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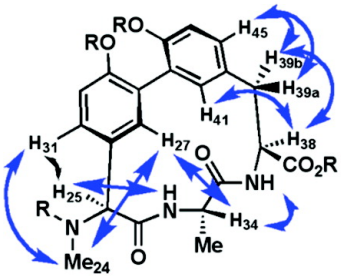

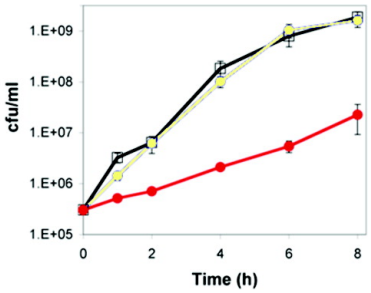

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Structural and Initial Biological Analysis of Synthetic Arylomycin A_2

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Abstract: The growing threat of untreatable bacterial infections has refocused efforts to identify new antibiotics, especially those acting by novel mechanisms. While the inhibition of pathogen proteases has proven to be a successful strategy for drug development, such inhibitors are often limited by toxicity due to their promiscuous inhibition of homologous and mechanistically related human enzymes. Unlike many protease inhibitors, inhibitors of the essential type I bacterial signal peptidase (SPase) may be more specific and thus less toxic due to the enzyme's unique structure and catalytic mechanism. Recently, the arylomycins and related lipoglycopeptide natural products were isolated and shown to inhibit SPase. The core structure of the arylomycins and lipoglycopeptides consists of a biaryl-linked, N-methylated peptide macrocycle attached to a lipopeptide tail, and in the case of the lipoglycopeptides, a deoxymannose moiety. Herein, we report the first total synthesis of a member of this group of antibiotics, arylomycin A_2. The synthesis relies on Suzuki−Miyaura-mediated biaryl coupling, which model studies suggested would be more efficient than a lactamization-based route. Biological studies demonstrate that these compounds are promising antibiotics, especially against Gram-positive pathogens, with activity against *S. epidermidis* that equals that of the currently prescribed antibiotics. Structural and biological studies suggest that both N-methylation and lipidation may contribute to antibiotic activity, whereas glycosylation appears to be generally less critical. Thus, these studies help identify the determinants of the biological activity of arylomycin A_2 and should aid in the design of analogs to further explore and develop this novel class of antibiotic.

1. Introduction

The emergence of bacteria that are resistant to all available antibiotics threatens to end the 'antibiotic era' and has reinvigorated efforts to identify new antibiotics, especially those acting by novel mechanisms. In 2002, a novel class of natural products, the arylomycins, was isolated from *Streptomyces* strain Tu 6075 and shown to inhibit the growth of several Gram-positive bacteria.[1] Subsequent studies demonstrated that the antibiotic activity of the arylomycins results from their inhibition of the bacterial type I signal peptidase (SPase), an essential serine protease required to process cell surface bound preproteins.[2] Although protease inhibitors have been extensively pursued as antimicrobials, they have been challenging to develop as drugs due to low discrimination against human proteases.[3] However, SPase utilizes a unique Ser-Lys catalytic dyad and appears to act via an unusual mechanism involving nucleophilic attack on its substrate from the *si*-face as opposed to the *re*-face attack characteristic of the more common Ser-His-Asp catalytic triad serine proteases.[4] Consistent with its unconventional mechanism, SPase is not inhibited by standard serine protease inhibitors.[5] These observations suggest that the arylomycins or related derivatives might be developed into a novel class of antibiotics.

SPase is an essential enzyme because it is needed to process proteins that are required for bacterial viability. However, SPase is also required to process proteins involved in a variety of other processes such as antibiotic resistance and virulence. For example, secretion of the β-lactamases that confer resistance to β-lactam antibiotics require SPase, and an arylomycin-like lipoglycopeptide (see below) has been shown to inhibit the secretion of these proteins in *Staphylococcus aureus*.[6] In addition, many components of bacterial secretion systems involved in toxin delivery and adhesion require processing by

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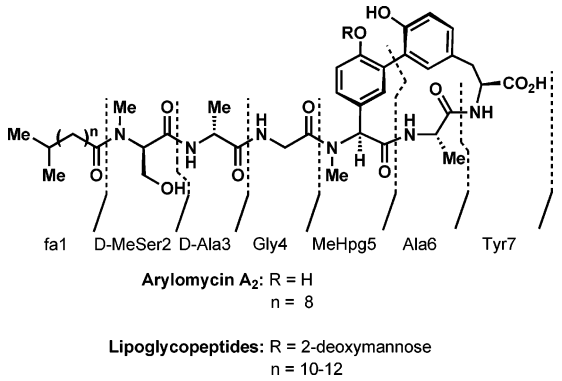

Figure 1. Arylomycin A_2 and lipoglycopeptide natural products.

SPase.[7] Thus, the arylomycin family of natural products not only have interesting antibacterial properties, but they may also have the ability to modulate virulence and sensitize bacteria to other antibiotics.[8]

There are two related series of arylomycins, the arylomycins A and B. The core structure of both series of compounds consists of a tripeptide macrocycle with a C−C biaryl linkage between a hydroxyphenylglycine (MeHpg5) residue and a tyrosine (Tyr7) residue (Figure 1). Attached to the core macrocycle is a lipopeptide tail comprised of a C_{11}−C_{15} fatty acid linked to the N-terminal tripeptide D-MeSer2-D-Ala3-Gly4. In addition, the peptide backbone is N-methylated at the second and fifth residues (MeSer2 and MeHpg5). The two series of arylomycins are differentiated by Tyr7 nitration in the B series.

The crystal structure of the *Escherichia coli* SPase−arylomycin A_2 complex has been reported.[9] The structure reveals that the C-terminal carboxylate of the natural product forms a hydrogen-bond with both residues of the catalytic Ser-Lys dyad, while the remainder of the peptide forms an extensive series of hydrogen-bonding and packing interactions with the enzyme. Sterically hindered biaryl-compounds, like the arylomycins, may exhibit two rotational isomers, or atproisomers, due to slow rotation about the interannular C−C bond. However, the crystal structure suggests that *E. coli* SPase binds only the S_a atropisomer. This binding selectivity appears to result from packing interactions between the protein side chains of Pro87 and Leu42 and the aryl rings of MeHpg5 and Tyr7.

Independently, in 2004 a group at Eli Lilly screened a library of *Streptomyces* sp. natural products for SPase inhibitors and identified a number of related lipoglycopeptides.[6] Interestingly, these lipoglycopeptides have the same core structural features as the arylomycins (Figure 1), but their macrocycles are modified by glycosylation and their conjugated fatty acid tails are generally longer, by up to five carbons. Although lipidation, glycosylation, and N-methylation are all common modifications of nonribosomally synthesized peptides, it is unclear how these modifications contribute to biological activity, in general, and to the antibacterial properties of the arylomycins and related lipoglycopeptides, in particular.

Both structure−activity relationship studies and the characterization of the arylomycins and related lipoglycopeptides as antibiotics or inhibitors of virulence would be greatly facilitated by their total synthesis. The syntheses of similar biaryl-bridged macrocycles have been the focus of much recent effort due to their diverse and remarkable biological activities.[10] A key aspect of these syntheses is macrocyclization, and multiple strategies have been developed. For example, Evans et al.[11] synthesized the biaryl-linked AB ring macrocycle of vancomycin via biaryl vanadium coupling, and both Nicolaou et al.[12] and Boger et al.[13] assembled the same ring system by intermolecular Suzuki cross-coupling followed by intramolecular macrolactamization. Similarly, biphenomycin[14] and the TMC-95[15] family of macrocycles were synthesized by first forming the biaryl-coupled intermediate via Negishi,[16] Stille,[17] or Suzuki[18] coupling reactions, followed by derivatization, and finally ring closure by macrolactamization. Derivatives of biphenomycin and TMC-95A have also been synthesized by first assembling the derivatized tripeptide and then cyclizing by intramolecular Suzuki−Miyaura cross-coupling.[19]

Herein, we report the first total synthesis of an arylomycin natural product, arylomycin A_2. The synthesis employed an intramolecular biaryl-bond forming macrocyclization for cyclic tripeptide ring closure, which model studies predicted would be more efficient than a macrolactamization-based route. Atropisomerism of arylomycin A_2 was observed and investigated through the synthesis of derivatized core macrocycles. Derivatives were also synthesized to address the contribution of N-methylation, glycosylation, and fatty acid tail length to antibiotic activity. Our data suggests that both N-methylation and lipidation make important contributions to antibiotic activity; however, the contributions are somewhat different for different bacteria. Perhaps most notably, we report that this class of natural product has very potent antibiotic activity against the important human pathogen *S. epidermidis*. Synthetic access to the arylomycins and related compounds should allow for further study of their unique mechanism of action and for their development as antibiotics.

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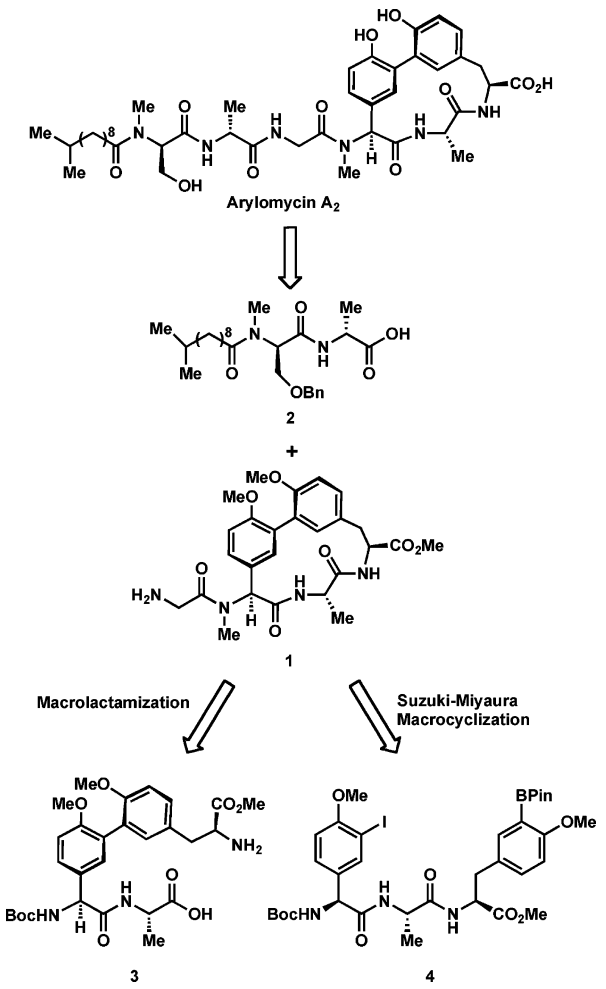
Scheme 1. Retrosynthesis of Arylomycin A_2

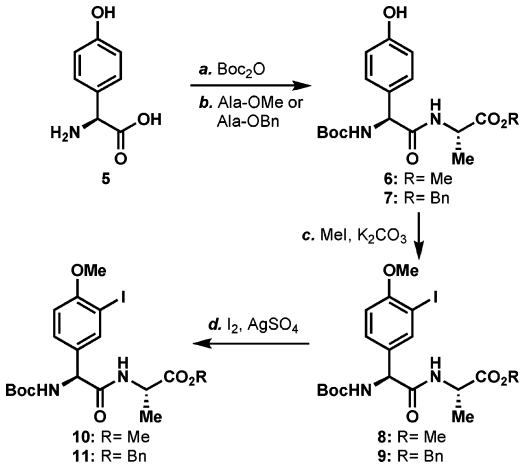


2. Results

Retrosynthetic analysis of arylomycin A_2 suggested a logical first disconnection between the macrocycle **1** and the lipopeptide tail **2** (Scheme 1). Lipopeptide tail synthesis using peptide and fatty acid couplings was expected to be straightforward, leaving macrocyclization as the key step in the synthesis. As discussed above, literature precedent suggested that the macrocycle might be successfully assembled by intermolecular biaryl coupling, followed by intramolecular macrolactamization, or by Suzuki−Miyaura macrocyclization of an assembled tripeptide. We first evaluated the potential of these approaches using model substrates, **3** and **4**.

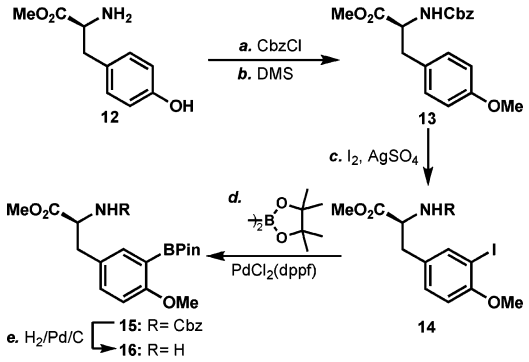
2.1. Model Studies. Testing the macrolactamization and the Suzuki−Miyaura macrocyclization routes required similar precursor substrates. The first precursors, phenylhydroxyglycine-alanine iododipeptides **10** and **11**, were synthesized as shown in Scheme 2. The synthesis began with Boc protection of commercially available 4-hydroxyphenylglycine **5**. The protected hydroxyphenylglycine was then coupled to either Ala-OMe or Ala-OBn with EDC/HOBt, yielding protected dipeptides **6** and **7**. The phenols were protected as methyl ethers with MeI and K_2CO_3 in acetone and selectively iodinated using Sy's conditions (I_2/$AgSO_4$).[20] Iododipeptides **10** and **11** were obtained in 54% and 42% yield, respectively, over four steps.

Scheme 2. Iododipeptide Synthesis[a]



[a] Reagents and conditions: (a) Boc_2O (1.0 equiv), $NaHCO_3$ (1.5 equiv), acetone:H_2O (1:1), overnight; (b) Ala-OMe or Ala-OBn (1.0 equiv), EDC (1.5 equiv), HOBt (1.0 equiv), TEA (1.3 equiv), DMF, overnight, 71% and 68% (2 steps) for **6** and **7**, respectively; (c) MeI (10.0 equiv), K_2CO_3 (5.0 equiv), acetone, reflux, 17 h; (d) I_2 (1.05 equiv), $AgSO_4$ (1.05 equiv), MeOH, 30 min, 76% (2 steps).

Scheme 3. Tyrosine Boronic Ester Synthesis[a]



[a] Reagents and conditions: (a) CbzCl (1.0 equiv), Na_2CO_3 (2.0 equiv), acetone:H_2O (1:1), overnight, 84%; (b) DMS (1.0 equiv), K_2CO_3 (2.0 equiv), acetone, 2 h, 94%; (c) I_2 (2.0 equiv), $AgSO_4$ (2.0 equiv), MeOH, 2 h, 77%; (d) bis(pinicolato)diboron (2.0 equiv), KOAc (12.0 equiv), $PdCl_2$(dppf) (0.2 equiv), DMSO, overnight, 81%; (e) H_2 (1 atm), 10% Pd/C, 95% EtOH. For (a)-(d) see references 18b and 20a for experimental details.

The pinacol boronate fragment **15** was synthesized following the protocols of Danishefsky[18d] and Hutton,[21] as shown in Scheme 3. The commercially available tyrosine methyl ester **12** was Cbz protected and then methylated using dimethyl sulfate, yielding **13**. I_2/$AgSO_4$ was used to mono-iodonate the aromatic ring *ortho* to the methoxy group, and the resulting iodo tyrosine was converted to boronic ester **15** using Miyaura boration conditions.[22]

2.1.1. Ring Closure via Macrolactamization. The macrolactamization precursor was assembled by coupling boronic ester **15** with iododipeptide **11** (Scheme 4). Exploratory Suzuki−Miyaura coupling studies using $PdCl_2$(dppf) as a catalyst showed moderate yields of compound **17** (∼30 to 40%). Compound **17** proved difficult to characterize by NMR spectroscopy, as the ^1H spectrum exhibited severely broadened and/or apparently doubled resonances. This likely results from interconverting atropisomers, which has been observed with similar biaryl

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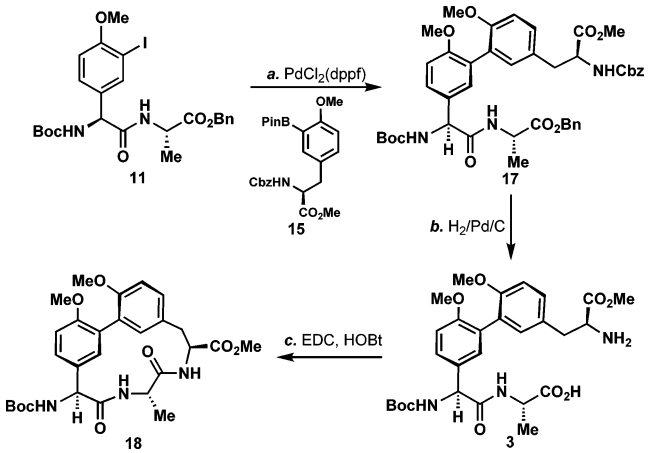
Scheme 4. Macrolactamization[a]



[a] Reagents and conditions: (a) **11** (1.0 equiv), **15** (1.2 equiv), $PdCl_2$(dppf) (0.2 equiv), K_2CO_3 (5.0 equiv), DMSO, 36 h, 36%; (b) H_2 (1 atm), 10% Pd/C (1/3 w/w), 95% MeOH, 3 h, quant.; (c) **3** (0.0005 M), EDC (4 equiv), HOBt (4 equiv), DMF, 48 h, <10%. Atropisomer assumed for **3**, **17**, and **18**.

systems.[23] Consistent with this conclusion, the reaction product was a single spot by thin layer chromatography and the ESI−MS determined mass matched precisely that predicted for the coupled product **17**. Simultaneous deprotection of the Cbz and Bn groups with H_2/Pd/C yielded the macrolactamization substrate **3**.

With compound **3** in hand, we examined a variety of macrolactamization conditions to produce macrocycle **18**, including HATU/DMAP, EDC/HOBt, FTAH/DIEA, and DPPA/DIEA. EDC/HOBt proved optimal; however, the reaction proceeded in low yield (∼25%) and produced a mixture of compounds, with no single component present in greater than 10% yield. In addition, optimal yields of the product mixture required low substrate concentrations (0.5 mM to 1 mM); at higher concentrations (5 mM) only cyclic multimers were observed. The low yields and prohibitively small scale on which the reaction must be run render the macrolactamization route impractical for arylomycin synthesis.

2.1.2. Ring Closure via Intramolecular Suzuki−Miyaura Coupling. Construction of model compound **4** to evaluate the alternative intramolecular Suzuki−Miyaura coupling-based route to **18** began with iododipeptide **10** (Scheme 5). Deprotection of **10** with LiOH in THF/H_2O yielded the free acid iododipeptide **19**. This compound was then coupled with boronic ester **16**, obtained from H_2/Pd/C reduction of **15**, to yield the desired cyclization substrate, tripeptide **4**.

With tripeptide **4** in hand, we proceeded to test conditions for the intramolecular Suzuki−Miyaura cross-coupling reaction. Preliminary macrocyclization coupling experiments gave higher yields than those observed for the macrolactamization route. During optimization of reaction conditions (Table 1), we found that solvent had the most significant effect on reaction efficiency, with a 49% yield of the desired macrocycle **20** (2 steps) obtained when the reaction was performed in acetonitrile. In contrast to the macrolactamization route, the yield was independent of

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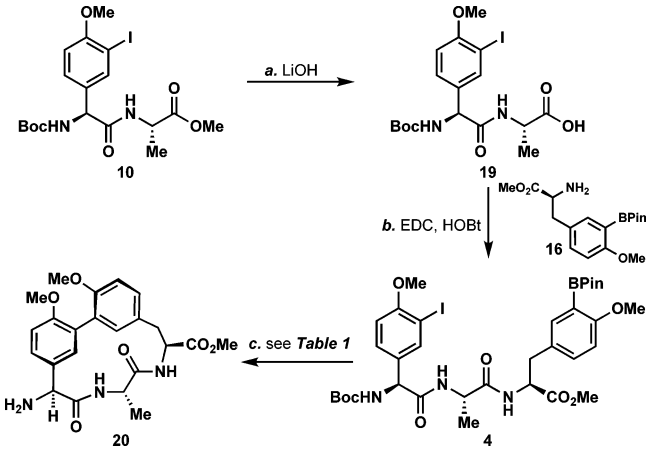
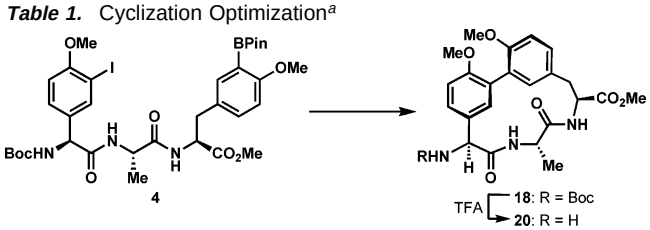
Scheme 5. Suzuki−Miyaura Macrocyclization[a]



[a] Reagents and conditions: (a) 0.2 N LiOH (1.7 equiv), THF, 2.5 h; (b) **19** (1.0 equiv), **16** (1.1 equiv), HOBt (2.5 equiv), EDC (2.2 equiv), $NaHCO_3$ (cat), AcCN:DMF (2.2:1), overnight. Atropisomer assumed for **20**.

Table 1. Cyclization Optimization[a]



entry	Pd	ligand	solvent	base	yield of **20**
1	$Pd(II)Cl_2$	dppf	30:1 Tol:H_2O	K_2CO_3	34%
2	$Pd(II)Cl_2$	dppf	MeCN	K_2CO_3	49%
3	$Pd(II)Cl_2$	dppf	DME	K_2CO_3	29%
4	$Pd(II)Cl_2$	dppf	DMSO	K_2CO_3	10%
5	$Pd(II)Cl_2$	dppf	MeCN	K_3PO_4	48%
6	$Pd(II)(OAc)_2$	dppf	MeCN	K_2CO_3	44%
7	$Pd(II)Cl_2$	Sphos	MeCN	K_2CO_3	37%
8	$Pd(II)Cl_2$	bis-tpp	MeCN	K_2CO_3	49%
9	$Pd_2(dba)_3$	bis-tpp	MeCN	K_2CO_3	46%
10	$Pd(II)(OAc)_2$	PCy_3	MeCN	K_2CO_3	34%
11	$Pd_2(dba)_3$	dppf	30:1 Tol:H_2O	K_2CO_3	38%

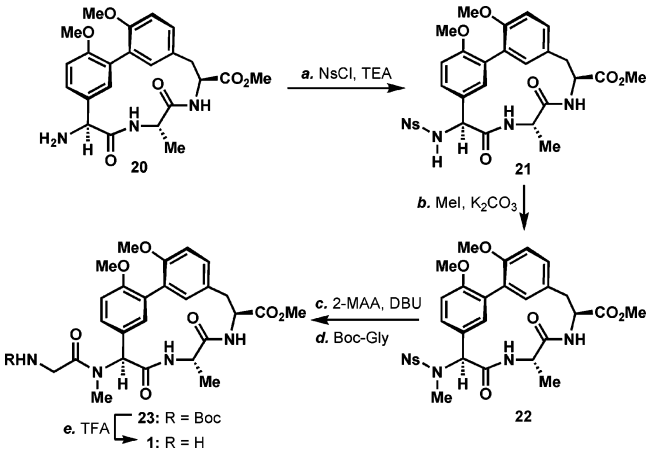
[a] All cyclization reactions were treated with 10 equiv of K_2CO_3, and heated to 80 °C for 18−22 h under Ar. After workup, all reactions were treated with a 4:1 mixture of CH_2Cl_2:TFA for approx 1.5 h and purified. Yields are isolated yields. Abbreviations: dppf, 1,1′ bis(diphenylphosphino)ferrocene; Sphos, 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl; PCy_3, tricyclohexylphosphine; bis-tpp, bis(triphenylphosphine); dba, dibenzylideneacetone; Tol, toluene; MeCN, acetonitrile; DME, dimethoxyethane.

substrate concentration between 1 and 20 mM. These results suggested that macrocyclization via Suzuki−Miyaura coupling would provide a viable route to the arylomycin class of natural products.

2.2. Synthesis of Arylomycin A_2. Synthesis of the natural product commenced with monomethylation of the amine terminus of macrocycle **20**. Monomethylation of **20** using cesium hydroxide[24] and iodomethane was unsuccessful, yielding only overmethylated macrocycle. Monomethylation of **18** with iodomethane and K_2CO_3 was also unsuccessful, yielding only starting material. Attempts to use the formyl imine of the Boc-deprotected macrocycle as a substrate for a Diels−Alder reaction with cyclopentadiene (which would then be followed by a tandem retro Diels−Alder imine reduction[25]) yielded no product.

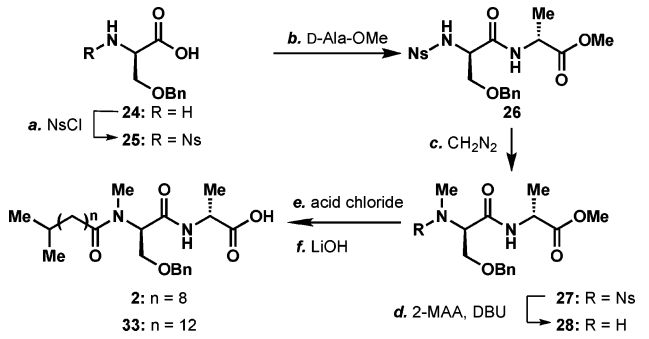
(24) Salvatore, R. N.; Nagle, A. S.; Jung, K. W. *J. Org. Chem.* **2002**, *67*, 674−683.

Scheme 6. Formation of Methylated Macrocycle[a]



[a] Reagents and conditions: (a) NsCl (1.5 equiv), TEA (3 equiv), AcCN, 2 h; (b) MeI (10 equiv), K_2CO_3 (10 equiv), acetone, overnight, 37% (2 steps); (c) 2-MAA (3 equiv), DBU (5 equiv), AcCN, 30 min; (d) Boc-Gly-OH (1.1 equiv), HOBt (3.3 equiv), EDC (3 equiv), CH_2Cl_2:DMF (3:1), overnight, 74% (2 steps); (e) TFA: CH_2Cl_2 (4:1), 2 h, 96%. Atropisomer assumed for **20**, **21**, and **22**.

Scheme 7. Lipopeptide Tail Construction[a]

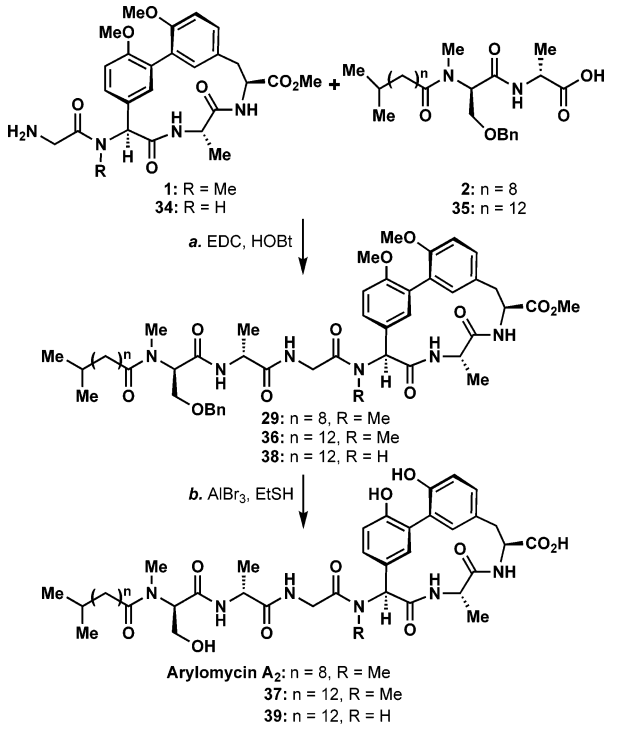


[a] Reagents and conditions: (a) NsCl (1.05 equiv), 1 N NaOH:THF (10:1), overnight, 62%; (b) D-Ala-OMe (1.0 equiv), HOBt (3.3 equiv), EDC (3 equiv), $NaHCO_3$ (1 equiv), CH_2Cl_2:DMF (3:1), overnight, 88%; (c) CH_2N_2 (12 equiv), CH_2Cl_2, 5 min, 90%; (d) 2-MAA (3 equiv), DBU (5 equiv), AcCN, 15 min; (e) C_{12} (for **2**) or C_{16} (for **33**) acid chloride (1 equiv), 9% $NaHCO_3$, CH_2Cl_2, 5 h; (f) 0.2 N LiOH (1.15 equiv), THF, 3 h, 19% and 43% for **2** and **33**, respectively (3 steps).

Attempted methylation of the 4-nitrobenzenesulfonyl (nosyl)-protected macrocycle **21** with diazomethane also yielded only starting material. However, treating compound **21** with 10 equiv of both iodomethane and K_2CO_3 in refluxing acetone yielded the desired monomethylated product **22** in 37% yield (2 steps) after column chromatography (Scheme 6). Nosyl deprotection of **22** with 2-mercaptoacetic acid and DBU gave the monomethylated macrocycle, which was coupled to Boc-protected glycine with EDC/HOBt, yielding compound **23** in 74% yield (2 steps). Boc deprotection of macrocycle **23** yielded compound **1**.

The N-terminal peptide residues and the fatty acid chain were assembled in the absence of the macrocycle due to the low solubility of the full peptide in organic solvent (Scheme 7). Benzyl ether protected D-serine **24** was first nosylated under aqueous conditions, yielding compound **25**. Coupling of **25** to D-Ala-OMe using EDC/HOBt yielded **26**, and subsequent sulfonamide methylation with diazomethane yielded compound **27**. Nosyl deprotection using 2-mercaptoacetic acid and DBU allowed for a one-pot coupling of **28** to isolauric acid chloride (the use of 2-mercaptoacetic acid facilitates removal of the reaction byproducts[26]). Methyl ester deprotection with lithium hydroxide afforded the lipopeptide tail **2**.

Scheme 8. Final Assembly of Macrocycle and Lipopeptide Tail[a]



[a] Reagents and conditions: (a) **1** (1.0 equiv), **2** (1.1 equiv), HOBt (3.3 equiv), EDC (3.0 equiv), $NaHCO_3$ (cat), AcCN:DMF (2.2:1), 5 h, 63%; (b) $AlBr_3$ (25 equiv), EtSH, 4 h, 52%.

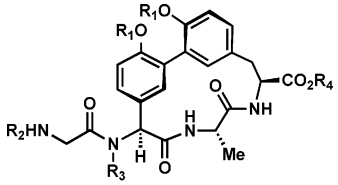
Compound **1** was coupled to lipopeptide **2** via amide formation, yielding the protected arylomycin A_2, **29**, in 63% yield (Scheme 8). Global deprotection with $AlBr_3$/EtSH[13c] at 50 °C afforded arylomycin A_2 in 52% yield. The authenticity of the natural product was confirmed by comparison with the NMR spectra and HPLC retention time of the authentic natural product (kindly provided by Prof. Hans-Peter Fiedler, University of Tubingen).

2.3. Atropisomerism. Restricted rotation, or atropisomerism, about the interannular bond of biaryl systems commonly results in conformational heterogeneity on the NMR time scale. As previously reported,[1] arylomycin A_2 shows two sets of doubled ^{1}H NMR resonances in DMSO. One doubling of the resonances was shown to arise from cis-trans isomerism within the lipopeptide tail.[1] Interestingly, we found that several simple macrocycle intermediates synthesized en route to the natural product show doubled resonances, as well, suggesting that the second resonance doubling in DMSO is due to atropisomerism about the macrocycle biaryl bond. To examine this potential atropisomerism, we synthesized several derivatives of the glycine homologated core macrocycle (Figure 2). These model macrocycles were chosen to focus on macrocycle dynamics in the absence of the conformational heterogeneity associated with the lipopeptide tail.

We first examined the protected macrocycle analog **23**. The 1D ^{1}H spectrum of compound **23** showed two sets of resonances

(25) Grieco, P. A.; Bahsas, A. *J. Org. Chem.* **1987**, *52*, 5746−5749.

(26) Di Gioia, M. L.; Leggio, A.; Le Pera, A.; Liguori, A.; Napoli, A.; Siciliano, C.; Sindona, G. *J. Org. Chem.* **2003**, *68*, 7416−7421.



23: R_1 = Me; R_2 = Boc; R_3 = Me; R_4 = Me
30: R_1 = H; R_2 = H; R_3 = Me; R_4 = H
31: R_1 = H; R_2 = H; R_3 = H; R_4 = H
32: R_1 = Me; R_2 = Boc; R_3 = H; R_4 = Me

Figure 2. Model macrocycles synthesized to evaluate atropisomerism.

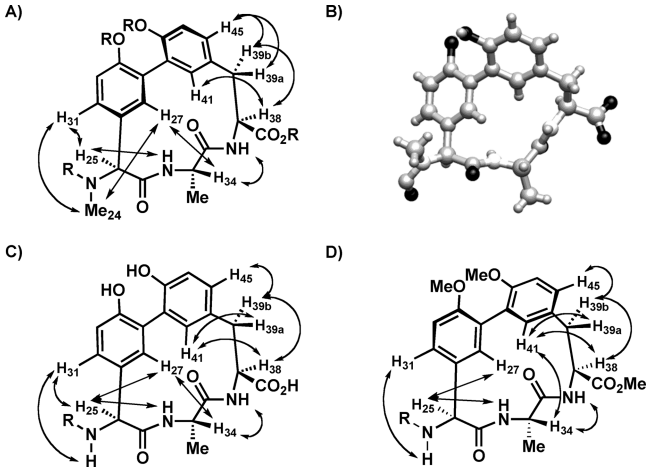


Figure 3. (A) NOEs suggesting that macrocycles **23** and **30** exist predominantly as the S_a atropisomer. (B) The conformation of arylomycin A_2 (truncated for clarity) bound in the active site of Spase (PDB ID: 1T7D[9]). (C) NOEs suggesting that macrocycle **31** exists as rapidly interconverting S_a and R_a atropisomers. (D) NOEs suggesting that macrocycles **32** exists predominantly as the R_a atropisomer.

in DMSO, with a ratio of approximately 3:1. The deprotected macrocycle **30** showed a similar 1D ^{1}H spectrum in DMSO, with resonances present in a ratio of approximately 4:1. Thus, the observation of two stable conformations is not an artifact of macrocycle protection, and deprotection favors the major atropisomer. ROESY spectra were next acquired for both model macrocycles in DMSO. Exchange cross-peaks between the different sets of resonances confirmed that they correspond to equilibrating isomers. For the major isomers of both **23** and **30**, NOEs were observed between H_{45} and both H_{39a} and H_{39b}; between NMe_{24} and both H_{31} and H_{27}; between H_{34} and H_{27}; and between H_{38} and H_{41} (Figure 3A). In addition, a stronger NOE was observed between H_{25} and H_{31} than between H_{25} and H_{27}. We also examined compound **30** in D_2O and MeOD and the results were virtually identical. These NOEs are uniquely consistent with the proton−proton distances of the S_a atropisomer, which is the atropisomer observed in the structure of arylomycin A_2 bound to SPase[9] (Figure 3B).

To examine the contribution of methylation at MeHpg5 to macrocycle structure and dynamics, we synthesized macrocycle **31**. Although the resonances of **31** are significantly overlapped in DMSO, it was apparent that, in contrast to **23** and **30**, **31** shows a single set of resonances and a distinctly different pattern of NOEs. To confirm this conclusion and to facilitate interpretation of the NOEs, spectra were acquired in MeOD; these spectra were similar to those acquired in DMSO, but were better resolved. In the ROESY spectrum of **31** in MeOD, H_{39a} shows an NOE only with H_{41}, while H_{39b} shows a strong NOE only with H_{45} (Figure 3C). This contrasts with **23** and **30** where both H_{39a} and H_{39b} show similar and relatively strong NOEs with H_{45}, but either no NOE or only a weak NOE with H_{41}. Furthermore, in **31**, H_{25} shows a similarly intense NOE with H_{31} and H_{27}, in contrast with **23** and **30**, where H_{25} shows a much stronger NOE with H_{31}. The structure of **31** most consistent with the data positions the aryl rings in the same plane (Figure 3C). This structure is unlikely to represent a stable conformation and is more likely to result from the presence of rapidly interconverting S_a and R_a atropisomers, which also explains why only a single set of NMR signals are observed for this macrocycle. The absence of the methyl group appears to allow the hydroxyphenylglycine ring to rapidly interconvert between two conformations, one in which H_{25} is proximal to H_{31} and one in which H_{25} is proximal to H_{27}. This, in turn, appears to cause changes in the structure of the coupled Tyr7 aromatic ring and a puckering of the associated methylene unit, which positions H_{45} closer to H_{39b} than to H_{39a}. Thus, in the absence of the N-methyl group, the macrocycle becomes more flexible. We conclude that N-methylation appears to rigidify the macrocycle and localize it preferentially to a conformation that is most appropriate for binding *E. coli* SPase.

Table 2. MICs (μM) of Selected Compounds

strain	arylomycin A_2	compd **37**	compd **39**
E. coli MG1655	>128	>128	>128
E. coli MG1655 w/PMBn	128	16	>128
S. aureus 8325	>128	>128	>128
S. epidermidis ATCC 35984	1	0.5	1
B. anthracis Sterne	n.d.	32	n.d.
E. faecium AEFA001[a]	n.d.	>64	n.d.
E. faecalis ATCC 29212	n.d.	>64	n.d.
E. faecalis ATCC 51299	n.d.	>64	n.d.

[a] Part of the Achaogen, Inc. strain collection.

To further study the effect of N-methylation, we examined macrocycle **32**, where the phenol moieties are protected but the exocyclic nitrogen is unmethylated. In the 1D ^{1}H spectrum we observed two sets of resonances in a ratio of approximately 4:1. Interestingly, the ROESY spectrum of **32**, suggests that it predominantly adopts the R_a atropisomer (Figure 3D). This conclusion is based on the strong NOEs between H_{25} and H_{27} (but not between H_{25} and H_{31}), between H_{39a} and H_{41}, and between H_{41} and H_{34} (but not between H_{27} and H_{34}). The chemical shifts and NOEs of the minor species of **32** suggest that it corresponds to the S_a atropisomer. This data further reinforces the conclusion that N-methylation pre-organizes the macrocycle for SPase binding by favoring the S_a atropisomer.

2.4. Biological Activity of Arylomycin A_2. With the synthetic natural product in hand, we tested its antibiotic activity against the major human pathogens *E. coli* (strain MG1655) and *S. aureus* (strain 8325). We found that high concentrations of arylomycin A_2 inhibit growth of polymyxin B nonapeptide permeabilized *E. coli* (minimum inhibitory concentration (MIC) of 128 μM), and as reported,[1b] have no effect on the growth of wild-type, non-permeabilized *E. coli* (Table 2). We also did not observe any growth inhibition of *S. aureus* with the natural product at concentrations up to 128 μM.

Because several lipoglycopeptides are known to inhibit the growth of *S. aureus*,[6] we were interested in deconvoluting the contributions of the fatty acid tail length and the phenylhydroxyglycine deoxymannose to antibacterial activity. Toward this goal, *iso*-C_{16} arylomycin **37**, possessing the longer *iso*-C_{16} fatty acid

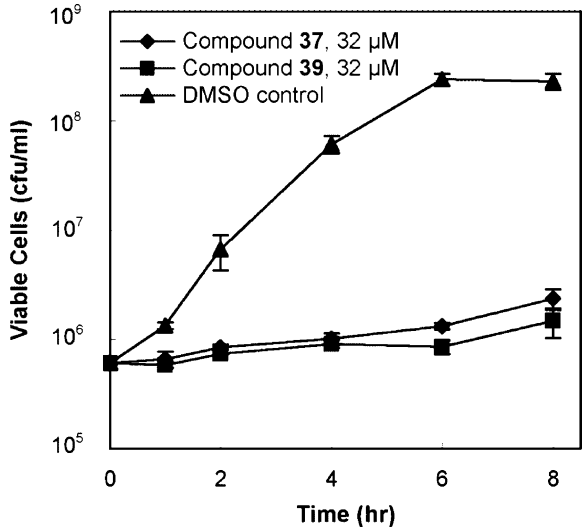


Figure 4. Inhibition of *S. aureus* growth. Bacterial cultures were subject to compound **37** or **39** in DMSO at the concentrations indicated or to DMSO alone. Colony forming units (cfu) were determined as described in the Supporting Information.

tail of the lipoglycopeptides and the sugar free biaryl linkage of arylomycin A_2, was synthesized by modification of the above-described procedure (Scheme 7). Isopalmitic acid chloride was coupled to nosyl deprotected D-Ser(OBn)-D-Ala-OMe, and the product was deprotected with LiOH yielding **35** in 43% yield (3 steps). This lipopeptide was then coupled to macrocycle **1** and globally deprotected, as described for the natural product (Scheme 8).

The antibiotic activity of the *iso*-C_{16} arylomycin **37** was examined with *E. coli* and *S. aureus*. Compound **37** inhibited the growth of permeabilized *E. coli* with an MIC of 16 μM. Exposure of *S. aureus* to up to 128 μM of **37** did not completely abolish growth; however, by visual inspection dramatically reduced cell densities were apparent at concentrations as low as 16 μM. To quantify these results, we determined the viable colony forming units of *S. aureus* as a function of time after exposure to **37** (Figure 4). In the absence of the inhibitor, *S. aureus* outgrew ~400-fold during an 8 h period, while in the presence of 32 μM of **37**, they outgrew only ~4-fold. Thus, like the analogous lipoglycopeptide (Shen-Bin, P., personal communication), **37** significantly inhibits the growth of *S. aureus*.

Encouraged by the results with *S. aureus*, we examined the antibacterial activity of compound **37** against a variety of other Gram-positive pathogens, including *Enterococcus faecalis* (ATCC 29212 and 51299), *Enterococcus faecium* (Achaogen Inc. strain AEFA001), *Bacillus anthracis* (Sterne), and *Staphylococcus epidermidis* (ATCC 35984). Compound **37** does not inhibit the growth of the enterococci *E. faecalis* or *E. faecium*. However, it does inhibit the growth of *B. anthracis*, with an MIC of 32 μM. Moreover, compound **37** is remarkably potent against *S. epidermidis*, inhibiting its growth with an MIC of 0.5 μM. To examine the contribution of fatty acid tail length to *S. epidermidis* toxicity, we determined the MIC of arylomycin A_2. Surprisingly, the natural product showed virtually identical inhibition of *S. epidermidis* (MIC = 1.0 μM). To better gauge the potency of arylomycin A_2 and **37**, we also determined the potency of vancomycin, linezolid, and daptomycin, which are currently the standard treatment options for *S. epidermidis* infections. We found that vancomycin, linezolid, and daptomycin kill *S. epidermidis* with MICs of 1.0, 0.5, and 1.0 μM, respectively. Thus, both arylomycin A_2 and **37** are as potent against *S. epidermidis* as the antibiotics that are currently prescribed for its treatment.

Finally, to test the prediction that exocyclic methylation pre-organizes the macrocycle for biological activity, we synthesized the derivative of **37** where the methyl group is removed. Compound **37** was selected, instead of arylomycin A_2, due to its increased potency against *E. coli*. The des-N-methyl analog **39** was synthesized by Boc-deprotection of macrocycle **32** and coupling to dipeptide **35**, followed by global deprotection (Scheme 7). Remarkably, removing the methyl group dramatically reduced the ability of the compound to inhibit the growth of permeabilized *E. coli* (Table 2). In contrast, removing the methyl group did not have a significant effect on growth inhibition with either of the Gram-positive bacteria, *S. aureus* or *S. epidermidis*.

3. Discussion

Nature is replete with biaryl- or biaryl ether-linked cyclic peptides that possess remarkable biological activities.[17] Modification by N-methylation, glycosylation, and/or lipidation can impart cyclic peptides with potent antibacterial activity, as exemplified by vancomycin[27] and teicoplanin.[28] Interestingly, the arylomycins and related lipoglycopeptides bear all three modifications; however, the specific contributions of these modifications to biological function are unknown. The ability of these natural products to inhibit SPase, a structurally and mechanistically unique bacterial Ser-Lys dyad protease required for protein export, makes them promising candidates for development as antibiotics. Synthetic access to these compounds and their derivatives was expected not only to allow for their evaluation as potential antibiotics, but also to provide a unique opportunity to study the contribution of N-methylation, lipidation, and glycosylation to antibacterial activity.

A modular strategy was implemented to synthesize arylomycin A_2 by independently constructing the C-terminal macrocycle and an N-terminal lipopeptide tail before joining them to form the natural product. The most significant obstacles were closure of the tripeptide macrocycle and subsequent methylation of the exocyclic amine. Macrolactamization, an efficient route to macrocyclization of similar compounds,[12b,13c,16−18] proceeded only at low substrate concentrations and with low yields. It is possible that formation of the 14-membered ring of the arylomycins is uniquely disfavored relative to the smaller and larger rings of the other biaryl-linked systems for which macrolactamization has been reported (12 atoms for the vancomycin AB ring system[12b,13c] and 17 atoms for the complestatin and TMC-95A systems[17,18,29]). The specific biphenyl linkage topology of the arylomycins may also prevent efficient macrolactamization. In contrast, macrocyclization by intramolecular Suzuki−Miyaura coupling proved more successful, possibly due to preorganization of the peptide backbone,[29] and accordingly, this strategy was used in the synthesis of the natural product.

(27) McCormick, M. H.; Stark, W. M.; Pittenger, G. E.; Pittenger, R. C.; McGuire, J. M. *Antibiot. Ann.* **1955**−**1956**, 606−611.
(28) (a) Bardone, M. R.; Paternoster, M.; Coronelli, C. *J. Antibiot.* **1978**, *31*, 170−177. (b) Parenti, F.; Beretta, G.; Berti, M.; Arioli, V. *J. Antibiot.* **1978**, *31*, 276−283.
(29) Elder, A. M.; Rich, D. H. *Org. Lett.* **1999**, *1*, 1443−1446.

Monomethylation of the macrocycle's exocyclic amine proved surprisingly challenging. Although this step was eventually accomplished using nosyl protection followed by MeI/K_2CO_3, the failures suggest that the amine environment is sterically encumbered. The steric congestion about the exocyclic amine is likely the reason why modifications at this site can influence the conformation of the proximal biaryl moiety (see below).

In addition to the peptide itself, there are four structural aspects of the arylomycins and related lipoglycopeptides that may contribute to biological activity, including, atropisomerism about the C−C biaryl bond, N-methylation, lipidation, and glycosylation. To characterize atropisomerism in these compounds, we examined macrocycles, **23** and **30**. These model macrocycles bear a glycine residue in lieu of the full lipopeptide tail, and thus allow for the characterization of atropisomerism dynamics of the macrocycle, free from the complexities associated with the known cis-trans isomerism of the lipopeptide tail.[1] The observation of doubled resonances and exchange peaks in the ROESY spectra of both macrocycles in DMSO confirmed that these simple macrocycles exist as two exchanging conformations, and that this heterogeneity does not result from the aryl protecting groups. On the basis of several key sets of NOEs (including H_{34} to H_{27} and/or to H_{41}; H_{41} to H_{39a} and/or H_{39b}; H_{45} to H_{39a} and/or H_{39b}; and H_{25} to H_{27} and/or H_{31}), the major conformation observed in both cases was the S_a atropisomer, which is the same as that observed in the crystal structure of the natural product bound to *E. coli* SPase (Figure 3B).

Methylation is a conspicuous modification of the natural product. It has already been reported that N-methylation at MeSer2 contributes to cis-trans isomerization within the lipopeptide tail,[1] an observation consistent with the effects of N-methylation on peptide structure in organic solvents.[30] Structural studies demonstrate that the MeHpg5 methyl group is oriented into solvent and does not mediate any interactions with SPase, nor does it cause isomerization about the amide bond. However, N-methylation is known to impact other aspects of peptide structure,[31] and as discussed above, the challenges associated with installation of the methyl group suggested that it resides in a well-packed environment and thus might influence biaryl ring structure. To examine the effect of N-methylation on arylomycin atropisomerism, we synthesized and characterized macrocycle **31**. The data suggests that **31** exists as an average of rapidly interconverting S_a and R_a atropisomers. Characterization of **32** revealed that the effect of methylation is even more pronounced when the interactions between the aryl hydroxy groups are exacerbated with methyl protecting groups; the phenol-protected macrocycle of **32** adopts predominantly the R_a atropisomer. Thus, N-methylation appears to favor the S_a atropisomer, the same atropisomer observed in the structure of the arylomycin A_2 complex with *E. coli* SPase.

With access to synthetic arylomycin A_2, we tested its antibacterial activity against the major human pathogens *E. coli* and *S. aureus*, representatives of Gram-negative and Gram-positive bacteria. As reported with the isolated natural product,[1] arylomycin A_2 had no effect on wild-type *E. coli*, despite binding *E. coli* SPase *in vitro* with a dissociation constant of approximately 800 nM.[19] This suggests that penetration of the outer cell membrane may limit activity. Indeed, arylomycin A_2 inhibits growth of an outer membrane permeabilized strain of *E. coli* with an MIC of 128 μM. Further supporting the suggestion that the activity of the natural product is limited by the outer membrane of Gram-negative bacteria, arylomycin A_2 has previously been shown to inhibit the growth of several Gram-positive aerobic soil bacteria, including *Arthrobacter pascens*, *Rhodococcus erythropolis*, and *Streptomyces viridochromogenes*.[1] However, we found that the natural product did not inhibit the growth of *S. aureus*, suggesting that additional factors must also play a role in the compound's biological activity.

To help understand the contribution of fatty acid tail length and macrocycle glycosylation to antibiotic activity, we synthesized the hybrid *iso*-C_{16} arylomycin **37**, which possesses the longer *iso*-C_{16} fatty acid tail of the lipoglycopeptides and the unglycosylated macrocycle of the arylomycins. Interestingly, elongation of the fatty acid tail produced a significantly more potent inhibitor of permeabilized *E. coli*, inhibiting growth with an MIC of 16 μM. This MIC is not significantly different from that observed with the lipoglycopeptides,[6] suggesting that tail length is the dominant factor differentiating the antibiotic activity of arylomycins and the lipoglycopeptides against permeablized Gram-negative bacteria. The more hydrophobic tail likely favors insertion into the hydrophobic inner membrane, possibly facilitating access to SPase. Unlike arylomycin A_2, compound **37** also significantly inhibits the growth of *S. aureus*, at concentrations as low as 16 μM, however, it fails to display an MIC due to a minimal but measurable growth at concentrations up to 128 μM (Figure 4). The behavior is qualitatively similar to that observed with the corresponding lipoglycopeptide (Sheng-Bin, P., personal communication). Thus, as with Gram-negative bacteria, increasing the length of the fatty-acid tail increases potency against *S. aureus* and glycosylation does not appear to make a significant contribution.

To help understand the contribution of methylation and atropisomerism to antibiotic activity, and to test the hypothesis that *N*-methylation favors the S_a atropisomer that binds *E. coli* SPase, we synthesized **39**, the des-N-methyl analog of compound **37**. Interestingly, whereas **37** inhibits the growth of permeablized *E. coli* with an MIC of 16 μM, compound **39** showed no MIC, up to 128 μM. Decreased inhibition of SPase by **39** is unlikely to result from decreased cell penetrance as SPase resides on the outer surface of the inner membrane and the outermembrane is essentially removed as a significant barrier by permeabilization. Thus, on the basis of these results, along with the structural studies described above, we tentatively conclude that N-methylation favors the S_a atropisomer and preorganizes the natural product for binding to *E. coli* SPase. In contrast, **37** and **39** showed identical inhibition of *S. aureus*. Whether the dissimilar consequences of methylation result from differences between the different SPase proteins, or other differences between the biology of these Gram-negative and Gram-positive pathogens, remains to be determined.

Having identified fatty acid chain length and methylation as important determinants of antibacterial activity, we proceeded with compound **37** to examine the inhibition of other Gram-positive pathogens. We found no antibacterial activity against the enterococci *E. faecalis* and *E. faecium*, but **37** shows promising activity against both *B. anthracis* and *S. epidermidis*. Most notably, **37** is as potent against *S. epidermidis* as

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(31) Manavalan, P.; Momany, F. A. *Biopolymers* **1980**, *19*, 1943−1973.

vancomycin, linezolid, and daptomycin, the antibiotics that are currently prescribed for the treatment of *S. epidermidis* infections. Interestingly, in contrast with *E. coli* and *S. aureus*, fatty acid chain length is not critical for activity against *S. epidermidis*, as arylomycin A_2 inhibited growth with a similar MIC. Also in contrast to *E. coli*, but similar to *S. aureus*, removal of the methyl group from **37** did not significantly reduce the potency against *S. epidermidis*. The potency of these compounds against *S. epidermidis* is particularly interesting, as this bacteria is known to be the primary cause of infections associated with indwelling medical devices, suggesting that these compounds may have well-defined and important therapeutic applications.

4. Conclusion

The identification of antibiotics that act via unique mechanisms is critical for combating bacteria, which are rapidly evolving resistance to all currently available antibiotics. The bacterial Ser-Lys dyad protease SPase is an attractive target for drug design because it is surface exposed, essential for bacterial viability, and it has a unique structure and catalytic mechanism. In addition, it is required for processing proteins involved in resistance to other antibiotics and virulence. We have reported the first total synthesis of the SPase inhibitor arylomycin A_2, as well as several of its derivatives. Suzuki−Miyaura coupling, as opposed to macrolactamization, was found to provide the most efficient route to the natural product. Synthetic access to arylomycin A_2 and several of its derivatives allowed us to determine that exocyclic N-methylation at MeHpg5 appears to pre-organize the macrocycle for binding *E. coli* SPase, where fatty acid tail length, but not glycosylation, is also important. With regard to *S. aureus*, fatty acid tail length, but not N-methylation or glycosylation, appears to be important. Finally, with *S. epidermidis*, neither increasing the length of the fatty acid chain, nor removal of the N-methyl group alters growth inhibition. Clearly, the impacts of methylation, lipidation, and potentially glycosylation on antibiotic activity depend on the target bacteria. Future studies aimed at increasing the potency and spectrum of these compounds will focus on understanding the bacteria-specific effects as well as on the optimization of the molecule, itself. The reported modular synthetic route to these natural products should facilitate these studies, and should also help further define the potential of this class of natural products as antibiotics and/or inhibitors of bacterial virulence.

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Supporting Information Available: Experimental procedures, characterization of new compounds, ^{1}H, ^{13}C, and ROESY NMR spectra, and complete ref 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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