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In vitro activity of the arylomycin natural product antibiotics against *Staphylococcus epidermidis* and other coagulase-negative staphylococci

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Short Title: Anti-CoNS activity of the arylomycins

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Abstract

The arylomycins are a class of natural product antibiotics that act via the inhibition of type I signal peptidase (SPase), and we have found in diverse bacteria that their activity is limited by the presence of a resistance-conferring Pro residue in SPase that reduces inhibitor binding. We have also demonstrated that *Staphylococcus epidermidis*, which lacks this Pro residue, is extremely susceptible to the arylomycins. Here, to further explore the potential utility of the arylomycins, we report an analysis of the activity of a synthetic arylomycin derivative, arylomycin C_{16}, against clinical isolates of *S. epidermidis* and other coagulase-negative staphylococci (CoNS) from distinct geographical locations. Against many important species of CoNS, including *S. epidermidis*, S. *haemolyticus*, *S. lugdunensis*, and *S. hominis*, we find that arylomycin C_{16} exhibits activity equal to or greater than that of vancomycin, the antibiotic most commonly used to treat CoNS infections. While the susceptibility was generally correlated with the absence of the previously identified Pro residue, several cases were identified where additional factors appear to also contribute.

Introduction

The coagulase-negative staphylococci (CoNS) are a heterogeneous group of at least 15 different species of Gram-positive bacteria that have emerged in recent decades as important nosocomial pathogens (10, 31). A particularly problematic species is *Staphylococcus epidermidis*, which is responsible for a growing number of infections among hospital patients with compromised immune systems and is especially notorious for forming biofilms that adhere to surgical equipment and other hospital surfaces and indwelling devices (3, 18). Methicillin was traditionally the first line antibiotic against CoNS, but its widespread use has resulted in resistance in 50% to 80% of CoNS infections and 75 to 90% of nosocomial *S. epidermidis* infections (18). As a result, vancomycin is now the first line agent for treating CoNS infections; however isolates with reduced susceptibility to vancomycin have also been observed (11, 26), and the emergence of enterococci harboring mobile elements that confer vancomycin resistance has raised concerns that resistance might be transferred to *S. epidermidis* and/or other CoNS (17, 28). These concerns continue to motivate the search for new antibiotics that are active against CoNS, and especially against *S. epidermidis*.

The arylomycins (Fig. 1) are a novel class of natural product antibiotics that act by inhibiting bacterial type I signal peptidase (SPase) (19, 25). SPase is a Ser-Lys dyad protease that removes N-terminal signal sequences from pre-proteins following their translocation across the cytoplasmic membrane (5, 20). SPase is an attractive target for antibiotic therapy because it is conserved, essential, and located in the relatively accessible outer leaflet of the cytoplasmic membrane. Furthermore, because bacterial SPase acts via a catalytic mechanism that is distinct from its eukaryotic homologues, the arylomycins are unlikely to exhibit mechanistic toxicity in humans (5, 20).

Despite the apparent accessibility, essentiality, and conservation of SPase, initial reports suggested that the arylomycins were active against only a few Gram-positive bacteria, including *Streptococcus pneumoniae*, *Rhodococcus opacus*, and *Brevibacillus brevis* (15, 25), and not against other important Gram-positive pathogens or against any Gram-negative bacteria. However, after reporting the first synthesis of an arylomycin, arylomycin A_2, as well as the synthetic derivative arylomycin C_{16} (Fig. 1), we found that each potently inhibits the growth of *S. epidermidis* (24), and that *S. epidermidis* evolves resistance to the arylomycins by mutating residue 29 of one of its two SPases, SpsIB, from Ser (Ser^{29}) to Pro (Pro^{29}) (29). Moreover, a Pro residue is naturally present at the analogous position in the homologous SPases of the pathogens *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa,* and we showed that it imparts resistance by reducing the affinity with which the arylomycins bind. Furthermore, we found that a remarkably diverse range of both Gram-positive and Gram-negative bacteria whose SPases lack a Pro at the analogous position are susceptible to the arylomycins, including *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Helicobacter pylori*, and *Chlamydia trachomatis,* and some strains of *Francisella tularensis* (29). In total, the results suggest that the identified SPase polymorphism is a major contributor to naturally occurring arylomycin resistance. However, we also showed that *Yersinia pestis* as well as some strains of *S. aureus* are susceptible to the arylomycins despite the presence of an analogous Pro, while others, such as many of the Lactobacillales, Clostridia and Bacteriodetes, are resistant, despite its absence, implying that in some cases susceptibility must depend on additional factors such as variable levels of toxicity associated with the inhibition of protein secretion.

The potent activity of the arylomycins against a strain of *S. epidermidis* (RP62A) suggests that they might be useful in the treatment of this and perhaps other CoNS. Here, to examine the spectrum of activity of the arylomycins against clinical isolates of *S. epidermidis* and other

CoNS, we report the activity of arylomycin C_{16} against two panels of isolates from hospitals in geographically diverse locations and compare the activity to that of vancomycin. The results reveal that the arylomcyins have potent antibacterial activity against a range of important CoNS species whose SpsIB orthologs lack the previously identified resistance-conferring Pro, while less activity is observed against species where Pro is present. While we generally observed similar susceptibilities for different isolates within a species, significant differences were observed in several cases, with one atypical susceptibility resulting from the presence of a Ser in place of the resistance-conferring Pro. Significant differences in susceptibility between isolates of the same species are usually observed with clinically deployed antibiotics where selection for resistance has occurred during therapy (2, 9, 14, 21), and therefore these results may be relevant to understanding the natural evolution of arylomycin resistance in nature.

Materials and Methods

A total of 282 non-duplicate, speciated clinical isolates of CoNS were obtained from the London Health Services Centre, London, Ontario, Canada (13). Of these, 143 isolates were *S. epidermidis*, while the remaining 139 consisted of 12 groups comprised of varying numbers of isolates of *Staphylococcus capitis, Staphylococcus caprae, Staphylococcus cohnii, Staphylococcus haemolyticus, Staphylococcus lugdunensis, Staphylococcus saprophyticus, Staphylococcus simulans, Staphylococcus warneri*, and *Staphylococcus hominis.* This panel of CoNS is a subset of a larger panel whose susceptibility to quinupristin/dalfopristin, linezolid, telithromycin, and vancomycin has been reported (13). A second set of non-duplicate, non-speciated clinical isolates of CoNS was obtained from the Department of Microbiology, University of California San Diego Medical Center Hillcrest, San Diego, CA. These strains were

collected from patients with various coagulase negative infections between April and June of 2008.

Arylomycin C_{16} was synthesized as described previously (24). MICs of arylomycin C_{16} were determined for isolates from both sets of CoNS using a modified CLSI agar dilution method. Bacterial inocula were prepared from log phase cultures or from suspensions of colonies grown on solid media, diluted appropriately, and spotted onto tryptic soy agar at a concentration of 1×10^4 cfu/spot. Spots were then incubated for 24 h at 37 ℃, and the MIC was defined as the concentration at which there was no visible growth. MICs of vancomycin were also determined in an identical manner to provide a reference activity. Several of the isolates demonstrated heterogeneous growth on solid media, and in these cases individual colonies of each morphotype were analyzed separately and speciated based on sequencing and phylogenetic analysis of a portion of the *dnaJ* gene, which has previously been demonstrated to serve as a sensitive measure of Staphylococcal speciation (Fig. S1) (27). Speciation was also confirmed by *dnaJ* sequence analysis for isolates whose arylomycin MICs deviated from the MIC_{50} by greater than 4–fold (Fig. S1). Additionally, the *spsIB* genes from these isolates were sequenced to determine whether any polymorphisms were present that could account for the observed variation in susceptibility. Primers for the PCR amplification of *spsIB* genes were designed based on regions of highly conserved sequence within the upstream ORF SERP0551 (*S. epidermidis* RP62A numbering) and the downstream *rexB* gene (Fig. S2). Degeneracies were included at positions that varied within the sequenced strains of the CoNS in order to maximize the likelihood of annealing (Table S1).

To determine with high confidence the history of speciation within the CoNS examined in this study, a phylogenetic analysis was performed using regions of four essential genes that have previously been validated as speciation markers (16S rDNA, *rpoB*, *groEL*, and *dnaJ*) (Table S2) (1, 6, 7, 27). For each protein coding gene, published DNA sequences from

129 speciated strains were translated into protein sequence, and aligned using MUSCLE (8). The
130 DNA sequences were then mapped onto the amino acid alignment using the program TranAlign
131 (23). Aligned 16S rDNA sequences were obtained from the Ribosomal Database Project (4).
132 For each organism, the four aligned sequences were concatenated, and phylogenetic analysis
133 was conducted by the method of Maximum Likelihood using PhyML 3.0 (12), with the HKY85
134 nucleotide substitution model, 20 rate categories, and SPR branch improvement. The
135 phylogeny of SPase genes was determined similarly using sequences deposited in the NCBI
136 database as well as sequences obtained in the present study. Trees were displayed using
137 MEGA4 (30).
138
139
140 **Results**
141
142 A total of 143 isolates of *S. epidermidis* from the London Health Services Center were analyzed.
143 The MIC_{50} and MIC_{90} of arylomycin C_{16} against these 143 *S. epidermidis* isolates were found to
144 be 0.5 µg/ml and 1 µg/ml, respectively (Table 1). These susceptibilities are similar to that
145 reported previously for the *S. epidermidis* laboratory strain RP62A (MIC = 0.5 µg/ml) (24), and
146 compare favorably to the MIC_{50} and MIC_{90} of vancomycin, both of which we found to be 2 µg/ml,
147 in agreement with values reported previously (13). Additionally the range of MICs observed
148 among the 143 isolates was narrow, with the most resistant isolate having an arylomycin C_{16}
149 MIC of 4 µg/ml.
150 The remaining 9 species of CoNS examined displayed arylomycin C_{16} susceptibilities
151 ranging from highly susceptible to resistant (Table 1). Specifically, we found that *S.*
152 *haemolyticus, S. lugdunensis*, and *S. hominis* are highly susceptible to arylomycin C_{16}, with
153 MIC_{50} and MIC_{90} values of 0.25 to 2 µg/ml. In contrast, we found that *S. capitis*, *S. caprae*, and

S. *cohnii* are only moderately susceptible, with MIC_{50} values between 8 and 16 µg/ml and MIC_{90} values between 16 and 32 µg/ml, and that *S. warneri* and *S. saprophyticus* are resistant, with MIC_{50} values equal to or greater than 64 µg/ml and MIC_{90} values of greater than 64 µg/ml. Finally, while many *S. simulans* isolates were sensitive to the arylomycins (MIC_{50} of 2 µg/ml), a significant number of isolates were highly resistant, resulting in a MIC_{90} of >64 µg/ml.

Under laboratory conditions, *S. epidermidis* RP62A evolves arylomycin resistance by mutation of Ser^{29} to Pro^{29} in SpsIB, one of two active SPases encoded by this organism, and in the related organism *S. aureus*, as well as in the more distantly related *E. coli* and *P. aeruginosa,* an analogous Pro^{29} is naturally present and contributes significantly to arylomycin resistance (29) (here and throughout the remainder of this manuscript, unless otherwise indicated, Ser^{29} and Pro^{29} refer to the residue in SpsIB orthologs at the position corresponding to 29 in *S. epidermidis*). In contrast, we did not observe mutations in the second *S. epidermidis* SPase gene, *spsI*, in any of the resistant isolates. To determine whether similar mutations might be responsible for the differences in arylomycin susceptibility observed among the different CoNS, we examined the sequences of their *spsI* and *spsIB* genes (Fig. 2 and Table S3). To identify SPase sequences, we used the NCBI BLAST interface to search the sequenced genomes of *S. capitis, S. haemolyticus, S. hominis, S. lugdunensis, S. saprophyticus,* and *S. warneri*, and found that each except *S. saprophyticus* possess both SpsI and SpsIB homologs. We also included the recently reported sequence of the *spsIB* gene from *Staphyloccus xylosus* (22), which does not encode an SpsI SPase (S. Leroy, personal communication). Because no correlation between susceptibility and the sequence of *spsI* was observed, and because *S. epidermidis* evolves resistance by mutation of *spsIB*, we PCR amplified and sequenced the *spsIB* orthologs from *S. caprae*, *S. cohnii*, and *S. simulans*, whose genomes have not been fully sequenced (see Supporting Information). Overall, the data support the model that Pro^{29} contributes significantly to arylomycin resistance within CoNS. *S.*

179 *lugdunensis*, *S. haemolyticus*, *S. hominis,* and a significant percentage of *S. simulans* isolates
180 are extremely susceptible and these species lack Pro^{29}. In contrast, the CoNS species most
181 resistant to the arylomycins, *S. warneri* and *S. saprophyticus*, each harbor a SPase with Pro^{29}.
182 The correlation between Pro^{29} and arylomycin susceptibility is less clear for species that display
183 intermediate susceptibilities; for example, while *S. caprae* and *S. capitis* have Pro^{29}, and *S.*
184 *cohnii* has Ser^{29}, all three display MIC_{50} values between 4 and 16 µg/ml.

185 Most of the species examined have the narrow range of susceptibilities to arylomycin C_{16}
186 expected for an antibiotic that has not been used clinically (2, 9, 14, 21) (Table 1). However, for
187 several species, we found a greater range in susceptibilities, or we found outliers with
188 susceptibilities that differed significantly from those of the other isolates (Table 1 and Fig. S3).
189 For example, one isolate of *S. lugdunensis* is significantly more susceptible than the species
190 average (MIC 8–fold below the MIC_{50}), while a second isolate is unusually resistant (MIC 128–
191 fold above the MIC_{50}). Sequencing of the *spsIB* genes revealed identical sequence at the
192 amino acid level (including at residue 29) (Fig. S4). In addition, a bimodal distribution of MICs
193 was observed for isolates of *S. warneri* and *S. simulans* with peaks at 16 µg/ml and >64 µg/ml
194 and at 2 µg/ml and >64 µg/ml, respectively (Fig. S3). Sequencing the *spsIB* genes from
195 representative isolates of each group, revealed nearly identical SPase sequences and no
196 variation at residue 29 (Fig. S4). Finally, we identified a particularly susceptible isolate of *S.*
197 *warneri* with an MIC that is 32–fold below the species MIC_{50}. Interestingly, the particularly
198 susceptible strain of *S. warneri* encoded Ser^{29} as opposed to Pro^{29}, which is typically present in
199 *S. warneri* isolates, further supporting the genotype/phenotype correlation.

200 The observed differences in MICs that cannot be accounted for by differences in SpsIB
201 sequence, both within and between species, suggest that additional factors contribute to
202 arylomycin susceptibility. To determine whether these additional factors are shared among
203 related organisms, we examined MICs as a function of phylogeny (Fig. 2). The phylogenetic

analysis based on four highly conserved genes (see Materials and Methods) suggests that *S. simulans* was the earliest species to diverge from the common ancestor of the examined CoNS, with the other species forming two groups, one comprised of *S. xylosus*, *S. saprophyticus*, and *S. cohnii*, and the second comprised of the remaining species. Notably, SpsI SPases have only been shown to be present in the second group, although their presence cannot be ruled out in the unsequenced species.

S. saprophyticus, which has Pro^{29} and is extremely resistant to the arylomycins (MIC_{50} > 64 μg/ml), is most closely related to *S. cohnii,* which lacks Pro^{29}, and yet is only moderately susceptible to the arylomycins (MIC_{50} = 8 μg/ml). To test whether other species that are closely related to *S. saprophyticus* and *S. cohnii* share this intrinsically lower susceptibility, we determined the MIC of a typed strain of *S. xylosus*, which is closely related to *S. saprophyticus*, but like *S. cohnii* lacks Pro^{29}. As expected, the strain of S. *xylosus* is moderately susceptible to the arylomycin (MIC = 4 μg/ml). Thus, this group of related bacteria appears to have a lower basal level of arylomycin susceptibility. In contrast, *S. epidermidis*, *S. hominis*, *S. lugdunensis*, and *S. haemolyticus*, which lack Pro^{29} and display extreme arylomycin susceptibility (MIC_{50} 0.25 – 2 μg/ml), as well as *S. capitis* and *S. caprae* which have Pro^{29} but remain moderately susceptible (MIC_{50} 8 – 16 μg/ml), are more related to one another than to the other CoNS species examined. Despite the fact that some *S. warneri* isolates are extremely resistant, the remaining isolates have MICs of ~16μg/mL, which is consistent with the level of resistance observed for the other CoNS with Pro^{29} within the more susceptible phylogenetic group.

Finally, we evaluated the activity of arylomycin C_{16} against strains from an unspeciated CoNS panel from University of California San Diego Medical Center, and observed MIC_{50} and MIC_{90} values of 2 μg/ml and >32 μg/ml, respectively (Fig. 3). In addition to revealing a portion of resistant isolates, the distribution of MICs shows significant variations in the susceptibility of the remaining strains. Although interpretation of these results is complicated by the lack of

speciation, the MIC distribution is consistent with a model in which both the presence of Pro^{29} and a second yet to be determined factor, combine to yield tiers of arylomycin susceptibility.

Discussion

Members of the arylomycin class of natural product antibiotics act via a novel mechanism of action, the inhibition of SPase, and thus, the inhibition of the essential process of protein secretion. We found that in addition to excellent activity against *S. epidermidis* (24), arylomycin C_{16} has potent activity against *S. haemolyticus*, *S. lugdunensis*, and *S. hominis*. Each of these CoNS encodes a SPase with Ser^{29}, consistent with the central role of this residue in determining arylomycin susceptibility that we observed previously with other bacteria (29).

The remaining species examined range from only moderately susceptible to extremely resistant, and Pro^{29} clearly makes a significant contribution to resistance in many of these cases. Interestingly, phylogenetic analysis suggests that Ser^{29} was prevalent during speciation, but that Pro^{29} evolved in at least two independent instances, once in *S. saprophyticus* and once in the common ancestor of *S. caprae* and *S. capitis* (Fig. 2). Pro^{29} in *S. warneri* may represent a third instance, although it might instead result from common ancestry with *S. caprae* and *S. capitis*, and this distinction is beyond the resolution of the current analysis. Interestingly, many synonymous and non-synonomous mutations differentiate the *spsIB* genes of the different staphylococcal species, suggesting that ample mutational diversity has been sampled, but only Ser and Pro appear to be tolerated at position 29. Importantly, the multiple, independent instances where Pro^{29} was introduced during staphylococci speciation suggests that there may be a natural selective pressure for Pro^{29}, and the variation within isolates of an extant species suggests that this pressure may have occurred recently and may even still be present. While

253 the nature of the selection pressure is currently unknown, it is tempting to consider that it may
254 be related to the presence of arylomycins in nature (29).

255 The observed differences in susceptibilities between some of the staphylococcal species
256 reveal that, in addition to SpsIB residue 29, there must be other factors that also contribute to
257 overall susceptibility, and these other factors generally appear to be shared among closely
258 related species, resulting in groups of species having higher or lower basal susceptibilities.
259 One possibility is that the presence of a second SPase gene, orthologs of *S. epidermidis spsI*, in
260 some species of CoNS could contribute to their different arylomycin susceptibilities. In principle,
261 expressing two arylomycin sensitive SPase proteins could result in hypersusceptibility if each
262 SPase recognized a different subset of essential pre-protein substrates. Alternatively,
263 expressing one sensitive and one resistant SPase could result in either a susceptible or
264 resistant phenotype, depending on the exact mechanism of arylomycin induced cell death,
265 which is yet to be determined. Interestingly, all of the species with higher basal susceptibility
266 have two SPases while, *S. xylosus* (S. Leroy, personal communication) and *S. saprophyticus*
267 have only one SPase and display lower basal susceptibility. Consistent with a possible role for
268 a second SPase contributing to arylomycin susceptibility, *S. aureus* strain 8325 is highly
269 resistant to arylomycin (MIC >128 µg/ml (24)) despite being closely related to the more
270 susceptible group of CoNS, and this species has only a single SPase, apparently due to a
271 recent deletion of its *spsI* homologue. Further experiments will be required to elucidate the
272 potential role of multiple SPases in CoNS susceptibilities, and/or to identify other factors
273 contributing to susceptibility such as differences in the composition of secreted proteins or the
274 presence of modifying enzymes. Comparison of resistant and susceptible isolates of the same
275 species may provide a valuable approach to identifying additional factors that contribute to
276 arylomycin susceptibility.

Regardless of the origins of arylomycin susceptibility and resistance, it is clear that a broad and clinically important (16) range of CoNS strains are susceptible to the arylomycins. In fact, in these cases the level of susceptibility compares favorably with the level of susceptibility to vancomycin, the antibiotic currently recommended for treatment of CoNS infections but for which resistance is a major concern. The availability of a novel antibiotic, with a novel mechanism of action, would significantly improve CoNS therapy. Along with previously reported data (29), the data also suggest that if the arylomycin class of natural products could be optimized to overcome the loss in affinity mediated by the presence of the resistance conferring Pro^{29}, then they would have broad activity against CoNS, and possibly other bacteria as well.

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386 **Figure Captions**

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388 FIG. 1. Structures of arylomycin A_2 (R = *iso*-C_{12}; $CH_2(CH_2)_7CH(CH_3)_2$) and arylomycin C_{16} (R =
389 *iso*-C_{16}; $CH_2(CH_2)_{11}CH(CH_3)_2$).

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391 FIG. 2. Phylogenetic relationship, SPase sequence, and arylomycin susceptibility of CoNS
392 species. *Macrococcus caseolyticus* was used as an outgroup to root the tree, and confidence
393 values were determined based on the alternative likelihood ratio test. For each species, the
394 amino acids in SpsIB and in SpsI at the positions corresponding to residue 29 in *S. epidermidis*
395 SpsIB are indicated. MIC values reflect the MIC_{50} except for *S. simulans* and *S. warneri* where
396 the values listed correspond to peaks in a bimodal distribution of MICs. [a]Presence and/or
397 sequence of SpsI homologue is unknown. [b]Species does not have an SpsI homologue.

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399 FIG. 3. Distribution of arylomycin susceptibilities of unspeciated CoNS isolates obtained from
400 the University of California, San Diego Medical Center.

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TABLE 1. MICs (µg/mL) of speciated coagulase-negative strains from the London Health Services Centre to arylomycin C_{16} and vancomycin[1]

Species	Number of isolates	Arylomycin C_{16}			Vancomycin		
		MIC_{50}	MIC_{90}	MIC Range	MIC_{50}	MIC_{90}	MIC Range
S. epidermidis	143	0.5	1	0.12 – 4	2	2	1 – 2
S. lugdunensis	10	0.25	0.25	0.03 – 32	2	2	2
S. hominis	17	0.25	0.5	0.06 – 0.5	2	4	1 – 4
S. haemolyticus	10	2	2	1 – 2	2	2	2 – 4
S. simulans	12	2	>64	2 – >64	1	2	1 – 2
S. caprae	10	8	16	4 – 16	2	2	1 – 2
S. cohnii	27	8	16	4 – 16	2	2	1 – 2
S. capitis	24	16	32	4 – 64	2	2	1 – 2
S. warneri	19	64	>64	2 – >64	4	4	1 – 4
S. saprophyticus	10	>64	>64	>64	1	2	1 – 2
S. xylosus[a]	-	-	-	4	-	-	-
S. aureus[a]	-	-	-	>64[b]	-	-	-

[a]Reference strains, *S. xylosus* ATCC 29971 and *S. aureus* NCTC 8325, are included for comparison. [b] Ref. (24).

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