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Synthetic Dihydropacidamycin Antibiotics: A Modified Spectrum of Activity for the Pacidamycin Class

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Abstract—Dihydropacidamycins having an antibacterial spectrum modified from that of the natural product pacidamycins and mureidomycins have been synthesized. Synthetic dihydropacidamycins with noteworthy antibacterial activity against wild-type and resistant *Escherichia coli* have been identified (MIC = 4–8 μg/mL). Some dihydropacidamycins are shown to have activity against multi-resistant clinical strains of *Mycobacterium tuberculosis*. Compounds of this class are inhibitors of the cell wall biosynthetic enzyme, MraY.

The increasing resistance of community- and hospital-acquired infections to standard antibiotic therapies has raised the need for new anti-infective treatments.[1] Strategies to this end have included the modification of existing classes of antimicrobial agents,[2] the use of fixed combinations of an old antibiotic with a second drug to potentiate the antimicrobial compound,[3] and the development of novel classes of antimicrobial drugs with unexploited modes of action.[4]

Our efforts in this area have focused on the pacidamycin antibiotics[5] (and related mureidomycins[6] and napsamycins,[7] collectively called uridyl peptide antibiotics or UPAs) for three reasons. First the UPAs have an unexploited mode of action; UPAs inhibit the cell-wall biosynthetic enzyme MraY (transferase, translocase I).[8] Consequently ofloxacin- and β-lactam-resistant strains have been shown to remain sensitive to compounds in the UPA family.[9] Second, compounds in the UPA class have shown, in addition to favorable toxicological[10] and pharmacokinetic properties, promising pharmacodynamic qualities against the highly refractory pathogen, *Pseudomonas aeruginosa*.[5b,6c] Finally, we recently published the stereochemical elucidation, total synthesis, and biological evaluation of UPA derivatives;[11] compounds such as dihydropacidamycin D (see Fig. 1) have been shown to have antibiotic activity equal to that of pacidamycin D,[5d] and dihydropacidamycin D is accessible by total synthesis using straightforward methodology. This methodology appeared to be flexible enough, by virtue of the modular nature of the amino acid components, to provide access to a wide variety of synthetic UPA analogues. A general synthetic scheme is outlined (Scheme 1).

The UPA class suffers from two liabilities that have prohibited the introduction of a drug from this class. First, they have a limited spectrum; while they are active against *P. aeruginosa*,[9] an organism with high intrinsic resistance to all current therapies, they are not active

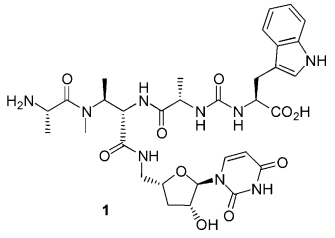

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Figure 1. Dihydropacidamycin D obtained by hydrogenation of the natural product, or by total synthesis.

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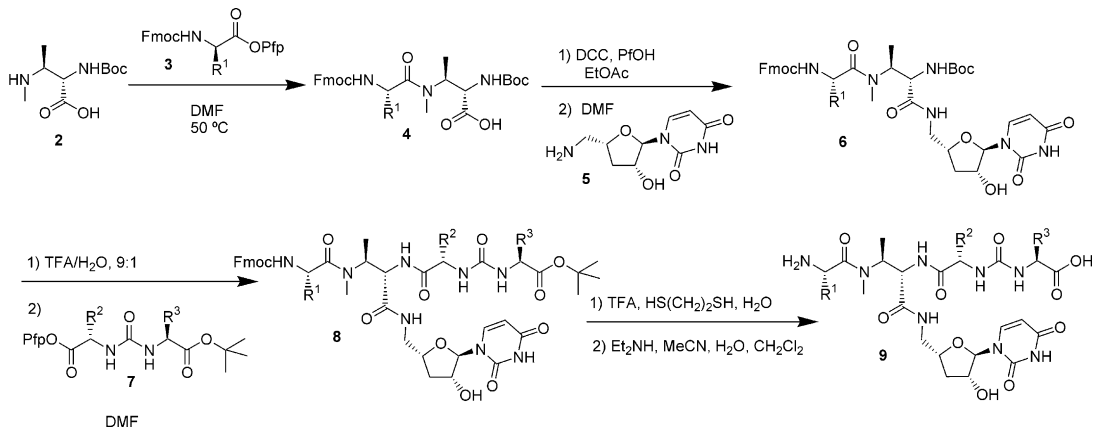


Scheme 1. Details found in ref 11.

against any other significant human pathogen. Second, *P. aeruginosa* becomes resistant to these compounds at a frequency (10^{-5}–10^{-6}) that would restrict the usefulness of a UPA-based drug.[5b,6c] We posited that these liabilities resulted from the physical properties of this class of molecules; for instance, the polarity or potential for extensive H-bonding to water may restrict passive access of compounds in the UPA class to the intracellular target in pathogenic species. Similarly, we reasoned that *P. aeruginosa*, a pathogen that has high intrinsic resistance to most antibiotics in the clinic, must be sensitive to these compounds by virtue of an active uptake system(s). Perhaps the high frequency of resistance of *P. aeruginosa* with respect to the UPAs can be explained as a result of some mutation in this uptake system(s) or change in its level of expression. We reasoned that through modulation of the physical properties of the synthetic UPAs by variation of the residues of the pseudopeptide backbone, we could either improve passive uptake, thus improving the antibacterial spectrum and reducing the frequency at which susceptible organisms become resistant, or at very least access active uptake mechanisms in additional pathogenic organisms in order to expand or modify the spectrum of the UPA class.

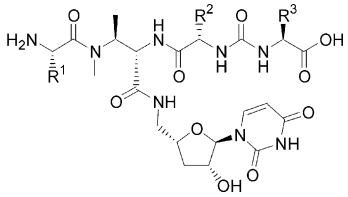
The methodology[11] reported for the synthesis of dihydropacidamycin D was applicable with little or no additional optimization to the synthesis of multiple varied derivatives of dihydropacidamycin D, and was facilitated by the commercial availability of appropriately protected amino acid derivatives. We tested the generality of the synthetic method and showed that it did indeed allow for the incorporation of multiple amino acid derivatives. Slight modification was required for the incorporation of the hydrogenation-incompatible methionine derivatives. For derivatives containing methionine at the urea portion of the UPA, benzyl ester protecting groups were replaced with allyl esters in the synthetic scheme described in ref 11; these were cleaved under catalytic conditions compatible with the thioether, n-$Bu_3SnH/Pd(PPh_3)_4$.

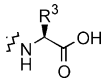
We initially synthesized approximately 50 compounds to probe the SAR against a panel of bacteria that included the gram-negative pathogens *P. aeruginosa*, *Escherichia coli*, *Pasteurella multocida*, and the gram-positive pathogen *Staphylococcus aureus*. Susceptibility tests were performed using a broth microdilution assay according to NCCLS reference method.[12]

The antimicrobial data from this exercise was used to bias our basis set amino acids used in the construction of a small set of dihydropacidamycins (200) made by solution-phase parallel synthesis. These initial 50 compounds incorporated hydrophobic, hydrophilic, polar, non-polar, aromatic, charged, natural and unnatural amino acids. We discovered that the amino acid at the R^3 position (see general structure in Table 1) needed to be aromatic, and preferably electron-rich. The R^1 and R^2 amino acids had fewer particular restrictions, but it was clear that charged and polar groups diminished or abolished antibacterial activity, while hydrophobic residues and unfunctionalized residues were generally favored. Thus, 200 synthetic dihydropacidamycins were constructed in a solution-phase parallel synthesis exercise in which the R^1 position amino acid was chosen from Gly, Ala, Leu, Phe, 4-fluorophenylalanine (4-FPA). The R^2 position amino acid was chosen from Phe, Met, Leu. Ala, 4-FPA. Finally, the R^3 amino acid was chosen from Tyr and Trp.

While the marjority of compounds synthesized performed as well as or worse than pacidamycin 4, with little or no change in antimicrobial spectrum, several noteworthy improvements from the UPA paradigm were identified; the antimicrobial activities of these derivatives are summarized below. We did observe significant increase in potency against *P. aeruginosa* in comparison with the natural product, pacidamycin 4 (Table 1). Compound **11** showed a 2-fold increase in potency while **12** showed a 4-fold improvement in potency over the natural product against this wild-type organism. Also, for the first time, a compound in the UPA family has been identified with activity against wild-type *E. coli*; compound **10** shows a striking 64-fold improvement over the natural product with respect to its activity against wild-type *E. coli*. Interestingly, however, this same compound lost potency against *P. aeruginosa*. No compounds were identified with any significant activity against *P. multocida*.

Table 1. Activity of selected synthetic pacidamycins against several gram-negative pathogens



Compd	Structure			Minimum inhibitory concentrations (MICs in μg/mL)[d]		
				*P. aeruginosa* wild-type	*E. coli* wild-type	*P. multocida* wild-type
Pacidamycin 4[a]	*m*-Tyr[b]	Ala	Trp	16	256	128
10	Ala	4-FPA[c]	Tyr	64	4	512
11	Gly	Phe	Trp	8	256	64
12	Gly	Leu	Trp	4	128	128

[a]The natural product pacidamycins all have a 3′-deoxyuracil with a C(4′)–C(5′) exocyclic olefin.
[b]*m*-Tyr, *meta*-tyrosine.
[c]4-FPA, 4-fluorophenylalanine.
[d]See ref 12.

Due to its interesting shift in spectrum, compound **10** was further profiled against various other gram-negative organisms using the spiral gradient endpoint (SGE) method for MIC determination (Spiral Biotech, Inc, see Table 2). Clinical strains of *E. coli* tested, regardless of resistance phenotype, were susceptible to compound **10** (MIC = 4–8 μg/mL); other organisms such as *Klebsiella pneumoniae* and *Acinetobacter baumanii* were clearly resistant. Wild-type *Citrobacter freundii* was susceptible to compound **10** (MIC = 1 μg/mL) while *C. diversus* was only marginally susceptible. In further testing of compound **10** against a more extensive set of *E. coli* clinical isolates, one multiply resistant strain was identified with an MIC of ≥64 μg/mL. As is the case with the natural products, no synthetic pacidamycins were identified with any significant activity against *S. aureus*. Additional profiling revealed the same to be true for *Streptococcus pneumoniae*, a primary causative agent of community-acquired pneumonia.[1a]

The compounds were also assayed for activity against mycobacteria, using *Mycobacterium smegmatis* and *Mycobacterium fortuitum* as indicator organisms, since these are easy to grow in a standard microbiology laboratory. Compounds that showed good activity against one or both of these organisms, or performed well in earlier panels were then assayed for activity against *Mycobacterium tuberculosis*, an organism for which there are limited therapeutic options. The compounds were assayed against four strains of *M. tuberculosis*, two of which, the W and P strains, are resistant to essentially all approved anti-tuberculosis treatments. We assayed these compounds against four strains of *M. tuberculosis* (Table 3), two of which, the W and P strains, are resistant to essentially all approved tuberculosis treatments. Interestingly, the natural product pacidamycin 4 had no significant anti-tuberculosis activity while three of the nine synthetic analogues tested had MICs of 4–8 μg/mL against four different strains of *M. tuberculosis*, including the multi-resistant strains. This result is intriguing considering the difficulty of identifying novel mechanism-specific anti-tuberculosis compounds.

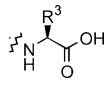
The mechanism of action of the mureidomycins[8] and pacidamycins has been shown to be inhibition of the cell wall biosynthetic enzyme MraY. Our colleagues have developed a high-throughput assay that measures MraY activity and is based on the incorporation of a fluorescent

Table 2. Activity of **10** against resistant gram-negative clinical isolates

Strain	Source	Resistance	MIC[b] (μg/mL)
E. coli	Proprietary	Wild-type	4
E. coli	Clinical	GEN, RIF, SM, SP, SU, TET, Hg	8
E. coli	Clinical	AMP, KAN, NM, RIF, TET, Hg	8
Salmonella spp.	Clinical	AMP, ERY	8
K. pneumoniae	Clinical	AMP, GEN, NAL, TET interm.[a]	≥64
K. pneumoniae	Clinical	N/A	32
C. diversus	Clinical	N/A	16
C. freundii	Clinical	GEN, ERY	1
E. cloacae	Clinical	ERY	≥64
A. baumanii	Clinical	Wild-type	≥64

GEN, gentamicin; RIF, rifampicin; SM, streptomycin; SP, spectinomycin; SU, sulfonamides; TET, tetracycline; Hg, mercury; AMP, ampicillin; KAN, kanamycin; NM, neomycin; ERY, erythromycin; NAL, nalidixic acid.
[a]This organism has intermediate-level susceptibility to TET.
[b]Measured by the spiral gradient endpoint (SGE) method, Spiral Biotech, Inc.

Table 3. Activity of selected compounds against *Mycobacterium tuberculosis*

Compd	Structure[a]			MICs[b] (μg/mL) against *M. tuberculosis* strains[c]			
				H37Rv	TN913	TN565 (W)	TN1618 (P)
Pacidamycin 4[d]	*m*-Tyr	Ala	Trp	>30	>30	>30	>30
12	Gly	Leu	Trp	>30	30	10	10
13	Ala	Met	Tyr	>30	>30	>30	>30
14	Ala	Phe	2-NAL	30	30	30	10
15	Leu	Phe	Trp	30	30	30	10
10	Ala	4-FPA	Tyr	10	8	8	10
16	Ala	4-BIP	Trp	8	4	4	10
17	Ala	Phe	Trp	8	8	4	8
18	Ala	4-FPA	Trp	8	8	4	8
19	Ala	4-TFM	Trp	8	4	4	8

[a]Refer to the general structure in Table 1.
[b]MICs were measured at 3 weeks and were done by increasing drug concentration by 2-fold dilution until 8 μg/mL, the next growth points were then 10 and 30 μg/mL.
[c]H37Rv and TN913 are susceptible to rifampicin (RIF), isoniazide (INH), ethambutol (EMB), streptomycin (STREP) ethionamide (ETH), kanamycin (KAN), capreomycin (CAP), ciprofloxacin (CIP), cycloserine. TN565 (W) is resistant to INH, RIF, KAN, STREP, EMB, ETH, CIP. TN1618 (P) is resistant to INH, RIF, STREP, EMB, ETH, CIP, CAP, *p*-aminosalicylic acid.
[d]The natural product pacidamycins all have a 3′-deoxyuracil with a C(4′)–C(5′) exocyclic olefin. *m*-Tyr, *meta*-tyrosine; 4-FPA, 4-fluorophenylalanine; 2-NAL, (2-naphthyl)alanine; 4-BIP, (4-biphenyl)alanine; 4-TFM, 4-trifluoromethylphenylalanine.

dansylated UDP-MurNAc-pentapeptide substrate in lipid vesicles containing decaprenol-phosphate.[13] They have shown these synthetic UPA derivatives, like the natural products, to be potent inhibitors of MraY isolated from *E. coli* (e.g., IC_{50} for **10** = 15 nM). A full disclosure of enzyme inhibition SAR along with the experimental details of their assay will be reported in due course.

For the first time, the spectrum of the UPAs has been modified to include additional gram negative pathogens including *E. coli* (both wild-type and multi-resistant clinical strains), and organisms of the genus *Citrobacter*. Additionally, a synthetic dihydropacidamycin 4-fold more potent against *P. aeruginosa* than the natural product pacidamycin 4 has been identified. The challenge of finding novel antituberculosis agents is a difficult one; thus it is striking that three synthetic dihydropacidamycins have activity in the 4–8 μg/mL range against both wild-type and multiply resistant strains, especially since the natural product pacidamycin 4 is shown to be inactive against these organisms.

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