SynBio-SynChem Approaches to Diversifying the Pacidamycins through the Exploitation of an Observed Pictet-Spengler Reaction

Christopher Cartmell,[a, b] Antoine Abou Fayad,[a, c] Rosemary Lynch,[a] Sunil V. Sharma,[a] Nils Hauck,[d] Bertolt Gust,[d] and Rebecca J. M. Goss*[a]

A nonenzymatic Pictet-Spengler reaction has been postulated to give rise to a subset of naturally occurring uridyl peptide antibiotics (UPAs). Here, using a combination of strain engineering and synthetic chemistry, we demonstrate that Pictet-Spengler chemistry may be employed to generate even greater diversity in the UPAs. We use an engineered strain to afford access to *meta*-tyrosine containing pacidamycin 4. Pictet-Spengler diversification of this compound using a small series of aryl-aldehydes was achieved with some derivatives affording remarkable diastereomeric control.

Introduction

The uridyl peptide antibiotics (UPAs) are a series of compounds with good activity against notorious pathogens including *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*. The UPAs include the pacidamycins,[1] napsamycins,[2] sansanmycins,[3,4] mureidomycins[5,6] tunicamycins[7] and liposidomycins.[8] These structurally similar and biosynthetically intriguing compounds employ series of previously unprecedented and rare enzymatic steps in their assemblies.[9–11] Napsamycins, pacidamycin 4 N (**2**) and sansanmycin F (**4**)[11] all contain a bicyclic amino acid, 6-hydroxy-1,2,3,4-tetrahydro-3-isoquinoline carboxylic acid (TIC), or its 1-methyl, or 1,1-dimethyl analogue at the N terminus of the pseudo-tetrapeptide backbone (Figure 1). This amino acid residue is derived from *meta*-tyrosine that is thought to have undergone a Pictet-Spengler like reaction introducing an *N*-methylene bridge and forming a bicycle.

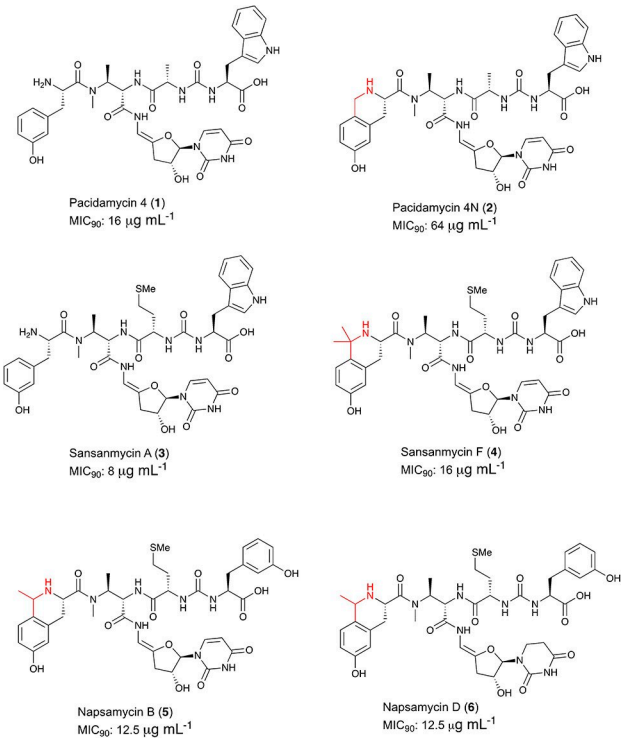

Figure 1. Naturally occurring UPAs: parent compounds and those containing bicyclic residues (TIC and its mono and dimethyl equivalents) at their N termini. Reported MIC_{90} values against *P. aeruginosa* are given.[2,3,11]

The UPAs exhibit a clinically unexploited mode of antibiotic action, inhibiting MraY, a crucial enzyme in peptidoglycan biosynthesis.[12–14] MraY is a structurally complex enzyme that spans the bacterial cell membrane with ten trans-membrane helices[14] and is responsible for uploading the peptidoglycan precursor formed within the cell, onto a membrane bound undecaprenyl phosphate carrier lipid, thereby generating lipid

[a] *Dr. C. Cartmell, Dr. A. Abou Fayad, Dr. R. Lynch, Dr. S. V. Sharma, Prof. Dr. R. J. M. Goss*
School of Chemistry and BSRC, University of St Andrews
St Andrews, Fife, KY16 9ST (UK)
E-mail: rjmg@st-andrews.ac.uk
[b] *Dr. C. Cartmell*
Department of Chemistry
University of Prince Edward Island Charlottetown
Prince Edward Island, C1A 4P3 (Canada)
[c] *Dr. A. Abou Fayad*
Department of Experimental Pathology, Immunology and Microbiology
Faculty of Medicine. Center of Infectious Disease Research (CIDR)
WHO Collaborating Center for Reference and Research on Bacterial Pathogens
American University of Beirut
Riad El-Solh/Beirut 1107 2020 Lebanon
[d] *Dr. N. Hauck, Dr. B. Gust*
Pharmazeutische Biologie, Pharmazeutisches Institut
Eberhard-Karls-Universität
Auf der Morgenstelle 8, 72076 Tübingen (Germany)

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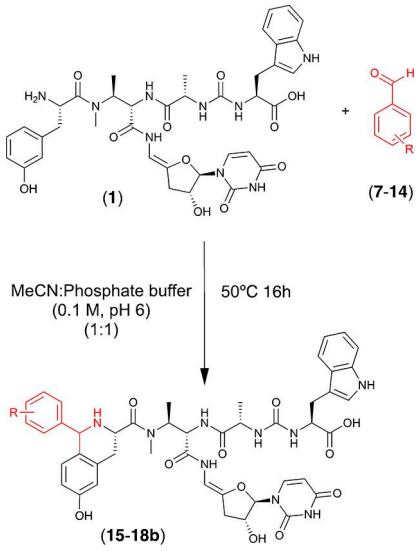
I.[15] It is postulated the UPAs inhibit MraY through a mechanism by which the N-terminal amine of the UPA pentapeptide competes with Mg^{2+} from the enzyme's active site.[16] Series of analogues of the pacidamycins have been generated through GenoChemetic, precursor-directed biosynthetic and total-synthetic approaches.[17–20]

The configuration of the N-terminal portion of the UPAs and its influence on the positioning of the terminal amine appears to be crucial. In the UPA analogues that bear TIC at the N terminus of the peptide the N-terminal primary amine is masked. For each of the *meta*-tyrosine containing UPAs and its TIC analogue, it is noteworthy that the analogue, though less potent, retains a level of activity. Curiously, the biggest difference in activity is observed in the pacidamycin 4 (**1**): pacidamycin 4 N (**2**) pairing,[21] (MIC_{90} 16 μgmL^{-1} and 64 μgmL^{-1} respectively) where the smallest and least sterically demanding Pictet-Spengler partner, formaldehyde is incorporated (Figure 1). We set out to explore whether this *meta*-tyrosine and its natural propensity to undergo a Pictet-Spengler reaction might be utilised to generate further unnatural analogues, and to investigate whether larger Pictet-Spengler partners result in altered activity.



Scheme 1. Pictet-Spengler modification of pacidamycin 4 (**1**) with a range of substituted benzaldehydes (**7**–**14**).

Results and Discussion

The pacidamycins are naturally generated as a complex suite of analogues with variations in the amino acids comprising the peptide backbone. To further diversify the UPA series, and perhaps gain greater insight into the biological relevance of this subset of UPAs, we set out to investigate whether we could use a semisynthetic approach to access an expanded suite of these modulated *meta*-tyrosine containing compounds. To initiate this study, it was necessary to first access sufficient quantities of pacidamycin 4 (**1**). The naturally occurring suite of pacidamycins are generated as a complex mixture, with some members being naturally more abundant. To optimize production of pacidamycin 4 (**1**) required for this study, we utilized a *Streptomyces coeruleorubidus* strain engineered to over produce pacidamycins. Therefore, the integrative plasmid pNH07 was constructed that harbours the positive regulator *npsM* of the napsamycin biosynthetic gene cluster under control of the strong promotor *pErmE**. pNH07 was integrated into the genome of the native producer generating *S. coeruleorubidus* NRRL18370/pNH07. Following fermentative culturing (see the Supporting Information), pacidamycins were purified using ion exchange followed by reverse phase chromatography resulting in yields of Pacidamycin 4 (**1**) at a level of ~1.2 mg/L (see the Supporting Information). Derivatization of this material with a range of aryl aldehydes (**7**–**10**) was subsequently explored.

To determine whether Pictet-Spengler chemistry would be effective in generating analogues of pacidamycin a small-scale screen, to be analysed by LC-HRMS, was initially carried out with a series of aryl aldehydes (**7**–**14**) (Scheme 1). The Pictet-Spengler reaction was first reported in 1911 reacting β-phenylethylamine with formaldehyde dimethyl acetal under acidic conditions, yielding 1,2,3,4-tetrahydroisoquinoline.[22] As a starting point for our investigations we looked toward the mild aqueous conditions developed by Pesnot et al. that had enabled β-arylethylamine derivatization generating tetrahydroisoquinolines as pairs of diastereoisomers.[23]

Analytical scale reactions were carried out on pacidamycin 4 using excess arylaldehyde (50 mM) in a 1:1 mixture of acetonitrile (MeCN) and phosphate buffer (0.1 M, pH 6) at 50 °C for 16 h. The initial screen allowed us to select the best aldehyde partners to take forward to larger scale. No conversion could be observed when 3-fluorobenzaldeyde, 4-dimethylaminobenzaldehyde and 4-nitrobenzaldehyde were used, and only low conversion could be seen in reactions with benzaldehyde, and so these compounds were not progressed.

Reactions where moderate to excellent levels of conversion were observed by LC-HRMS were scaled up to 1 mg of purified pacidamycin 4 (**1**) in order to afford sufficient compound to characterize the product by NMR spectroscopy, as well as to explore the compound by bioassay. The selection of the four aryl aldehydes 5-bromosalicyldehyde (**7**), 4-bromobenzaldehyde (**8**), 4-bromo-3-nitrobenzaldehyde (**9**) and 4-chlorobenzaldehyde (**10**) provided the opportunity to explore whether any stereo-chemical control might exist in this Pictet-Spengler reaction, perhaps conferred by the peptide backbone. Reaction conditions were as before.

Moderate to high conversions were achieved, as previously observed on the smaller scale reactions, and the products were purified by reverse phase HPLC. Moderate to good isolated yields were achieved from all reactions apart from the reaction using 4-chlorobenzaldehyde (**10**) (where isolation was not possible). To enable interpretation of diastereomeric ratio of the Pictet-Spengler analogues, and further insight into the diastereoselectivity, reactions of each aldehyde individually with L-*meta*-tyrosine were carried out (Figure 2 and Supporting Information).

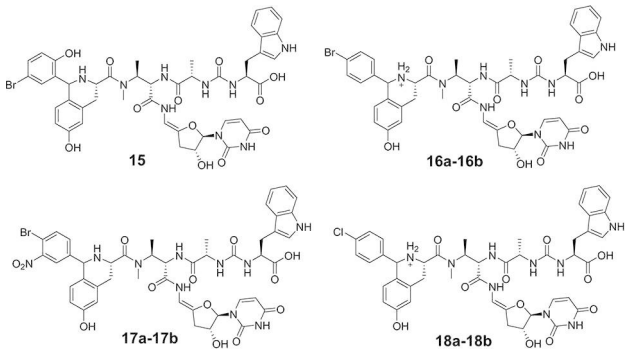


Figure 2. Structures of the products for reaction of pacidamycin 4 (**1**) with aryl-aldehydes (**7**–**10**).

Reaction of pacidamycin 4 (**1**) with both 5-bromosalicylaldehyde (**7**) and 4-bromo-3-nitrobenzaldehyde (**8**), gave excellent conversions. The conversions observed are dependent upon a complex combination of solubility, and electronics. Successively lower conversions are seen for 4-bromobenzaldehyde (**9**), followed by 4-chlorobenzaldehyde (**10**) and benzaldehyde (**11**), in this case with the more lipophilic compounds resulting in the higher conversions (see Table 1). For the pacidamycin analogues, diastereomeric ratios were established by combining NMR analysis and LC–MS analysis. Notably, reaction of pacidamycin 4 (**1**) with 5-bromo-salicylaldehyde (**7**) appeared to be fully diastereoselective, though it was not possible to directly identify which diastereoisomer had been generated. However,

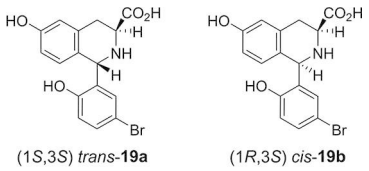


Figure 3. Structures of products for L-*meta*-tyrosine Pictet-Spengler reaction with 5-bromo-salicylaldehyde.

NMR chemical shifts of the structurally similar tetrahydroisoquinolines can be used to determine *cis*/*trans* diastereomers.[24] We synthesised a series of tetrahydroisoquinolines using L-*meta*-tyrosine and aldehydes **7**–**10** (see Sections 3.5–3.9 in the Supporting Information) to aid assignment of our pacidamycin derivatives. We observed NOE correlation between H-1 and H-3, at 5.66 and 4.01 ppm respectively, for **19a**–**19b**, thus, indicating to be (1*R*,3*S*) *cis* diastereomer (Figure 3). Conversely, the downfield H-1 at 5.96 ppm, without NOE correlation, would represent the (1*S*,3*S*) *trans* diastereomer. By comparing the ^{1}H chemical shifts of 5-bromo-salicylaldehyde products **19a**–**19b** and **15**, the later matched well with the *trans* diastereomer (see the Supporting Information). Potential reasons for the observed diastereoselectivity with bromo-salicylaldehyde could include hydrogen bonding from the aromatic hydroxyl to the peptidic backbone, locking the system into a preferred configuration. A low level of selectivity was observed within the other pacidamycin/aldehyde reactions, and similarly with reaction of

Table 1. Conversion and isolated yields obtained from the reaction with pacidamycin 4 (**1**) and selected benzaldehyde derivatives. Conditions: Pacidamycin 4 100 μg (1 mg for scale up), 25 mM of the aldehyde partner in a solution of 1 : 1 MeCN/KH_2PO_4 (0.1 M, pH 6) at 50 °C for 16 h.

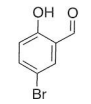
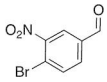
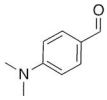
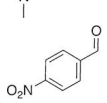
Aryl-aldehyde		Conversion [%] (Isolated yield)
	5-bromosalicylaldehyde (**7**)	99 (73)
	4-bromo-3-nitrobenzaldehyde (**8**)	99 (64)
	4-bromobenzaldehyde (**9**)	52 (39)
	4-chlorobenzaldehyde (**10**)	33
	benzaldehyde (**11**)	< 10
	3-fluorobenzaldehyde (**12**)	0
	4-dimethylaminobenzaldehyde (**13**)	0
	4-nitrobenzaldehyde (**14**)	0

Table 2. Diastereomeric ratio analyses of three Pictet-Spengler derivatized pacidamycin analogues calculated by integration of peak areas of the new inserted stereogenic centre.

Compound	Diastereomeric ratio by ^{1}H NMR
5-bromo-salicylaldehyde derivative of pacidamycin 4 (**15**)	1:0
4-bromobenzaldehyde derivative of pacidamycin 4 (**16a**–**16b**)	1:0.85
4-bromo-3-nitrobenzaldehyde derivative of pacidamycin 4 (**17a**–**17b**)	1:0.4

L-*meta*-tyrosine and aldehydes **8**–**10** a level of diastereoselectivity could also be observed (see Table 2 and Supporting Information).

It is possible that the biosynthetic pathways that generate the UPAs have evolved to produce and incorporate an unusual *meta*-tyrosine residue as a mechanism for attenuating the activity of the antibiotic conferred by the free primary amine, perhaps providing a tuneable self-resistance mechanism. This residue is predisposed to undergo a Pictet-Spengler reaction under environmental, nonenzymatic, conditions, and under certain medium/solvent conditions conversion of pacidamycin 4 (**1**) to 4 N (**2**) may be seen. It is curious that the genes encoding generation of the *meta*-tyrosine and its activation as the amino adenylate/incorporation into the nonribosomal peptide lie outside the boundaries of the pacidamycin biosynthetic cluster, whilst an analogous alanine activating adenylation domain is embedded within the cluster, giving rise to the N-terminal alanine suite of pacidamycins.[25] MIC_{90} testing was performed on pacidamycin and three Pictet-Spengler derivatives: 5-bromosalicylaldehyde, 4-bromobenzaldehyde, 4-bromo-3-nitro benzaldehyde (**15**–**17**) with *Pseudomonas aeruginosa* ATCC 15 442 at a concentration of 64 $\mu g\,mL^{-1}$. The derivatization of these analogues could indeed be seen to confer protection for *P. aeruginosa*, in line with this hypothesis, with only pacidamycin 4 (1), included as a control, presenting activity against *P. aeruginosa* at a concentration of 64 $\mu g\,mL^{-1}$. All pacidamycin Pictet-Spengler analogues were found to be inactive at concentrations up to 128 $\mu g\,mL^{-1}$.

Conclusion

A series of Pictet-Spengler products are reported as naturally occurring across the uridyl peptide antibiotic series, however, unlike in alkaloid biosynthetic pathways no "Pictet Spenglerase" associated with any of the uridyl peptide antibiotic biosynthetic gene clusters has been identified.[3,25] Our observation of the nonenzymatic conversion of the pacidamycins to pacidamycin N series, and our ability to promote Pictet-Spengler product formation under mild conditions implies that these analogues may not be enzyme generated. The natural formation of Pictet-Spengler analogues of the UPAs may be key to playing a role in resistance and strain protection. From our MIC_{90} testing we found the new Pictet-Spengler derivatives conferred protection on the antibiotic. This result demonstrates the importance of the N terminus to bioactivity of this natural product, and that modifications of this nature can result in detrimental effects to bioactivity. The incorporation of *meta*-tyrosine may therefore be seen to enable the attenuation of compound activity through Pictet-Spengler diversification. It is intriguing, in the light of these results, that the "outsider" adenylation gene, that is selective for *meta*-tyrosine (pac21h) dominates the portfolio of the pacidamycins produced in both the WT and heterologous expression systems, with the *meta*-tyrosine containing analogues being the predominant product when both adenylation domains are present.[26] Horizontal gene transfer of the central BGC, without the additional pac21h would not afford compounds containing N termini *meta*-tyrosine and as such, utilisation of a synthetic Pictet-Spengler reaction to reduce compound toxicity would not be possible. Studies to further understand the interplay of the regulation of this complex pathway and resistance mechanisms would be interesting, but far beyond the scope of this project focussed on analogue access.

Could this *meta*-tyrosine and its natural propensity to enable masking of the primary terminal amine, hold some clues as to natural self-resistance mechanisms?

Beyond the UPAs, for which the *meta*-tyrosine analogues are produced only in low titre and are hard won, incorporation of *meta*-tyrosine, into the N-terminal of a peptide or natural product, opens up an opportunity for reversibly modulating a peptide or natural product and tuning its conformation and activity. The promise of such an approach is made even more attractive in the light of the notable diastereoselectivity that can be achieved.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: compound diversification · natural products · pacidamycin · Pictet-Spengler · semisynthesis · uridyl peptide antibiotic

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