by RAINEY *et al.*[8] using the primer pair 27f (5′ GAGTTTGATCCTGGCTCAG 3′) and 1500r (5′ AGAAAGGAGGTGATCCAGCC 3′)[8]. Analysis of the 16S rDNA sequence obtained from the isolate followed the method described by RAINEY *et al.*[9] using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems model 373A automated DNA sequencer. Sequence of the 16S rDNA was manually aligned and compared to the sequences published previously. These were stored in DSMZ-internal database consisting of more than 6000 16S rDNA sequence entries, including those from the Ribosomal Database Project and EMBL. All analyses were done on SUN SparcII workstation.

Fermentation

For production of arylomycins strain Tü 6075 was cultivated in a 20-liter fermenter (type b20, Giovanola) equipped with an intensor system. The production medium consisted of glucose 0.5%, starch 1%, malt extract 1%, yeast extract 0.3%, casein peptone 0.3%, NH_4NO_3 0.3%, and $CaCO_3$ 0.2% in tap water (pH 7.2, adjusted with 5 N NaOH). The fermenter was inoculated with 5 vol-% of shaking cultures, grown at 27°C in the same medium for 72 hours in 500 ml-Erlenmeyer flasks with one buffle on a rotary shaker at 120 rpm.

Isolation

Hyphlo Super-cel (2%) was added to the fermentation broth which was separated by multiple sheet filtration into the culture filtrate (18 liters) and mycelium. The culture filtrate was subjected to an Amberlite XAD-16 column

(40×8 cm; Rohm and Haas), impurities were eluted with H_2O - MeOH (4 : 6), and arylomycins were desorbed with 5 liters of MeOH. The mycelium cake was extracted three times with 2 liters of MeOH - acetone (1 : 1). The eluates and extracts containing arylomycins were combined and concentrated *in vacuo*. The aqueous residue was adjusted to pH 5 (1 N HCl) and extracted six times with the same volume of EtOAc in total. The organic extract was concentrated *in vacuo* and subjected to a diol-modified silica gel column (LiChroprep Diol, 40~63 μm, 400×25 mm; Merck). Arylomycins were desorbed by a linear gradient elution using CH_2Cl_2 - MeOH starting at 0% MeOH to 100% MeOH within 4 hours at a flow rate of 4.5 ml/minute (medium pressure pump Mod. 381, gradient controller Mod. 388, Büchi). The arylomycin containing fractions were concentrated to dryness and dissolved in a small volume of MeOH. Arylomycins of A (colourless) and B (yellow) series were separated on a Sephadex LH-20 column (900×25 mm) using MeOH as eluent. Each arylomycin complex of A and B series was separated to a single component by preparative reversed-phase HPLC using a stainless steel column (250×16 mm) filled with 10-μm LiChrospher RP Select B and a linear gradient elution with 0.5% HCOOH - ACN starting at 40% ACN to 100% ACN within 15 minutes at a flow rate of 20 ml/minute. Finally, pure arylomycins were obtained after preparative reversed-phase HPLC using 10-μm Nucleosil-100 C-18 material and a linear gradient elution with 0.1% CH_3COOH - ACN starting at 40% ACN to 70% ACN 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), 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 290 nm by a Gilson spectrophotometer Mod. 116, equipped with a preparative cell. Arylomycins A series were obtained as white powders and arylomycins B series as yellow powders after lyophilisation.

HPLC-DAD-Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode-array detector and a HP Kayak XM 600 ChemStation using software revision A.08.03 (Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435 and 500 nm; the spectrum range was from 200 to 600 nm with a 2-nm step and a sampling rate of 640 milliseconds.

A 10-ml aliquot of the fermentation broth was centrifuged (10 minutes, 13,000 *g*). The supernatant was adjusted to pH 5 and extracted with the same volume of ethyl acetate. The organic layer was concentrated to dryness and resuspended in 1 ml MeOH. The mycelium pellet was extracted with MeOH - acetone (1 : 1), the extract was concentrated to dryness and resuspended in 1 ml MeOH. A 10 μl aliquot of the samples was injected onto a HPLC column (125×4.6 mm) fitted with a guard-column (20×4.6 mm) which were packed with 5-μm Nucleosil-100 C-18 (Maisch). The samples were analysed by a linear gradient elution using 0.1% *o*-phosphoric acid - ACN starting at 0% ACN to 100% ACN within 15 minutes at a flow rate of 2 ml/minute with a 1-minute hold at 100% ACN, followed by a 5-minute post-time under initial conditions.

HPLC-ESI-MS-Analysis

The system consisted of an ABI 140A HPLC gradient pump (Applied Biosystems) and an API III Taga 6000 E mass spectrometer equipped with a nebulizer-assisted electrospray source (Sciex). The HPLC column eluate was split by a T and introduced into the ion source at a constant flow rate of 40 μl/minute. A 4 μl aliquot of the samples was injected onto an HPLC column (100×2 mm) packed with 3-μm Nucleosil-100 C-18 (Grom). Samples were separated by a linear gradient elution using 0.01% TFA - ACN starting at 0% ACN to 100% ACN within 60 minutes at a flow rate of 200 μl/minute.

Biological Assays

The broth dilution method was used to determine the minimal inhibition concentrations of arylomycins A_1, A_2, A_3, A_4, A_5, B_1, B_4, B_6, B_7, and a mixture of arylomycins B_2 and B_3. The antibiotics were dissolved in DMSO at the final concentrations of less than 5% DMSO. Bacteria were grown in the medium consisted of nutrient broth 0.8% and NaCl 0.5% in deionized water. Streptomycetes were grown in the medium consisted of malt extract 1%, glucose 0.4% and yeast extract 0.4% in tap water (pH 7.3). Bacterial cells and spores (10^6/ml) were used as inoculum, and growth inhibition was evaluated after incubation at 27°C and 37°C for 24 and 96 hours, respectively, on a rotary shaker.

Results

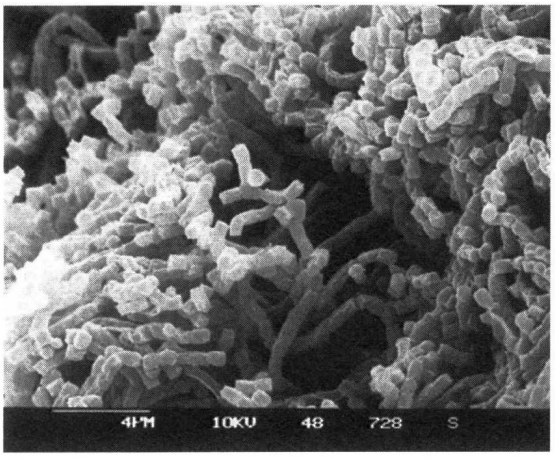
Taxonomy

The presence of LL-diaminopimelic acid in the peptidoglycan together with the morphological characteristics permitted affiliation of strain Tü 6075 to the

genus *Streptomyces*. The substrate mycelium was pale yellowish and the spore mass colour beige-yellowish. Spore chains were straight (Fig. 2). No melanin production was observed.

Nearest phylogenetic neighbours of strain Tü 6075 were determined by 16S rDNA sequence analysis. The sequence of strain Tü 6075 was found to be closely related to those of *Streptomyces sindenensis* DSM 40255^T (99.8%), *Streptomyces badius* DSM 40139^T (99.8%), *Streptomyces setonii* DSM 40395^T (99.7%), *Streptomyces albovinaceus* DSM 40136^T and *Streptomyces griseus* ssp. *alpha* DSM 40937^T (99.5%). However, DNA : DNA hybridisation studies are necessary for the exact affiliation at the species level.

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. Tü 6075.

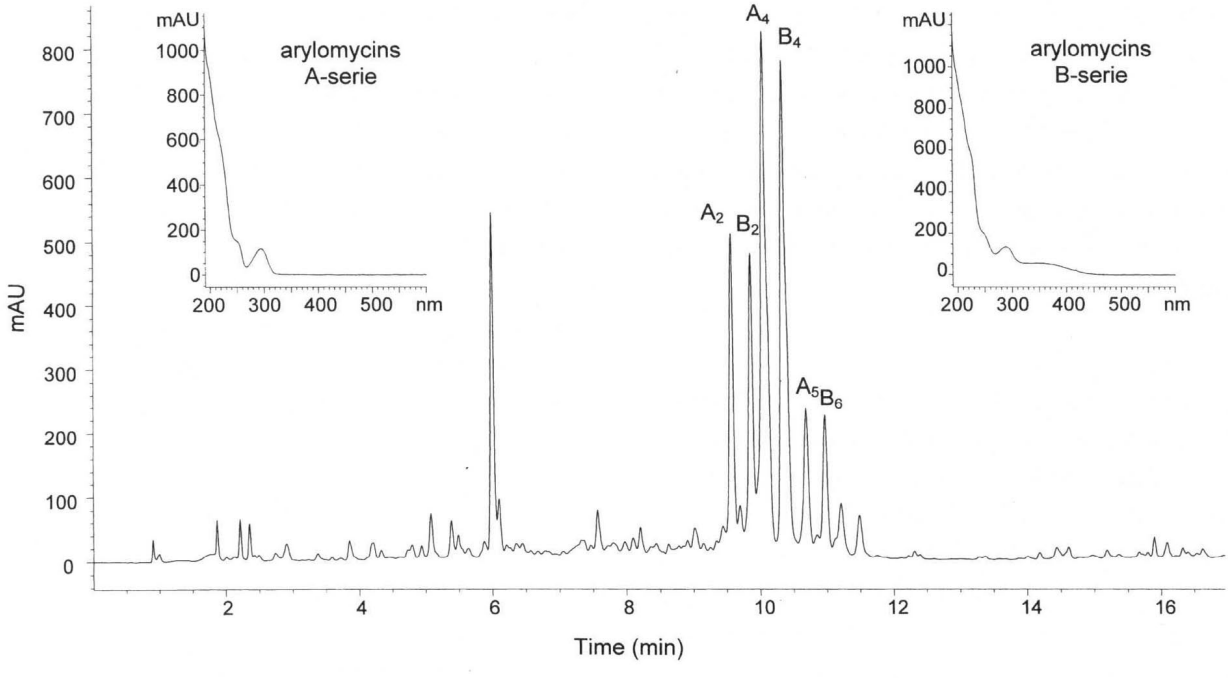


Bar represents 4 μm.

Fermentation and Isolation

Maximal production of arylomycins was obtained in batch fermentations after incubation of 120 hours, reaching 14 mg/liter of arylomycin A_4 and 10 mg/liter of arylomycin A_2, respectively. Arylomycins were isolated from both the culture filtrate and mycelium. HPLC analysis of the mycelium extract is shown in Fig. 3. The eluates and extracts containing arylomycins were concentrated, extracted and purified by adsorption chromatography using diol-modified silica gel. Separation of A and B series was achieved by Sephadex LH-20 chromatography. Finally, pure arylomycins were obtained after preparative reversed-phase HPLC, resulting in five fractions containing each single component of A series as white powders, and four fractions

Fig. 3. HPLC analysis of the mycelium extract of *Streptomyces* sp. Tü 6075 monitored at 210 nm.



Inserts: UV-visible spectra of arylomycins A and B.

Table 1. Antimicrobial spectrum of arylomycins, determined by the agar plate diffusion test.

Organism	A_1	A_2	A_3	A_4	A_5	B_1	B_2/B_3	B_4	B_6	B_7
Arthrobacter globiformis DSM 20124^T	–	–	+	+	10	10	12	9	22	23
Arthrobacter oxydans DSM 6612	–	–	10	12	14	14	12	10	20	15
Arthrobacter pascens DSM 20545^T	–	–	14	9	13	13	+	12	23	22
Rhodococcus erythropolis DSM 1069	+	15	18	22	20	20	12	18	26	25
Streptomyces viridochromogenes Tü 57	–	–	–	7	15	7	–	–	13	11
Brevibacillus brevis DSM 30^T	–	–	–	–	–	–	–	–	8	11
Mucor hiemalis Tü 179/180	+	+	+	+	9	–	–	–	9	–

+, slight inhibition

Table 2. Minimal inhibition concentrations (MIC) of arylomycins as determined by the broth dilution method.

Organism	A_1	A_2	A_3	A_4	A_5	B_1	B_2/B_3	B_4	B_6	B_7
Arthrobacter globiformis DSM 20124^T	30	>100	100	100	100	30	100	100	30	3
Arthrobacter oxydans DSM 6612	100	>100	>100	>100	100	30	>100	>100	>30	30
Arthrobacter pascens DSM 20545^T	30	100	100	100	30	30	30	30	30	10
Rhodococcus erythropolis DSM 1069	10	10	10	3	1	10	30	10	3	1
Streptomyces viridochromogenes Tü 57	30	30	30	30	10	10	30	30	10	10
Brevibacillus brevis DSM 30^T	10	n.d.	10	30	10	1	1	3	<0.1	<0.1

containing each single component of B series, and one fraction containing a mixture of arylomycins B_2 and B_3 as yellow powders, after lyophilisation.

Biological Properties

The minimal inhibitory concentrations of arylomycins determined by a broth dilution method are shown in Table

1. Arylomycins showed antibiotic activities against Gram-positive bacteria. The most sensitive bacteria tested were *Rhodococcus erythropolis* DSM 1069 and *Brevibacillus brevis* DSM 30^T. Arylomycins of B series are characterized by significantly higher antibacterial activities than their A series analogues. Not only nitro substitution influenced the activity but also the length of the saturated fatty acid side-chain.

Gram-negative bacteria, such as *Escherichia coli* K12, *Proteus mirabilis* ATCC 35501, and *Pseudomonas fluorescens* DSM 50090^T were not inhibited. Arylomycins of A series and arylomycin B_6 showed a weak antifungal activity against *Mucor hiemalis* Tü 179/180. Other eucaryotic organisms tested, such as *Saccharomyces cerevisiae* ATCC 9080, *Botrytis cinerea* Tü 157, the green algae *Chlorella fusca*, and the duckweed *Lemna minor*, were not sensitive to arylomycins (data not shown).

Discussion

Arylomycins represent the first example for biaryl-bridged lipopeptides produced by actinomycetes. The search for biaryl-bridged antibiotics in a commercial database[10] resulted only in a few hits: the cyclic tripeptides biphenomycins[11,12] produced by *Streptomyces griseorubiginosus*, and glycopeptides of the vancomycin-type[13,14] produced by various *Amycolatopsis* species. In the case of biphenomycins the biaryl bridge is formed by two 2-hydroxyphenylalanines, and in the case of vancomycin-type glycopeptides by hydroxyphenylglycine at position 5 and 3,5-dihydroxyphenylglycine at position 7.

Most of the lipopeptide-type antibiotics are produced by bacilli. Only a few members of this group were isolated so far from actinomycetes, and they are characterised by a great variety of biological activities. Considering these manifold activities it would be worthwhile to illuminate the mode of action of arylomycins.

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References

1) BERTASSO, M.; M. HOLZENKÄMPFER, A. ZEECK, F. DALL'ANTONIA & H.-P. FIEDLER: Bagremycin A and B, novel antibiotics from *Streptomyces* sp. Tü 4128. J. Antibiotics 54: 730~736, 2001
2) FIEDLER, H-P.: Biosynthetic capacities of actinomycetes. 1. Screening for secondary metabolites by HPLC and UV-visible absorbance spectral libraries. Nat. Prod. Lett. 2: 119~128, 1993
3) HÖLTZEL, A.; D. G. SCHMID, G. J. NICHOLSON, S. STEVANOVIC, J. SCHIMANA, K. GEBHARDT, H.-P. FIEDLER & G. JUNG: Arylomycins A and B, new biaryl-bridged lipopeptide antibiotics produced by *Streptomyces* sp. Tü 6075. II. Structure elucidation. J. Antibiotics 55: 571~577, 2002
4) EL-NAKEEB, M. A. & H. A. LECHEVALIER: Selective isolation of aerobic actinomycetes. Appl. Microbiol. 11: 75~77, 1963
5) HÜTTER, R.: Systematik der Streptomyceten, pp. 1~382. Karger, Basel, 1967
6) STANECK, J. L. & G. D. ROBERTS: Simplified approach to the identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. 28: 226~231, 1974
7) PUKALL, R.; E. BRAMBILLA & E. STACKEBRANDT: Automated fragment length analysis of fluorescentyl-labeled 16S rDNA after digestion with 4-base cutting restriction enzymes. J. Microbiol. Meth. 32: 55~64, 1998
8) LANE, D. J.: 16S/23S rRNA sequencing. *In* Nucleic Acid Techniques in Bacterial Systematics. *Ed.*, E. STACKEBRANDT & M. GOODFELLOW, pp. 125~175. Wiley, Chichester, 1991
9) RAINEY, F. A.; N. WARD-RAINEY, R. M. KROPPENSTEDT & E. STACKEBRANDT: The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. Int. J. Syst. Bacteriol. 46: 1088~1092, 1996
10) Dictionary of Natural Products on CD-ROM, Version 10:1, Chapman & Hall, Hampden Data Services, London, 2001
11) EZAKI, M.; M. IWAMI, M. YAMASHITA, S. HASHIMOTO, T. KOMORI, K. UMEHARA, Y. MINE, M. KOHSAKA, H. AOKI & H. IMANAKA: Biphenomycins A and B, novel peptide antibiotics. I. Taxonomy, fermentation, isolation and characterization. J. Antibiotics 38: 1453~1461, 1985
12) UCHIDA, I.; N. SHIGEMATSU, M. EZAKI, M. HASHIMOTO, H. AOKI & H. IMANAKA: Biphenomycins A and B, novel peptide antibiotics. II. Structural elucidation of biphenomycins A and B. J. Antibiotics 38: 1462~1468, 1985
13) MCCORMICK, M. H.; W. M. STARK, G. E. PITTENGER, R. C. PITTENGER & J. M. MCGUIRE: Vancomycin, a new antibiotic. I. Chemical and biological properties. Antibiot. Annu. 1955-1956: 606~611, 1956
14) NADKARNI, S. R.; M. V. PATEL, S. CHATTERJEE, E. K. S. VIJAYAKUMAR, K. R. DESIKAN, J. BLUMBACH & B. N. GANGULI: Balhimycin, a new glycopeptide antibiotic produced by *Amycolatopsis* sp. Y-86,21022. J. Antibiotics 47: 334~341, 1994