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Initial Efforts toward the Optimization of Arylomycins for Antibiotic Activity

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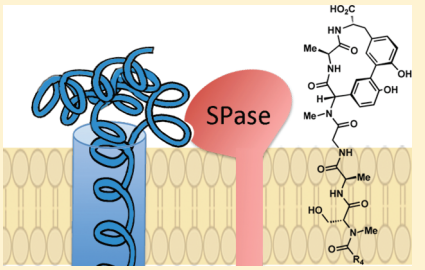
Supporting Information

ABSTRACT: While most clinically used antibiotics were derived from natural products, the isolation of new broad-spectrum natural products has become increasingly rare and narrow-spectrum agents are typically deemed unsuitable for development because of intrinsic limitations of their scaffold or target. However, it is possible that the spectrum of a natural product antibiotic might be limited by specific resistance mechanisms in some bacteria, such as target mutations, and the spectra of such "latent" antibiotics might be reoptimized by derivatization, just as has been done with clinically deployed antibiotics. We recently showed that the spectrum of the arylomycin natural product antibiotics, which act via the novel mechanism of inhibiting type I signal peptidase, is broader than previously believed and that resistance in several key human pathogens is due to the presence of a specific Pro residue in the target peptidase that disrupts interactions with the lipopeptide tail of the antibiotic. To begin to test whether this natural resistance might be overcome by derivatization, we synthesized analogues with altered lipopeptide tails and identified several with an increased spectrum of activity against *S. aureus*. The data support the hypothesis that the arylomycins are latent antibiotics, suggest that their spectrum may be optimized by derivatization, and identify a promising scaffold upon which future optimization efforts might focus.



INTRODUCTION

The evolution of bacteria that are resistant to multiple antibiotics poses a serious threat to human health.[1,2] Although much effort has been focused on the discovery and development of fully synthetic antibiotics,[3] most of the antibiotics used in the clinic were derived from natural products, which evolved over eons of time to penetrate bacteria, avoid efflux, and inhibit essential biochemical processes.[4] Unfortunately, broad-spectrum natural product antibiotics have become increasingly difficult to isolate and the more plentiful narrow-spectrum agents are limited either by unknown factors or by factors that are intrinsic to the compound, such as poor penetration or targeting proteins that are not sufficiently conserved and that are viewed as challenging to overcome by optimization.[3] In contrast, there is much precedent for reoptimizing antibiotics after their spectrum has been compromised by specific resistance mechanisms acquired during clinical use, as evidenced by the development of many "next generation" antibiotics.[5−8]

Nature is replete with antimicrobial peptides that hold promise as therapeutics.[9,10] The arylomycins are a class of nonribosomally synthesized lipopeptide antibiotics that inhibit bacterial type I signal peptidase (SPase), an essential serine−lysine dyad protease that is anchored to the outer leaflet of the cytoplasmic membrane and that removes N-terminal signal peptides from proteins that are transported out of the cytoplasm.[11−13] Three related series of arylomycins have been identified, the arylomycins A and B and the lipoglycopeptides, which have similar core macrocycles but different substituents and fatty acid tails (Figure 1).[14,15] On the basis of their novel mechanism of action, there was originally much enthusiasm for these compounds, but despite their ability to inhibit SPase in vitro and their in vivo activity against the soil bacteria *Rhodococcus opacus* and *Brevibacillus brevis* and the human pathogen *Streptococcus pneumoniae*, they were found to have no activity against a variety of other important human pathogens.[15,16] This apparently narrow spectrum is surprising considering that SPase is located in an accessible, external site and appears to be present and essential in all eubacteria.[12,17−19] To explore the origins of their narrow spectrum, we synthesized and evaluated arylomycin A_2, as well as several derivatives, including arylomycin C_{16} (Figure 1).[20] Interestingly, we found that the arylomycins are as active against *Staphylococcus epidermidis* as the antibiotics used for its treatment, and importantly, we determined that *S. epidermidis* evolves resistance by introducing a Pro residue into SPase at position 29,[21] which is predicted to interact with the P5 residue[22] of a bound peptide.[23−25] Remarkably, all bacteria that had been shown to be resistant to the arylomycins have a Pro at the corresponding position, and we identified a wide variety of bacteria that lack this residue and showed that the majority of them are sensitive to the arylomycins, including the Gram-positive pathogens *Streptococcus pyogenes* and *Staphylococcus hemolyticus* and the Gram-negative pathogens *Helicobacter pylori* and *Chlamydia trachomatis*. Moreover, while the arylomycins slow the growth of *Staphylococcus aureus* strain 8325, they do not actually prevent growth,[20] even at concentrations

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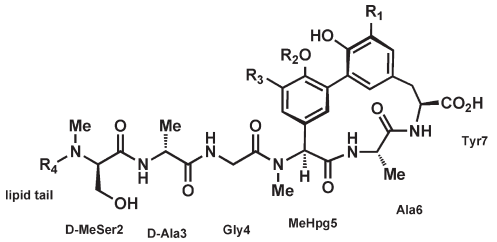


Arylomycin A family: R_1 = H, R_2 = H
R_3 = H, R_4 = acyl-*n*-C_{12}, acyl-*anteiso*-C_{13}, acyl-*iso*-$C_{11\text{-}12}$ or acyl-*iso*-C_{14}

Arylomycin A_2: R_1 = H, R_2 = H,
R_3 = H, R_4 = acyl-*iso*-C_{12}

Arylomycin C_{16}: R_1 = H, R_2 = H,
R_3 = H, R_4 = acyl-*iso*-C_{16}

Arylomycin B family: R_1 = NO_2, R_2 = H,
R_3 = H, R_4 = acyl-*n*-C_{12}, acyl-*anteiso*-C_{13}, acyl-*anteiso*-C_{15} or acyl-*iso*-$C_{11\text{-}14}$

Lipoglycopeptides: R_1 = H, R_2 = 6-deoxy-α-mannose
R_3 = H or OH, R_4 = acyl-*n*-$C_{14\text{-}16}$ or acyl-*iso*-$C_{14\text{-}16}$

1: R_1 = H, R_2 = H
R_3 = H, R_4 = alkyl-C_{16}

Figure 1. Arylomycin class of natural products and compound **1**.

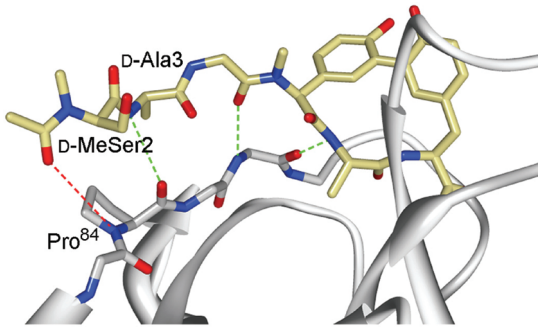
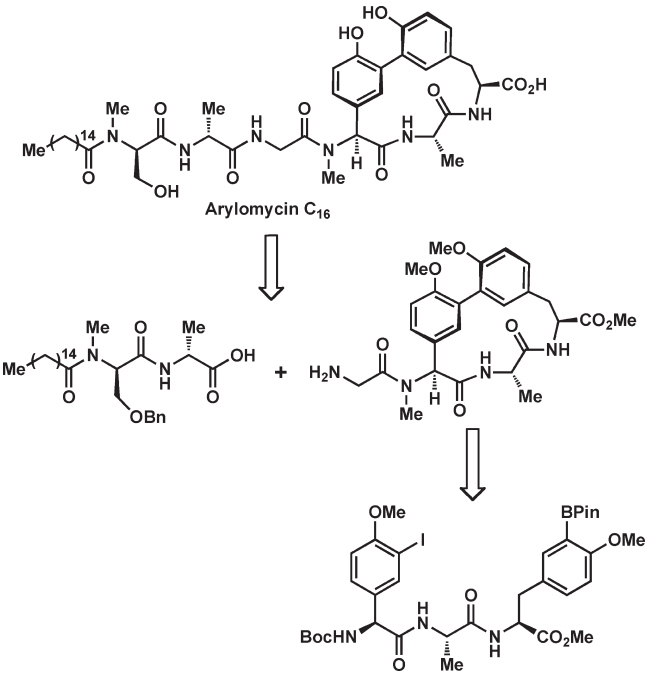


Figure 2. The crystal structure of arylomycin A_2 bound to *E. coli* SPase (PDB code 1T7D) reveals that Pro84 is located in a surface depression where it appears to preclude formation of a stabilizing H-bond with the fatty acid carbonyl oxygen (shown in red) and possibly to preclude accommodation of the lipid tail.[21,23] D-MeSer2 and D-Ala3 of the arylomycin are labeled. The resistance-conferring Pro is also labeled (using *E. coli* numbering). Note that because of a discrepancy in the numbering system used, Pro84 in Paetzel et al.[23] and in the structure 1T7D is denoted Pro83.

as high as 128 μg/mL; however, they do prevent the growth of the epidemic MRSA isolate USA300 with an MIC of 16 μg/mL. While this might result from unique features associated with methicillin resistance, it suggests that the arylomycin scaffold has the potential for broader spectrum *S. aureus* activity. Importantly, we showed that the Pro residue imparts resistance by reducing the affinity with which the arylomycin binds and that removing this residue is sufficient to render resistant *S. aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* highly sensitive. These data suggest that if the arylomycins could be optimized to bind SPases regardless of the resistance-conferring Pro, then they would have a remarkably broad spectrum of activity.

Scheme 1. Retrosynthesis of Arylomycin C_{16}[a]



[a] Arylomycin derivatives were synthesized by modification of previously reported protocols.[20,34] Briefly, tripeptide macrocycle precursor was synthesized by solution phase peptide couplings and then cyclized via Suzuki−Miyaura macrocyclization. The various lipopeptide tails were assembled via solution phase peptide couplings and then coupled to the macrocyclic core or the glycine-homologated macrocycle core.

Despite not having activity against wild type *E. coli*, two crystal structures of arylomycin A_2 bound to a soluble fragment of *E. coli* SPase have been reported (Figure 2).[23,26] The arylomycin is seen to bind in an extended β-sheet conformation that likely mimics the binding of membrane bound pre-protein substrates.[23] The C-terminal macrocycle of the arylomycin binds in a deep hydrophobic cleft and makes multiple H-bonds and hydrophobic interactions with the protein, while the C-terminal carboxyl group forms a critical salt bridge with the catalytic residues. The peptide tail extends down a shallow cleft in the surface of SPase and forms two H-bonds with backbone residues of the protein. The critical resistance-conferring residue, Pro^{84}, interacts with the N-terminal end of the peptidic tail and appears to preclude the formation of a H-bond to a carbonyl oxygen of the arylomycin and possibly to alter the trajectory of the lipid moiety as it enters the membrane (Figure 2). While the crystal structures are likely to reveal little information about the biologically relevant structure of the lipid tail because of the use of a soluble truncated fragment of SPase and the absence of a membrane bilayer, it most likely adopts an extended conformation to maximize packing within the outer leaflet of the cytoplasmic membrane.

As with a variety of other antibiotics that interact with membranes or that have membrane-associated targets,[27−33] the lipopeptide tail of the arylomycins has been shown to play an important role in their activity.[15,16,27−30] Here, we report the first structure−activity relationship study of synthetic arylomycins, focusing on derivatives with altered lipopeptide tails. The activities of the arylomycin derivatives were evaluated with

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S. epidermidis, *S. aureus*, *E. coli*, and *P. aeruginosa*. With each pathogen, the derivatives were evaluated in the context of SPases with and without the critical resistance-conferring Pro, to identify the changes in activity that result from altered interactions with this resistance-conferring residue and to identify the types of modifications that might be pursued to overcome resistance and thereby instill the arylomycin scaffold with broad-spectrum antibacterial activity.

RESULTS

The arylomycin derivatives were synthesized by modification of previously reported protocols[20,34] as illustrated in Scheme 1 (see Experimental Section and Supporting Information for details). Briefly, the tripeptide macrocycle precursor was assembled from ortho-iodinated hydroxyphenylglycine, alanine, and a tyrosine boronic ester. The tripeptide was cyclized via Suzuki−Miyaura macrocyclization and then methylated via a nosylated amine and either directly coupled to a lipopeptide tail or first coupled to glycine and then to a lipopeptide tail. The different lipopeptide tail derivatives were assembled using solution phase peptide couplings of the corresponding natural or unnatural amino acids, followed by lipidation. Methylation of the peptide backbone of the tail was accomplished prior to coupling to the lipid in the case of D-Ser2 methylation or after, in the case of the D-Ala3 methylation. Finally, all derivatives were globally deprotected using $AlBr_3$ and ethanethiol.

The minimal inhibitory concentration (MIC) of each derivative was determined using a standard broth dilution method. Test strains included wild type *S. epidermidis* (strain RP62A), as well as mutant strains of *S. aureus* (strain PAS8001), *E. coli* (strain PAS0260), and *P. aeruginosa* (strain PAS2006) that were rendered sensitive to the arylomycins by mutation of the resistance-conferring Pro to a residue that does not confer resistance (P29S in the *S. aureus* SPase, and P84L in the *E. coli* and *P. aeruginosa* SPase).[21] MICs were also determined with the isogenic wild type strains of *S. aureus* (strain NCTC 8325), *E. coli* (strain MG1655), and *P. aeruginosa* (strain PAO1), as well as an isogenic mutant strain of *S. epidermidis* where the resistance-conferring Pro has been introduced (strain PAS9001).[21] The term "resistant" is used to refer to the reduced sensitivity of a strain harboring the resistance-conferring Pro relative to the isogenic strain without the Pro and not to the absolute sensitivities of the different bacteria (e.g., resistant *S. epidermidis* is inhibited with an MIC of 8 μg/mL and is significantly more sensitive to arylomycin C_{16} than the resistant mutants of the other pathogens, which are not inhibited at concentrations as high as 128 μg/mL).

SPase and the N-terminal portion of its natural substrates are embedded within the bacterial cell membrane,[35−37] suggesting that some part of the inhibitor's lipopeptide tail must also be accommodated within the membrane, although the absence of a lipid membrane in the crystal structure makes determining precisely what part of the tail is embedded difficult. To probe this interaction and also to determine whether a positive charge at the lipid−peptide junction of arylomycin is capable of interacting with the negatively charged headgroups of the phospholipid bilayer, we synthesized derivative **1** (Figure 1), which replaces the lipid tail amide with a charged tertiary amine. This derivative has significantly reduced activity against *S. epidermidis* and sensitized *S. aureus* (MICs of 32 and 64 μg/mL, respectively) and no activity against any of the wild type or mutant Gram-negative strains examined. The decreased activity likely results from the inability of a hydrophobic environment to accommodate the charge, suggesting that this portion of the tail is embedded in the membrane or within the interface between the membrane and SPase.

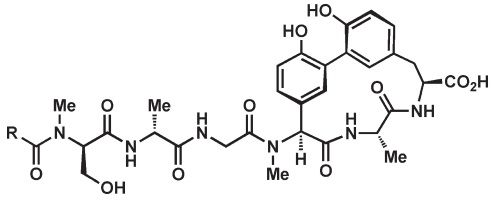
To explore the minimal tail length required for activity and to determine whether there is a limit to the tail length that can be accommodated within the cytoplasmic membranes of the different bacteria, we synthesized and characterized derivatives **2**−**5** (Table 1). None of these derivatives gained activity against any of the resistant bacteria relative to arylomycin C_{16}, but significant differences were apparent with *S. epidermidis* and the genetically sensitized strains. With the sensitive strains, the C_8 derivative **2** has no activity, but the C_{10} derivative **3** has activity against *S. epidermidis*, *S. aureus*, and *E. coli*, while only **4** and **5** show activity against *P. aeruginosa*, revealing that at minimum a C_{12} tail is required. In each case, activity increased with increasing tail length until it plateaued with the C_{16} fatty acid tail (i.e., arylomycin C_{16}), and activity decreased slightly with the C_{18} derivative **5** with all but *P. aeruginosa*.

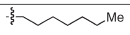
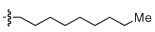
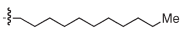
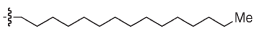
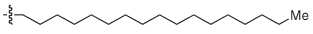
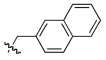
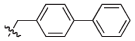
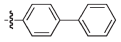
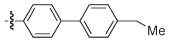
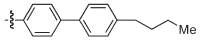
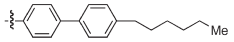
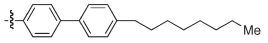
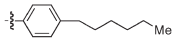
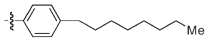
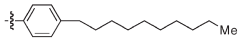
To further explore the effects of increased hydrophobicity, we synthesized and characterized derivatives with tails that contain one or more aromatic rings (Table 1). We first examined the series of napthyl and biphenyl derivatives **6**−**8**. The napthyl derivative **6** shows no activity against any of the bacteria tested, while the biphenyl derivative **7** retains some activity against wild type *S. epidermidis*. We found that compound **8**, which lacks the methylene spacer between the fatty acid carbonyl and the biphenyl moiety, also retains some activity against *S. epidermidis*, suggesting that flexibility of the biphenyl moiety is not essential. To further explore this biphenyl architecture, we synthesized the *p*-alkyl substituted biphenyl derivatives **9**−**12**. We observed an increase in activity with increasing alkyl substituent length against wild type *S. epidermidis* that plateaued with the C_6 and C_8 derivatives **11** and **12**, which are also active against resistant *S. epidermidis*. Interestingly, several of the compounds in this series are also active against both sensitized and wild type *S. aureus*, with relative activities similar to those observed with *S. epidermidis* but with absolute activities that were somewhat lower. None of the biphenyl derivatives have activity against the wild type or sensitized strains of *P. aeruginosa*, but they do maintain activity against sensitized *E. coli*, again showing trends that are similar to those observed with *S. epidermidis* and *S. aureus*.

We next examined the series of phenyl substituted tail mimetics **13**−**15** (Table 1). With *S. epidermidis* and the genetically sensitized strains, we again observed an increase in activity with increasing alkyl chain length. Moreover, the decylphenyl derivative **15** has activity against wild type *S. aureus*. Because the number of carbon atoms in this derivative is similar to that of arylomycin C_{16}, which has no activity against wild type *S. aureus*, the data suggest that at least some of the activity is mediated by the interaction of the polarizable aromatic moiety with the membrane or with SPase.

To explore the effects of lipopeptide methylation and to begin a more focused exploration of modifications that might overcome the deleterious effects of the resistance-conferring Pro, we synthesized and characterized derivatives with altered N-methylation at D-MeSer2 and D-Ala3 (Table 2), arylomycin residues that are proximal to this critical residue when bound to SPase (Figure 2). The absence of the D-MeSer2 *N*-methyl group in **16** results in a slight decrease in activity against both the wild type and resistant *S. epidermidis* strains and a more pronounced loss of

Table 1. Activity (MICs (μg/mL)) of Arylomycin Derivatives with Altered Fatty Acid Tails against Strains of *S. epidermidis*, *S. aureus*, *E. coli*, and *P. aeruginosa* Harboring SPase without (Sensitive) and with (Resistant) the Arylomycin-Resistance Conferring Pro Residue



No.	R	Sensitive[a]				Resistant[b]			
		S. epidermidis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. epidermidis*	*S. aureus*	*E. coli*	*P. aeruginosa*
2		>64	>64	>64	>64	>64	>64	>64	>64
3		16	64	32	>64	>64	>64	>64	>64
4		0.5	16	8	64	>64	>64	>64	>64
–[c]		0.5	2	0.5	8	8	>64	>64	>64
5		1	4	2	8	16	>64	>64	>64
6		>64	>64	>64	>64	>64	>64	>64	>64
7		32	>64	>64	>64	>64	>64	>64	>64
8		64	>64	>64	>64	>64	>64	>64	>64
9		8	64	16	>64	>64	>64	>64	>64
10		1	16	4	>64	>64	>64	>64	>64
11		0.5	8	1	>64	32	64	>64	>64
12		1	8	1	>64	16	16	>64	>64
13		8	64	32	>64	>64	>64	>64	>64
14		1	8	8	64	>64	>64	>64	>64
15		0.5	4	2	16	16	32	>64	>64

[a] Sensitive strains include wild type *S. epidermidis* RP62A, *lepB*(P29S) *S. aureus* NCTC 8325, *lepB*(P84L) *E. coli* MG1655, and *lepB*(P84L) *P. aeruginosa* PAO1. See text for details. [b] Resistant strains include *spsB*(S29P) *S. epidermidis* RP62A, and wild type strains, *S. aureus* NCTC 8325, *E. coli* MG1655, and *P. aeruginosa* PAO1. See text for details. [c] Arylomycin C_{16}, included for reference.

activity against each of the other strains that is most pronounced with *P. aeruginosa*. The loss in activity is even more pronounced with compound **17** where methylation of D-Ala3 ablates activity against all organisms tested.

To explore the effects of lipopeptide tail rigidity and to further explore modifications that might directly compensate for the resistance conferred by Pro29/84 of SPase, we designed the hydroxyproline derivative **18** (Table 2). In this compound, the side chain of D-MeSer2, which interacts with the side chain and backbone of the SPase residue at position 29/84,[23] is homologated by a methylene unit and fused with the methyl group of the neighboring *N*-methyl amide bond. We found that this modification results in a complete loss of activity against the Gram-negative organisms but only little to moderate loss in activity against the Gram-positive organisms. Interestingly, because **18** retained full activity against resistant *S. epidermidis*, the disparity in activities against the wild type and resistant variants is greatly decreased, suggesting that at least for this organism, **18** recognizes both the Ser- and the Pro-variant SPases similarly.

To further explore the effects of decreased rigidity, we synthesized derivative **19** (Table 2), which lacks the peptide bond between the serine and the fatty acid tail (and thus should impart the tail with greater rotational freedom). Compared to arylomycin C_{16} this molecule exhibited significantly less activity against all organisms tested, with no observable activity against *E. coli*, *S. aureus*, or *P. aeruginosa* and only moderate activity against *S. epidermidis*.

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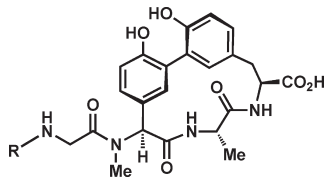
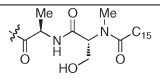
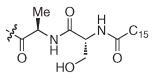
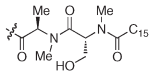
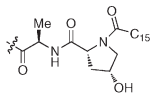
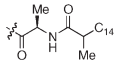
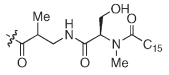
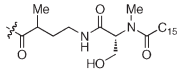
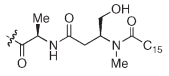
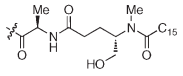
Table 2. Activity (MICs (μg/mL)) of Arylomycin Derivatives with Altered Lipopeptide Tails against Strains of *S. epidermidis*, *S. aureus*, *E. coli*, and *P. aeruginosa* Harboring SPase without (Sensitive) and with (Resistant) the Arylomycin-Resistance Conferring Pro Residue



No.	R	Sensitive[a]				Resistant[b]			
		S. epidermidis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. epidermidis*	*S. aureus*	*E. coli*	*P. aeruginosa*
–[c]		0.5	2	0.5	8	8	>64	>64	>64
16		1	16	4	>64	32	>64	>64	>64
17		>64	>64	>64	>64	>64	>64	>64	>64
18		4	4	>64	>64	8	>64	>64	>64
19		8	>64	>64	>64	>64	>64	>64	>64
20		8	>64	8	16	16	>64	>64	>64
21		8	8	>64	>64	8	>64	>64	>64
22		4	16	>64	>64	8	64	>64	>64
23		8	4	>64	>64	16	16	>64	>64

[a] Sensitive strains include wild type *S. epidermidis* RP62A, *lepB*(P29S) *S. aureus* 8325, *lepB*(P84L) *E. coli* MG1655, and *lepB*(P84L) *P. aeruginosa* PAO1. See text for details. [b] Resistant strains include *spsB*(S29P) *S. epidermidis* RP62A, and wild type strains, *S. aureus* 8325, *E. coli* MG1655, and *P. aeruginosa* PAO1. See text for details. [c] Arylomycin C_{16}, included for reference.

To increase flexibility without introducing or deleting other peptidic functionalities that might contribute to binding SPase, we synthesized and evaluated derivatives with one or two methylene units inserted immediately N-terminal or C-terminal to the amide bond linking D-MeSer2 to D-Ala3 (**20**–**23**, Table 2). These compounds did not gain activity against either of the wild type Gram-negative bacteria. With the sensitized Gram-negative strains, activity was observed only with **20**, which relative to the parent compound is 16-fold less active against *E. coli* but only 2-fold less active against *P. aeruginosa*. The effects of methylene addition were significantly different with the Gram-positive bacteria. Relative to arylomycin C_{16}, derivatives **20**–**23** lost 8- to 16-fold activity against sensitive *S. epidermidis* but retained activity against the resistant strain. This demonstrates that once the H-bond donor of the protein is removed (by mutation to Pro), perturbing the H-bond acceptor does not further decrease activity. The results were somewhat more complicated with *S. aureus*. As expected, relative to arylomycin C_{16}, addition of the methylene units decreased activity against the sensitized strain of *S. aureus*, 2- to 8-fold for **21**–**23** and at least 64-fold for **20**. In the case of wild type *S. aureus*, however, no activity is observed with **20** or **21**, but interestingly, **22** and especially **23** gain activity.

Compounds **11**, **12**, **15**, **22**, and **23** have increased activity against wild type *S. aureus* relative to arylomycin C_{16}. Because these compounds also have increased hydrophobicity and/or aromaticity, the increased gains could result from nonspecific effects such as membrane destabilization. While no mutants of *S. aureus* are available that are resistant to the arylomycins via a mechanism other than Pro29, *S. epidermidis* is closely related and the S31P mutation confers high levels of arylomycin resistance. Derivatives whose activity results from nonspecific effects should

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still be active against this mutant; however, no activity was observed (MIC > 64 μg/mL). These results suggest that the increased activity of the derivatives against *S. aureus* does indeed result specifically from SPase inhibition.

DISCUSSION

It is widely accepted that most if not all of the broad-spectrum antibiotic scaffolds produced by the bacteria cultured to date have been discovered[38] and that the reduced spectrum of the more commonly identified narrow spectrum agents is a limitation that is intrinsic to their structure or mechanism of action and thus difficult to overcome via scaffold derivatization. However, just as resistance plays a role in the clinical arms race between humans and pathogens, resistance might also play a role in the natural arms race between microbes for which many natural product antibiotics were evolved, via selection or genetic drift.[39−44] This suggests that the reduced spectrum of some narrow spectrum natural products might not be an intrinsic limitation of their scaffold or target but rather the result of specific resistance mechanisms of the sort that medicinal chemists have overcome via derivatization. We have termed these natural products "latent" antibiotics. Previously, we showed that the arylomycins have a broader spectrum of activity than previously appreciated and that both innate and acquired resistance is commonly caused by the presence of a specific Pro residue that reduces the affinity of inhibitor binding.[21] This specific mechanism of resistance suggests that the arylomycins may be latent antibiotics, and thus that their spectrum of activity might be optimized by derivatization, and also focused our efforts to optimize them on increasing the affinity with which they bind SPase.

We began testing the hypothesis that the arylomycins are latent antibiotics by exploring tail modifications, as interactions with the lipopeptide tail are disrupted by the resistance-conferring Pro. We first focused attention on the lipid portion of the lipopeptide tail which likely embeds into the cytoplasmic membrane and increases the effective concentration of the inhibitor in the vicinity of SPase, as has been suggested with other antibiotics that possess lipid tails and inhibit membrane-bound targets.[45−55] However, how far submerged the arylomycin lipopeptide is within the membrane is not known, nor is the optimal length of the fatty acid alkyl chain that may be inserted into the membranes of Gram-positive or Gram-negative bacteria. Our data suggest that the lipopeptide tail enters the hydrophobic environment of the membrane, or the membrane-SPase interface, C-terminal to the fatty acid carbonyl, that among the saturated fatty acid derivatives the C_{16} analogue is optimal for activity, and that the inhibition of *P. aeruginosa* generally requires slightly longer fatty acid tails.

The phenyl- and biphenyl-fatty acid tail series showed similar activities against *S. epidermidis*, *S. aureus*, and *E. coli*, with the longer *p*-alkyl derivatives having activity against both sensitive and resistant strains of *S. aureus*. Interestingly, *P. aeruginosa* again shows unique behavior, as it is not inhibited by any of the biphenyl-modified derivatives. This is particularly noteworthy considering that it is inhibited by the C_8- and C_{10}-substituted phenyl analogues, which in some cases are less hydrophobic. While some of the differences may result from altered outer membrane penetration or in vivo stability, the data likely reflect suboptimal insertion of the arylomycin into the plasma membrane of *P. aeruginosa*. It is interesting to speculate that this might result from unique aspects of the phospholipids that comprise the plasma membrane of *P. aeruginosa*, such as the presence of phosphatidylcholine,[56−60] or from different constituent fatty acids.[61−64] For example, *P. aeruginosa* appears to employ a higher percentage of cis-vaccenic acid (a C_{18} fatty acid) relative to palmitic and palmitoleic acids (which are C_{16} fatty acids),[61−64] possibly resulting in a slightly thicker plasma membrane and possibly accounting for the generally longer fatty acid tail lengths that were observed to be required for activity against *P. aeruginosa*. Overall, the data collected with the different tail derivatives suggest that the phenyl-modified derivatives are likely better scaffolds for arylomycin optimization than the natural, saturated fatty acid chains. This is most clearly highlighted by compound **15**, which retains all of the activities of the parent compound arylomycin C_{16} but also gains activity against *S. aureus*.

N-Methylation is common with nonribosomally synthesized peptides such as the arylomycins and is generally thought to optimize hydrophobicity, H-bonding potential, conformation, and/or resistance to proteases.[65−68] The peptide portion of the arylomycin lipopeptide tail is backbone methylated at D-MeSer2 and MeHpg5 but not at D-Ala3 or Gly4. Previously, we showed that the methyl group at MeHpg5 preorganizes the biaryl ring system for recognition of SPase.[20] When we altered the backbone methylation state of D-MeSer2 and D-Ala3, which are both proximal to the critical resistance-conferring Pro in the *E. coli* SPase-arylomycin A_2 complex,[23] activity was lost against both Gram-positive and Gram-negative bacteria. The slight decrease in activity that results from removal of the D-MeSer2 *N*-methyl group is unlikely to result from specific deleterious interactions with the lipid membrane, because of membrane fluidity, or from any interactions with SPase, as the structure of the *E. coli* SPase−arylomycin A_2 complex suggests that this region of the lipopeptide tail is either disordered or oriented away from the protein (although as discussed above, the N-terminally truncated form of SPase used in the structural studies renders this conclusion somewhat speculative).[23] Thus, the observed decrease in activity is likely the result of decreased hydrophobicity, outer membrane penetration, or protease resistance. The more pronounced loss in activity observed upon methylation of D-Ala3 likely results from replacement of a stabilizing H-bond with a destabilizing steric clash. Whatever the specific origins of the decreased activity at the two sites examined, the data suggest that natural lipopeptide tail methylation pattern is already optimized for activity.

In an effort to more directly compensate for the negative interactions introduced by the resistance-conferring Pro, we synthesized several derivatives with increased or decreased flexibility around D-MeSer2 and D-Ala3. While none of these derivatives gained activity against the wild type Gram-negative bacteria or against *S. epidermidis*, **22** and especially **23** gain significant activity against wild type *S. aureus*. While the precise mechanism by which these derivatives gain activity against *S. aureus* remains to be determined, the data nonetheless support the possibility that the spectrum of the arylomycins may be optimized by derivatization.

CONCLUSION

From a practical perspective, the data reveal that both the methylation state and the length of the straight chain fatty acid of the lipopeptide tail of the natural arylomycins are already optimized for activity but that the unnatural phenyl analogues are more promising scaffolds. From a conceptual perspective, the identification of derivatives with an expanded spectrum against

wild type *S. aureus* strains, most notably **12** and **23**, supports the hypothesis that arylomycins are latent antibiotics and focuses attention on the types of lipopeptide tail modifications that may be most likely to optimize their spectrum. Finally, this study identified derivatives that provide an improved scaffold for further optimization efforts focused on other parts of the molecule, for example, the macrocyclic ring core that interacts with parts of SPase that are proximal to the S1 and S3 binding pockets,[13,22,24] which are more traditional targets for peptidase inhibitor optimization. Such efforts are currently underway.

EXPERIMENTAL SECTION

General Methods. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX 400, Bruker DRX 500, or Bruker DRX 600 spectrometers. Chemical shifts are reported as δ values (parts per million, ppm) relative to chloroform (δ 7.26), methanol (δ 3.31), or dimethylsulfoxide (DMSO) (δ 2.50) for ^{1}H NMR and to chloroform (δ 77.16), methanol (δ 49.00), or DMSO (δ 39.52) for ^{13}C NMR. High-resolution time-of-flight mass spectra (HRMS) were measured at the Scripps Center for Mass Spectrometry. ESI mass spectra were measured on either an HP series 1100 MSD (accuracy of 0.1 amu) or a PESCIEX API/Plus (accuracy of 0.5 amu) instrument. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated, with the purity of all compounds determined to be >95% by HPLC. Mixtures were magnetically stirred, and reactions were monitored by thin layer chromatography (TLC) with 0.25 mm Whatman precoated silica gel (with fluorescence indicator) plates. Flash chromatography was performed with silica gel (particle size 40−63 μm, EMD Chemicals). Dry solvents were purchased from Acros. Anhydrous 1-hydroxybenzotriazole (HOBt) was purchased from Chem-Impex. All other chemicals were purchased from Fisher/Acros or Aldrich.

All preparative reverse phase chromatography was performed using two Dynamax SD-200 pumps connected to a Dynamax UV-D II detector (monitoring at 220 nm) on a Phenomenex Jupiter C_{18} column (10 μm, 2.12 cm × 25 cm, 300 Å pore size). All solvents contained 0.1% TFA: solvent A, H_2O; solvent B, 90% acetonitrile/10% H_2O. All samples were loaded onto the column at 0% B, and the column was allowed to equilibrate for ~10 min before a linear gradient was started. Retention times are reported according to the linear gradient used and the % B at the time the sample eluted.

Compound **19** was synthesized racemically, and the two diastereomers were separated and subjected to biological assay independently. The stereochemistry of each of the compounds was unknown, but the MICs for the most active compound are reported and discussed in the main text. Compounds **20** and **21** were also synthesized racemically; however, the diastereomers were not separated and the compounds were assayed as racemic mixtures.

Biological Methods. Minimum inhibitory concentrations (MICs) were performed in at least triplicate using a modified CLSI broth microdilution method in cation adjusted Mueller−Hinton broth for all strains. Briefly, by use of cultures inoculated from a fresh overnight plate scrape, 96-well flat bottom plates containing media and compounds at the appropriate concentrations were inoculated to a final density of 5×10^5 cfu/mL. Plates were sealed with oxygen permeable membranes and incubated for 24 h at 37 °C. Following incubation, seals were removed and optical density at 590 nm was measured using a Perkin-Elmer Envision 2103 multilabel reader. MICs were then confirmed by measuring viable cell count by serial dilution in phosphate buffered saline and plating on Mueller−Hinton II agar (BD Diagnostics). The MIC values given are the median of at least three independent replicates with no more than 2-fold deviation.

General Procedure A: Macrocycle and Tail Coupling. Example: Arylomycin C_{16}. The glycine homologated macrocycle (Scheme 1)[20] (80 mg, 0.16 mmol) was taken up in CH_3CN (7.2 mL) and DMF (3.2 mL) and treated sequentially with HOBt (64 mg, 3 equiv), the lipopeptide tail (Scheme 1)[20] (81.3 mg, 1 equiv), and EDC (90.3 mg, 3 equiv). The mixture was allowed to stir overnight, after which water, saturated $NaHCO_3$, and EtOAc were added, the aqueous phase was extracted 3× with EtOAc, and the combined organic layers were washed with 5% citric acid (pH 3) and brine. The organics were dried over sodium sulfate and concentrated. The crude material was purified by column chromatography (5.5% MeOH in DCM) to give the protected arylomycin C_{16} (72.4 mg, 45% yield).

General Procedure B: Global Deprotection. Example: Arylomycin C_{16}. The fully protected arylomycin[20] (72.4 mg, 72 μmol, 1 equiv) was dissolved in ethanethiol (2 mL) under Ar and treated with 1.0 M $AlBr_3$ in CH_2Br_2 (1.79 mL, 25 equiv). The reaction vial was sealed and heated to 50 °C and stirred for 4 h. The mixture was cooled to room temperature, MeOH was added (0.5 mL), and the volatiles were evaporated under a stream of nitrogen. MeOH was added again and was evaporated under a stream of nitrogen, and the crude product was dried under vacuum. The crude product was then dissolved in MeOH and purified by HPLC (linear gradient, 0.67% B/min, product eluted at 80% B) to give arylomycin C_{16} (32.6 mg, 51% yield). For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{46}H_{69}N_6O_{11}$ $[(M + H)^+]$, 881.5019; found, 881.5021.

General Procedure C: Macrocycle and Tail Coupling. Example: Compound 5. The procedure is based on the conditions reported previously.[69] The lipid tripeptide tail (23.5 mg, 52 μmol, 1 equiv) and the tripeptide macrocycle (70 mg, 2.2 equiv) were dissolved in THF (2 mL) under Ar and treated with TEA (7 μL, 1 equiv) and DEPBT (39 mg, 2.5 equiv). The mixture was allowed to stir overnight. Then the volatiles were evaporated under a stream of nitrogen, the residue was dried under vacuum, and EtOAc and saturated $NaHCO_3$ were added. The aqueous layer was extracted. Then the organic layer was washed with 0.1 N HCl, dried over sodium sulfate, and concentrated.

Compound **1** was synthesized using general procedures A and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{46}H_{70}N_6O_{10}$ $[(M + H)^+]$, 867.5226; found, 867.5207.

Compound **2** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{38}H_{53}N_6O_{11}$ $[(M + H)^+]$, 769.3767; found, 769.3770.

Compound **3** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{40}H_{57}N_6O_{11}$ $[(M + H)^+]$, 797.408; found, 797.4070.

Compound **4** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{42}H_{61}N_6O_{11}$ $[(M + H)^+]$, 825.4393; found, 825.4386.

Compound **5** was synthesized as described in general procedure C and then subjected to general procedure B to give the product (20.6 mg, 58% yield). For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{48}H_{72}N_6O_{11}$ $[(M + H)^+]$, 909.5332; found, 909.5328.

Compound **6** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{43}H_{54}N_6O_{11}$ $[(M + H)^+]$, 811.3297; found, 811.3300.

Compound **7** was synthesized using general procedures A and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{44}H_{48}N_6O_{11}$ $[(M + H)^+]$, 837.3454; found, 837.3443.

Compound **8** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{43}H_{46}N_6O_{11}$ $[(M + H)^+]$, 823.3297; found, 823.3296.

Compound **9** was synthesized using general procedures A and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{45}H_{50}N_6O_{11}$ $[(M + H)^+]$, 851.361; found, 851.359.

Compound **10** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{47}H_{54}N_6O_{11}$ $[(M + H)^+]$, 879.3923; found, 879.3924.

Compound **11** was synthesized using general procedures A and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{49}H_{58}N_6O_{11}$ $[(M + H)^+]$, 907.4236; found, 907.4246.

Compound **12** was synthesized using general procedures A and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{51}H_{62}N_6O_{11}$ [$(M + H)^+$], 935.4549; found, 935.4548.

Compound **13** was synthesized using general procedures A and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{43}H_{54}N_6O_{11}$ [$(M + H)^+$], 831.3923; found, 831.3917.

Compound **14** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{45}H_{58}N_6O_{11}$ [$(M + H)^+$], 859.4236; found, 859.4231.

Compound **15** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{47}H_{62}N_6O_{11}$ [$(M + H)^+$], 887.4549; found, 887.4539.

Compound **16** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{45}H_{66}N_6O_{11}$ [$(M + H)^+$], 867.4862; found, 867.4873.

Compound **17** was synthesized using general procedures A and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{47}H_{70}N_6O_{10}$ [$(M + H)^+$], 895.5175; found, 895.5190.

Compound **18** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{47}H_{68}N_6O_{11}$ [$(M + H)^+$], 893.5019; found, 893.5014.

Diastereomer A of compound **19** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{43}H_{63}N_5O_9$ [$(M + H)^+$], 794.4698; found, 794.4705. Diastereomer B of compound **19** was synthesized using general procedures A and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{43}H_{63}N_5O_9$ [$(M + H)^+$], 794.4698; found, 794.4689.

Compound **20** was synthesized racemically using general procedures C and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{47}H_{70}N_6O_{11}$ [$(M + H)^+$], 895.5175; found, 895.5180.

Compound **21** was synthesized racemically using general procedures C and B. For ^{1}H NMR see Supporting Information. ESI HRMS calcd for $C_{48}H_{72}N_6O_{11}$ [$(M + H)^+$], 909.5332; found, 909.5334.

Compound **22** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{47}H_{70}N_6O_{11}$ [$(M + H)^+$], 895.5175; found, 895.5178.

Compound **23** was synthesized using general procedures A and B. For ^{1}H NMR and ^{13}C NMR see Supporting Information. ESI HRMS calcd for $C_{48}H_{72}N_6O_{11}$ [$(M + H)^+$], 909.5332; found, 909.5305.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures and ^{1}H, ^{13}C, MS, and HRMS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

SPase, signal peptidase I; MIC, minimal inhibitory concentration; THF, tetrahydrofuran; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; EtOAc, ethyl acetate; Hex, hexanes; Ar, argon; TFA, trifluoroacetic acid

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