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

## II. Structure Elucidation†

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The structures of new lipopeptide antibiotics, arylomycins A and B, were elucidated by a combination of ESI-FTICR-mass spectrometry, NMR spectroscopy, EDMAN sequencing, and fatty acid and chiral amino acid analyses. The colourless arylomycins A share the peptide sequence of D-*N*-methylseryl$^2$(D-MeSer$^2$)-D-alanyl$^3$-glycyl$^4$-*N*-methyl-4-hydroxyphenylglycyl$^5$-(MeHpg$^5$)-L-alanyl$^6$-tyrosine$^7$ cyclised by a [3,3]biaryl bond between MeHpg$^5$ and Tyr$^7$. The yellow arylomycins B differ from arylomycins A by nitro substitution of Tyr$^7$. The *N*-termini of arylomycins A and B are acylated with saturated $C_{11}$~$C_{15}$ fatty acids (fa$^1$) comprising *n*, *iso*, and *anteiso* isomers. Arylomycins A and B represent the first examples of biaryl-bridged lipopeptides.

Arylomycins were isolated from the fermentation broth of *Streptomyces* sp. Tü 6075 (Fig. 1). The taxonomy of the producing organism, fermentation, isolation, and biological activities of arylomycins are described in the preceding paper[1]. Here, we present the structure elucidation of these novel compounds.

### Experimental

Electrospray Ionisation (ESI) Fourier Transform Ion Cyclotron Resonance (FTICR) Mass Spectrometry (MS)

Mass spectra were recorded on a passively shielded 4.7 Tesla Apex™ II ESI/MALDI-FTICR-mass spectrometer (Bruker Daltonik). ESI (Analytica of Branford) was performed in the positive mode with a grounded capillary sprayer needle mounted 60° off-axis. The software XMASS 5.0.10 (Bruker Daltonik) was used for data acquisition and processing. Mass calculation was performed with the standard elemental mass compilation of AUDI and WAPSTRA[2]. In general 512 k data points were acquired. A mass range of *m/z* 200~2000 Da was covered by performing excitation from *m/z* 150~2500 Da. Samples (1 μg/ml) were introduced in a continuous stream of 50% (v/v) aqueous acetonitrile containing 1% (v/v) AcOH at a flow rate of 1 μl/minute. An internal four-point-calibration was performed with a mixture of known compounds.

For fragmentation studies, high resolution FTICR-MS/MS and -MS$^3$ were acquired by sustained off-resonance irradiation (SORI)[3] collision induced dissociation (CID)

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using argon as collision gas. Before adding the collision gas, correlated sweep isolation was used to isolate precursor ions and eject all other ions from the analyser cell.

### NMR Spectroscopy

1D and 2D NMR spectra of arylomycins $A_2$ and $B_2$ were recorded on a Bruker AMX2-600 spectrometer operating at a proton frequency of 600.13 MHz using a 5-mm triple resonance probehead equipped with z-gradients. Spectra of arylomycins $A_2$ and $B_2$ were recorded in DMSO-$d_6$ solution at 305 K and in MeOH-$d_4$ solution at 300 K.

The data set acquired for each sample consisted of 1D $^1$H NMR, cleanTOCSY, CW-ROESY, NOESY (DMSO-$d_6$), gradient selected (gs)-HSQC and gs-HMBC experiments. The spectra were referenced to the signal of DMSO-$d_6$ at $\delta(^1H)=2.50$ ppm and $\delta(^{13}C)=39.5$ ppm, and of MeOH-$d_4$ at $\delta(^1H)=3.35$ ppm and $\delta(^{13}C)=49.0$ ppm, respectively.

### Fatty Acid and Chiral Amino Acid Analyses by GC-MS

Approx 100 $\mu$g of each sample was hydrolysed in 6 N HCl under vacuum at 110°C for 24 hours. The hydrolysate was extracted with diethyl ether and the organic layer derivatised to methyl esters (1.5 N methanolic HCl, 110°C, 15 minutes). Fatty acid methyl esters were extracted with *n*-pentane and analysed on both a Chrompack CP 9001 gas chromatograph (flame ionisation detection) and an Agilent 6890/5973 GC-MS (electron impact ionisation), both equipped with a 20 m×0.22 mm SGE HT-5 fused silica capillary. The components were identified by comparison of their mass spectra with library spectra and, where possible, by comparison of retention times[4].

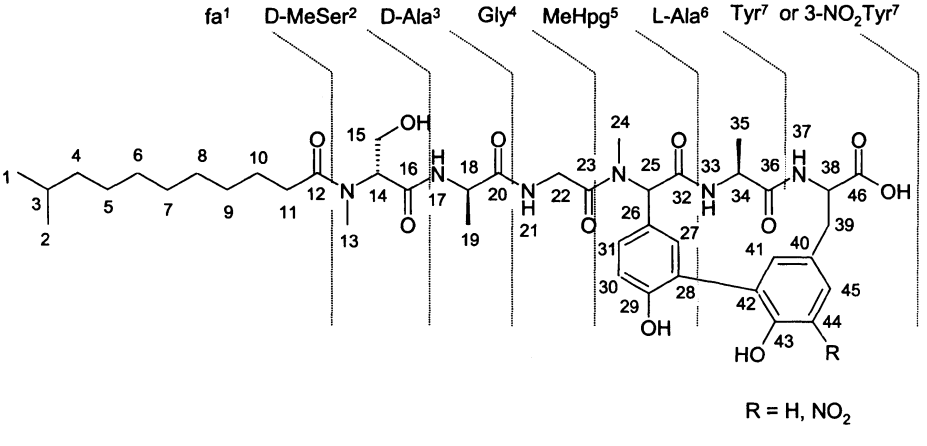
The amino acids in the aqueous fraction of the hydrolysate were derivatised to their *N*-trifluoroacetylated ethyl esters. These were analysed on the Agilent GC-MS using a home made 25 m×0.25 mm fused silica capillary coated with 30% octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-$\gamma$-cyclodextrin/70% PS 255 (dimethylpolysiloxane)[5,6]. In order to detect the biaryl-linked bis-amino acids, the *N*-trifluoroacetylated ethyl esters of the amino acids were subsequently trimethylsilylated (HMDS/BSTFA 1 : 1, 100°C for 30 minutes) prior to GC-MS analysis on the HT-5 capillary.

### Partial Hydrolysis and EDMAN Sequencing of Arylomycins $A_2$ and $B_2$

Approx 1 mg each of arylomycins $A_2$ and $B_2$ in 100 $\mu$l 6 N HCl were allowed to stand at room temperature for 24 hours, then HCl was removed by evaporation under vacuum. The extent of degradation was checked by ESI-FTICR-MS. The partial hydrolysates were then subjected to EDMAN sequencing.

Samples were applied onto a TFA treated glass filter disc coated with 0.75 mg of BioBrene Plus (Perkin-Elmer Applied Biosystems), and sequenced in a protein sequencer 494A 'Procise' (Perkin-Elmer Applied Biosystems). After one and two cycles of degradation, MeOH/TFA 1 : 1 (v/v) was delivered to the reaction cartridge for about 30 seconds and then purged through the cartridge outlet tubing by an argon stream. The samples were recovered in a tube fixed below the outlet tubing.

Fig. 1. Chemical structure of arylomycins exemplified on $A_2$ (R=H) and $B_2$ ($R=NO_2$).



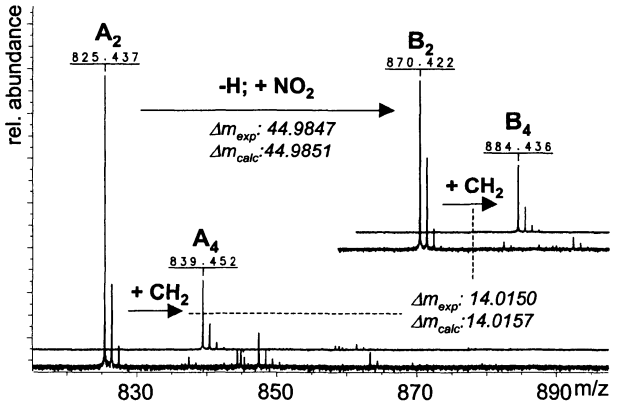

The *N*-termini of arylomycins $A_n$ and $B_n$ are acylated with saturated *n*, *iso*, and *anteiso* $C_{11}$~$C_{15}$ fatty acids (Table 1).

## Results

Arylomycins A and B (Fig. 1) were isolated from the fermentation broth of *Streptomyces* sp. Tü 6075 in five colourless and seven yellow fractions. Analysis of each fraction by ESI-FTICR-MS afforded molecular masses between 810 and 911 Da (Table 1) with very high mass accuracy. The mean exact mass differences of 14.0154 Da between fractions of one series indicated homologous compounds (calc. mass of $CH_2$: 14.0157 Da), whereas the mean exact mass difference of 44.9849 Da between A and B series suggested nitro substitution of the latter (calc. mass difference: 44.9851 Da) (Fig. 2).

Arylomycins $A_2$ and $B_2$ were selected for NMR spectroscopic studies. Both compounds exhibited in DMSO-$d_6$ solution two signal sets for all amino acid residues as well as for $CH_2$-10, $CH_2$-11 and C-12 of fa[1] in an approx ratio of 7 : 3. The twofold signal sets of residues Ala[3] and Gly[4] were further split up to give four sets for those residues altogether in an approx ratio of 5 : 2 : 2 : 1. The appearance of up to four resonances for one proton complicated the assignment procedure. Therefore, a second set of NMR spectra in MeOH-$d_4$ solution was acquired for each of the two compounds (data not shown). Possibly due to the exchange of amide protons in MeOH-$d_4$, only a twofold signal set (approx ratio of 8 : 2) for the residues Ala[3] and Gly[4] was observed.

Fig. 2. Characteristic exact mass differences observed by ESI-FTICR-MS between selected homologous compounds within one series ($\Delta m$=14 Da) and between A and B series ($\Delta m$=45 Da).




One Gly and two Ala residues were identified in TOCSY spectra by their unique spin systems. MeSer, MeHpg, as well as Tyr (arylomycin $A_2$) and 3-$NO_2$Tyr (arylomycin $B_2$)

Table 1. Mass and fatty acid analysis data of arylomycins A and B.

| isolated fractions | *m/z* $[M+H]^+$ | | error | formula | fatty acid |
|---|---|---|---|---|---|
| arylomycin | exp. | calc. | [ppm] | | |
| $A_1$ | 811.4228 | 811.4236 | 1.1 | $C_{41}H_{58}N_6O_{11}$ | *iso*-$C_{11}$ |
| $A_2$ | 825.4386 | 825.4393 | 0.9 | $C_{42}H_{60}N_6O_{11}$ | *iso*-$C_{12}$ |
| $A_3$ | 825.4386 | 825.4393 | 0.9 | $C_{42}H_{60}N_6O_{11}$ | *n*-$C_{12}$ |
| $A_4$ | 839.4537 | 839.4549 | 1.4 | $C_{43}H_{62}N_6O_{11}$ | *anteiso*-$C_{13}$ |
| $A_5$ | 853.4695 | 853.4706 | 1.3 | $C_{44}H_{64}N_6O_{11}$ | *iso*-$C_{14}$ |
| $B_1$ | 856.4088 | 856.4087 | 0.1 | $C_{41}H_{57}N_7O_{13}$ | *iso*-$C_{11}$ |
| $B_2$ | 870.4233 | 870.4244 | 1.2 | $C_{42}H_{59}N_7O_{13}$ | *iso*-$C_{12}$ |
| $B_2$, $B_3$ | 870.4233 | 870.4244 | 1.2 | $C_{42}H_{59}N_7O_{13}$ | *iso*-$C_{12}$, *n*-$C_{12}$ |
| $B_4$ | 884.4382 | 884.4400 | 2.1 | $C_{43}H_{61}N_7O_{13}$ | *anteiso*-$C_{13}$ |
| $B_4$, $B_5$ | 884.4382 | 884.4400 | 2.1 | $C_{43}H_{61}N_7O_{13}$ | *anteiso*-$C_{13}$, *iso*-$C_{13}$ |
| $B_6$ | 898.4539 | 898.4557 | 1.9 | $C_{44}H_{63}N_7O_{13}$ | *iso*-$C_{14}$ |
| $B_7$ | 912.4698 | 912.4713 | 1.6 | $C_{45}H_{65}N_7O_{13}$ | *anteiso*-$C_{15}$ |

Table 2. $^{1}H$ and $^{13}C$ chemical shifts (ppm) of arylomycin $A_2$ (DMSO-$d_6$, 305 K).†

| | | δ($^{1}$H) | δ($^{13}$C) | | | δ($^{1}$H) | δ($^{13}$C) |
|---|---|---|---|---|---|---|---|
| fa[1] | 1,2 | 0.85 | 22.2 | MeHpg[5] | 24 | 2.69 | 31.2 |
| | 3 | 1.50 | 27.0 | | 25 | 6.29 | 58.9 |
| | 4 | 1.14 | 38.2 | | 26 | - | 127.0 |
| | 5 | 1.25 | 26.4 | | 27 | 6.88 | 133.3 |
| | 6-8 | 1.30-1.23 | 28.2-29.3 | | 28 | - | 127.1 |
| | 9 | 1.28 | 28.5 | | 29 | - | 153.3 |
| | 10 | 1.51 | 24.3 | | 30 | 6.91 | 116.7 |
| | 11 | 2.32 | 32.5 | | 31 | 6.96 | 128.4 |
| | 12 | - | 172.8 | | 32 | - | 169.3 |
| MeSer[2] | 13 | 2.93 | 31.4 | Ala[6] | 33 | 8.54 | - |
| | 14 | 4.96 | 57.8 | | 34 | 4.78 | 47.2 |
| | 15 | 3.82/3.67 | 58.7 | | 35 | 1.18 | 18.7 |
| | 16 | - | 168.8 | | 36 | - | 171.4 |
| Ala[3] | 17 | 7.90 | - | Tyr[7] | 37 | 8.88 | - |
| | 18 | 4.35 | 47.8 | | 38 | 4.62 | 50.9 |
| | 19 | 1.26 | 17.8 | | 39 | 3.23/2.99 | 32.8 |
| | 20 | - | 171.8 | | 40 | - | 128.6 |
| Gly[4] | 21 | 7.92 | - | | 41 | 6.92 | 129.9 |
| | 22 | 4.03/3.96 | 40.5 | | 42 | - | 125.4 |
| | 23 | - | 168.5 | | 43 | - | 152.0 |
| | | | | | 44 | 6.84 | 116.2 |
| | | | | | 45 | 7.06 | 128.7 |
| | | | | | 46 | - | 172.7 |

† Data refer to the major signal set.

residues were assigned by means of ROESY, NOESY, HSQC, and HMBC spectra. The [3,3]biaryl-bond of MeHpg[5] and Tyr[7] in arylomycin $A_2$ and the respective [3,5]biaryl-bond of MeHpg[5] and 3-$NO_2$Tyr[7] in arylomycin $B_2$ were established from the signal pattern and multiplicities of the aromatic protons together with HMBC crosspeaks and ROEs or NOEs. Particularly indicative of the linkage were HMBC connectivities from H-27 to C-42 and from H-41 to C-28 as well as ROESY crosspeaks between H-27 and H-41 and between H-25 and H-41. The sequence of both compounds was established from sequential crosspeaks ($H^{N}_{(i+1)}$ or $CH_3{}^{N}_{(i+1)}\leftrightarrow H^{\alpha}_{i}$, $H^{\alpha}_{(i+1)}\leftrightarrow H^{\alpha}_{i}$) observed in NOESY and ROESY spectra as well as from $H^{\alpha}_{(i+1)}\leftrightarrow C'_{i}$ connectivities detected in the HMBC spectra. Fatty acid signals were assigned from TOCSY, HSQC, and HMBC spectra. The *iso* constitution of the fatty acids in arylomycins $A_2$ and $B_2$ was reflected by a doublet at $\delta=0.78$ in the $^{1}H$ NMR spectrum corresponding to the terminal methyl groups. The linkage of the fatty acid (fa[1]) to MeSer[2] was proven by HMBC connectivities from $H_3$-13 and H-14 of MeSer[2] to C-12 of fa[1]. $^{1}H$ and $^{13}C$ chemical shifts of the major signal sets of arylomycins $A_2$ and $B_2$ in

Table 3. $^{1}H$ and $^{13}C$ chemical shifts (ppm) of arylomycin $B_2$ (DMSO-$d_6$, 305 K).†

| | | δ($^{1}$H) | δ($^{13}$C) | | | δ($^{1}$H) | δ($^{13}$C) |
|---|---|---|---|---|---|---|---|
| fa[1] | 1,2 | 0.85 | 22.1 | MeHpg[5] | 24 | 2.69 | 31.0 |
| | 3 | 1.50 | 27.0 | | 25 | 6.23 | 59.0 |
| | 4 | 1.14 | 38.1 | | 26 | - | 124.2 |
| | 5 | 1.25 | 26.3 | | 27 | 6.92 | 131.2 |
| | 6-8 | 1.30-1.23 | 28.2-29.3 | | 28 | - | 127.6 |
| | 9 | 1.28 | 28.5 | | 29 | - | 158.0 |
| | 10 | 1.50 | 24.2 | | 30 | 6.65 | 117.7 |
| | 11 | 2.32 | 32.4 | | 31 | 6.81 | 127.5 |
| | 12 | - | 172.7 | | 32 | - | 169.4 |
| MeSer[2] | 13 | 2.93 | 31.4 | Ala[6] | 33 | 8.57 | - |
| | 14 | 4.96 | 57.7 | | 34 | 4.83 | 46.9 |
| | 15 | 3.81/3.67 | 58.6 | | 35 | 1.17 | 18.4 |
| | 16 | - | 168.6 | | 36 | - | 171.2 |
| Ala[3] | 17 | 7.90 | - | 3-$NO_2$Tyr[7] | 37 | 8.89 | - |
| | 18 | 4.35 | 47.6 | | 38 | 4.49 | 51.8 |
| | 19 | 1.25 | 17.8 | | 39 | 3.10/2.94 | 32.4 |
| | 20 | - | 171.6 | | 40 | - | 119.0 |
| Gly[4] | 21 | 7.90 | - | | 41 | 7.14 | 133.3 |
| | 22 | 4.01/3.96 | 40.4 | | 42 | - | 133.8 |
| | 23 | - | 168.3 | | 43 | - | 160.4 |
| | | | | | 44 | - | 138.8 |
| | | | | | 45 | 7.44 | 123.5 |
| | | | | | 46 | - | 172.5 |

† Data refer to the major signal set.

DMSO-$d_6$ solution are summarised in Tables 2 and 3.

The observation of exchange peaks in ROESY and NOESY spectra between the signal sets indicated the existence of a conformational equilibrium. The upfield shift observed for $CH_3$-13 ($\Delta\delta(^{13}C)$=3.1 ppm, $\Delta\delta(^{1}H)$= 0.16 ppm) and H-14 ($\Delta\delta(^{1}H)$=0.45 ppm) of MeSer[2] in the minor signal set was indicative of the *cis-trans* isomerisation of the fa[1]-MeSer[2] amide bond[7]. The geometry of the fa[1]-MeSer[2] amide bond was assigned as *trans* for the major signal set based on an NOE between $H_2$-11 and $H_3$-13 and as *cis* for the minor signal set based on an NOE between $H_2$-11 and H-14[8].

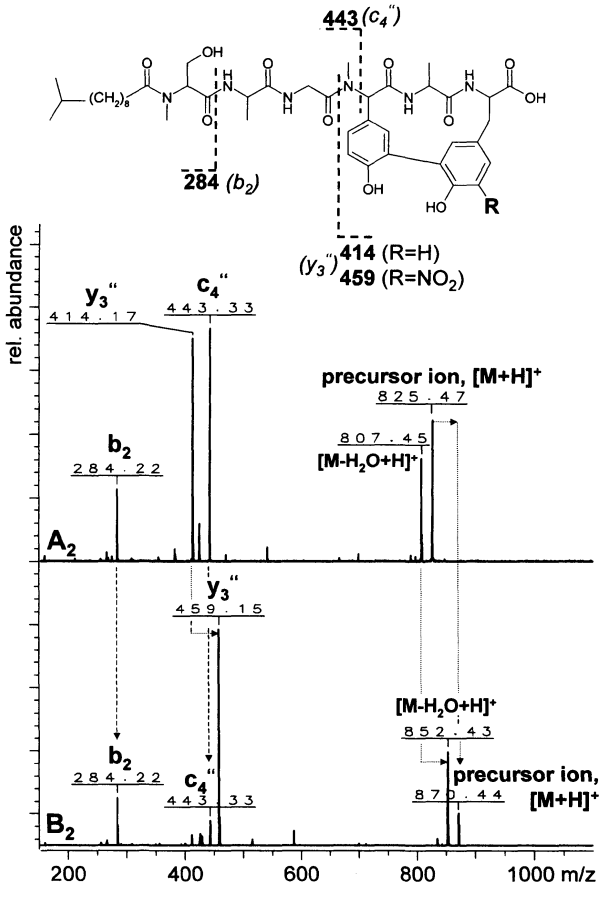
ESI-FTICR-MS/MS- and $MS^3$ experiments confirmed the structures of arylomycins $A_n$ and $B_n$ (Fig. 3). All compounds showed relatively intense $b_2$-fragments (nomenclature according to ROEPSTORFF and FOHLMAN[9]) due to the *N*-alkylation of the internal amino acid residue MeSer[2] (Fig. 3). *N*-Alkylation of internal amino acid residues activates the *C*-terminal side to form corresponding b-ions[10]. Within an arylomycin series, these $b_2$-ions differ by 14 Da due to the different chain lengths of the fatty acids. A shift in mass of 45 Da of the abundant

$y_3''$-ion between arylomycin series A and B is indicative of the nitro substitution of the $Tyr^7$ residue in arylomycins B (Fig 3) as is the difference in the relative abundance of the $c_4''$-ion.

All isolated arylomycins fractions were subjected to total hydrolysis and analysed for their fatty acid and amino acid composition by (chiral) GC-MS. Equimolar amounts of L-Ala, D-Ala, Gly, and D-MeSer were detected in all fractions. The biaryl-bridged bis-amino acids, with or without nitro substitution (B or A series respectively), were also identified by their mass spectra. Determination of the configuration of biaryl-linked bis-amino acids was not possible, primarily because of their high molecular weight and thus insufficient volatility, but also because reference substances are not available. The fatty acid analysis revealed the presence of saturated fatty acids in the range of $C_{11}$~$C_{15}$ comprising *n*, *iso*, and *anteiso* isomers (Table 1).

For assignment of the absolute configurations of $Ala^3$ and $Ala^6$, partial hydrolysis, ESI-FTICR-MS, EDMAN sequencing and chiral amino acid analysis were successively applied. ESI-FTICR-MS of the partial hydrolysates of arylomycins $A_2$ and $B_2$ revealed the hexapeptide cleaved from the fatty acid to be the main component. The partial hydrolysates were subjected to two cycles of EDMAN sequencing in order to cleave the first Ala ($Ala^3$) residue from the sequence. After each cycle, the residual substance was recovered by extraction, subjected to total hydrolysis and derivatisation, and analysed by chiral GC-MS. After one cycle of EDMAN sequencing of the arylomycin $A_2$ partial hydrolysate, L-Ala with an enantiomeric excess (ee) of 36.8% was found. The enantiomeric excess of L-Ala increased to 92.4% after the second cycle. The corresponding ee values for the arylomycin $B_2$ partial hydrolysate were 40.1% and 86.4% after one and two cycles, respectively. Removal of D-Ala during EDMAN degradation of the partial hydrolysates thus allowed assignment of the Ala residues as D-$Ala^3$ and L-$Ala^6$.

Fig. 3. ESI-FTICR-MS/MS of arylomycins $A_2$ and $B_2$.




Among numerous other signals all samples showed intense $b_2$-ions by cleavage of the *C*-terminal bond of $MeSer^2$. In addition, the loss of water and formation of intense $y_3''$- and $c_4''$-ions can be seen clearly. The mass shift of $\Delta m$=45 Da (marked with dotted arrows) of fragment $y_3''$ reflects nitro substitution within the biaryl-bridged moiety. The $c_4''$-ions do not differ by mass (marked with dashed arrows) but by magnitude, which shows the influence of nitro substitution of $Tyr^7$ on the activation of the N-$C^{\alpha}$ bond of $MeHpg^5$ for fragmentation.

## Discussion

Arylomycins A and B are lipohexapeptides containing saturated $C_{11}$~$C_{15}$ *n*, *iso*, and *anteiso* fatty acids, D- and L-amino acids, *N*-methylated amino acids, and a biaryl-bridged tripeptide segment. Arylomycins exist in a conformational equilibrium in MeOH and DMSO solution which involves *cis-trans* isomerisation around the $fa^1$-$MeSer^2$ amide bond. The occurrence of *cis-trans* isomerism around *N*-methylated amide bonds has been reported for natural products[11,12]. Whereas a similar biphenyl-bridged peptide structure was found in biphenomycin A and B[13~15], the combination of biaryl-bridging and fatty acid acylation is a novel feature in a natural product as are the respective bis-amino acid residues [3,3]$MeHpg^5$-$Tyr^7$ and [3,5]$MeHpg^5$-3-$NO_2Tyr^7$.

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