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New Pacidamycin Antibiotics Through Precursor-Directed Biosynthesis

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Pacidamycins, mureidomycins and napsamycins are structurally related uridyl peptide antibiotics that inhibit translocase I, an as yet clinically unexploited target. This potentially important bioactivity coupled to the biosynthetically intriguing structure of pacidamycin make this natural product a fascinating subject for study. A precursor-directed biosynthesis approach was employed in order to access new pacidamycin derivatives. Strikingly, the biosynthetic machinery exhibited highly relaxed substrate specificity with the majority of the tryptophan analogues that were administered; this resulted in the production of new pacidamycin derivatives. Remarkably, 2-methyl-, 7-methyl-, 7-chloro- and 7-bromotryptophans produced their corresponding pacidamycin analogues in larger amounts than the natural pacidamycin. Low levels or no incorporation was observed for tryptophans substituted at positions 4, 5 and 6. The ability to generate bromo- and chloropacidamycins opens up the possibility of further functionalising these compounds through chemical cross-coupling in order to access a much larger family of derivatives.

Introduction

The quest for new antibiotics, especially those with activity against Gram negative bacteria, is urgent. Each year, over 13 million lives world wide are currently claimed by infectious diseases, including 100 000 in the USA. Over the last decade this figure has doubled due to the emergence of multidrug-resistant strains. Conversely, very few new antibiotics have been marketed in the last 40 years, with this limited number falling into only four new structural classes.[1]

Two complementary approaches to developing novel antibiotics exist. These are the structural modification of existing antibiotic skeletons in order to defeat resistance mechanisms, and a quest for antibiotics with new structures or, better still, with new bacterial targets. The pacidamycins (**1**),[2–4] mureidomycins (**2**),[5,6] and napsamysins (**3**)[7] are structurally related uridyl peptide antibiotics which exhibit an as yet clinically unexploited mode of action (Figure 1). These compounds inhibit the enzyme translocase I, a membrane protein involved in bacterial cell wall biosynthesis.[8,9] Translocase I (MraY) catalyses the penultimate cytoplasmic event in peptidoglycan biosynthesis, the loading of the precursor uridine diphosphate-*N*-acetyl muramic acid (UDP-MurNAc)-pentapeptide onto undecaprenylphosphate to give lipid intermediate I with concomitant release of UMP (Scheme 1). The small number of natural products known to share this novel and potentially useful mode of antibacterial activity include structurally diverse groups such as the liposidomycins, tunicamycins and the muraymycins.[9,10] The uridyl peptide antibiotics pacidamycin, mureidomycin and napsamycin all consist of a pseudo tetra- or pentapeptide backbone, the sense of which is inverted twice by incorporation of 2,3-diaminobutyric acid and a urea motif (Figure 1). The peptide backbone is linked through the carboxy group of the branching diamino acid to the nucleoside through an exocyclic enamide, a moiety unique to this family of natural products. The structure of the uridyl peptide antibiotics was elucidated through extensive NMR studies.[4,6] These studies assigned the *cis* geometry of the exocyclic enamide double bond based on an NOE signal between the vinylic proton and the methylene protons of the amino sugar, whilst the absolute stereochemistry of the diamino acid was later established as (2*S*, 3*S*)-2,3-diaminobutyric acid through chemical synthesis by Boojamra et al.[11] Bugg and co-workers demonstrated the remarkable stability of the enamide present in the uridyl peptide antibiotics.[12]

The molecular basis for the mode of action of uridyl peptide antibiotics remains unclear due to a lack of structural information on translocase I. However, it has been demonstrated that the exocyclic enamide does not substantially contribute to the antibacterial activity of the mureidomycins or pacidamycins.[11,13] For example, dihydropacidamycin, which results from the catalytic hydrogenation of the enamide of pacidamycin, shows little difference in biological activity to its parent compound.[11] A series of uridyl dipeptide analogues mimicking the amino terminal portion and their full length analogues had been synthesised in order to determine features that are crucial for biological activity.[14] In vitro studies with purified translocase I demonstrated the importance of the N-terminal amino

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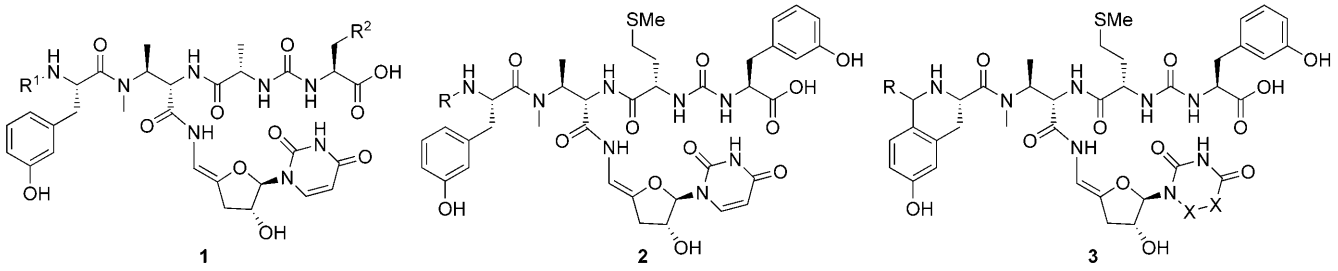


Pacidamycins

Pacidamycin 1:	R^1 = Ala	R^2 = indolyl
Pacidamycin 2:	R^1 = Ala	R^2 = phenyl
Pacidamycin 3:	R^1 = Ala	R^2 = 3-hydroxyphenyl
Pacidamycin 4:	R^1 = H	R^2 = indolyl
Pacidamycin 5:	R^1 = H	R^2 = phenyl
Pacidamycin 5T:	R^1 = H	R^2 = 3-hydroxyphenyl
Pacidamycin 6:	R^1 = Gly	R^2 = indolyl
Pacidamycin 7:	R^1 = Gly	R^2 = phenyl

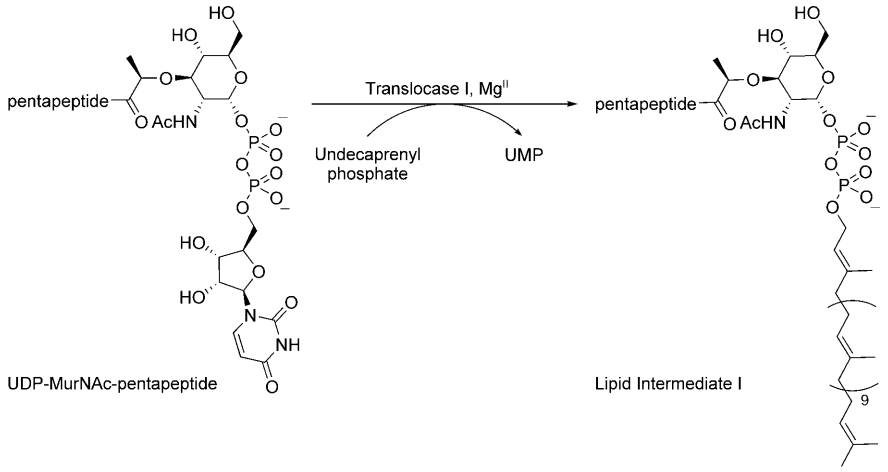
Mureidomycins

Mureidomycin A:	R = H	X-X = CH=CH
Mureidomycin B:	R = H	X-X = CH_2-CH_2
Mureidomycin C:	R = Gly	X-X = CH=CH
Mureidomycin D:	R = Gly	X-X = CH_2-CH_2

Napsamycins

Napsamycin A:	R = H	X-X = CH=CH
Napsamycin B:	R = H	X-X = CH_2-CH_2
Napsamycin C:	R = CH_3	X-X = CH=CH
Napsamycin D:	R = CH_3	X-X = CH_2-CH_2

Figure 1. The uridyl peptide family of antibiotics.



Scheme 1. The formation of lipid intermediate I catalysed by translocase I.

acid and its orientation to activity of the analogue. The amino terminus is implicated in binding to translocase I at a site occupied by Mg^{II} in the enzyme whereas the uridyl moiety is believed to compete for the UDP binding site (Figure 2). The above in vitro experiments indicated that the N-terminal portion, the branching diamino acid and the uridyl moiety made the strongest contributions to the biological activity of uridyl peptide antibiotics. This was further substantiated through assessment of the biological activity of a library of synthetic dihydropacidamycins against whole cells. These experiments demonstrated that a higher degree of structural variability was tolerated at the C-terminal portion of the molecule.[15] The natural uridyl peptide antibiotics exhibit a very narrow spectrum of antibacterial activity, acting specifically against *Pseudomonas aeruginosa*. This exquisite specificity of antibacterial activity is of potential use in the treatment of cystic fibrosis sufferers; nevertheless, it is an appealing challenge to increase the spectrum of activity of these antibiotics.

As an alternative approach to accessing pacidamycin derivatives through total synthesis, we envisaged a combination of biotransformation and precursor-directed biosynthesis to obtain the analogues. The utilization of biosynthetic pathways in order to generate new natural products is an area of growing interest. The simplicity of this approach in administering a simple synthetic precursor analogue is attractive. Utilising this approach, new analogues of clinically important natural products such as the immunosuppressant rapamycin have been prepared in a matter of days compared to a more lengthy and costly synthetic approach.[16,17] Here, we report the first manipulation of the biosynthesis of a peptidyl nucleoside antibiotic.

Results and Discussion

Our study focuses on interrogating the natural degree of flexibility of the pacidamycin biosynthetic pathway. *Streptomyces coeruleorubidus* AB 1183F-64 produces a suite of over ten pacidamycins including pacidamycins **1**–**7** (Figure 1). Variations are mainly observed at the N terminus, where an additional alanine or glycine residue can be found, and at the C terminus, where either tryptophan, phenylalanine or *meta*-tyrosine is incorporated. Assuming that the same biosynthetic machinery is responsible for the generation of all pacidamycins, the enzymes responsible for the selection and installation of the C-terminal residue appear to have a high degree of innate flexibility; this flexibility renders this position ideal for modification through precursor-directed biosynthesis. This provided encouragement that incorporation of a variety of tryptophan derivatives into pacidamycin might be feasible, and that it might

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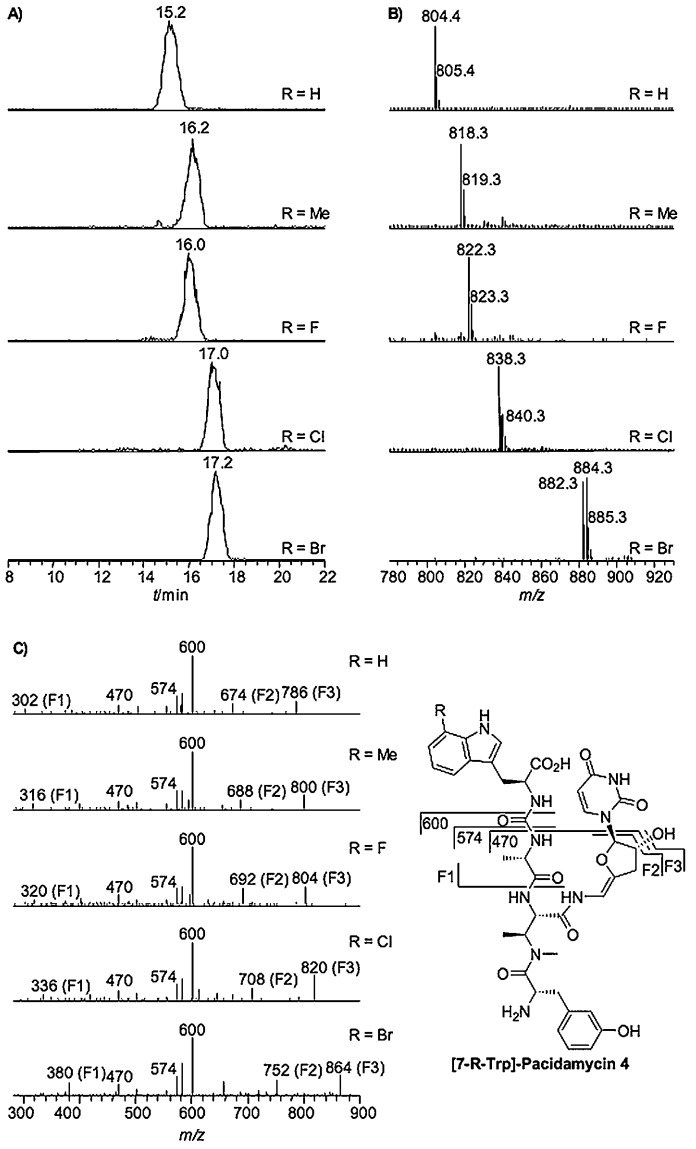


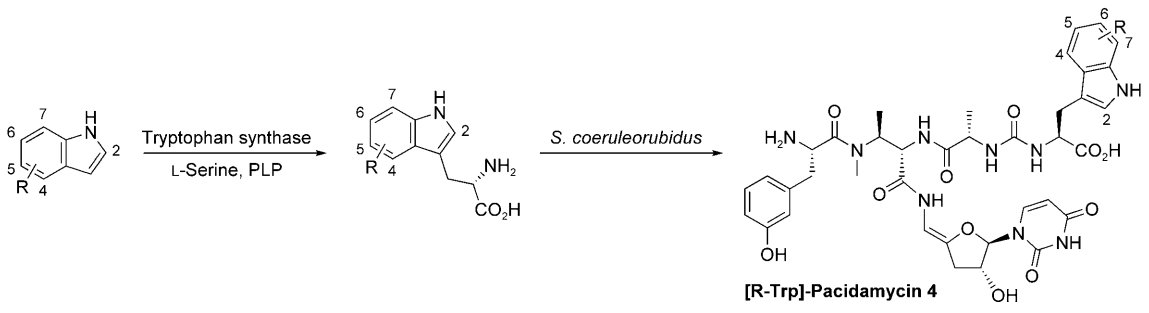
Figure 2. LC–MS analysis of [7-R-Trp]-pacidamycin 4 derivatives in comparison to the parent compound pacidamycin 4 (R = H). A) Extracted ion chromatograms. The retention times are indicated. B) MS spectra showing the molecular ion peaks. The spectra were averaged across the relevant retention times shown in A. C) MS–MS spectra. The assignments of selected fragment ions are shown.

even be possible to achieve reasonable levels of incorporation of these aromatic amino acids without the need to genetically modify the producing organism. Furthermore, it was postulated that modification of the carboxy terminal residue would minimise adverse effects on binding to translocase I, as most of the interactions are thought to span from the N terminus to the nucleoside portion.[13,14]

The media reported for the isolation of the pacidamycins consisted of a range of complex materials from which the purification of the pacidamycins was laborious.[2] In order to optimize incorporation of our tryptophan analogues into pacidamycin and to ease purification of the resultant analogues, we tested the pacidamycin production profile in minimal medium. Using lactose as the carbon source, we observed pacidamycins 4, 5 and 5T (Figure 1) as the major components at an approximate ratio of 3:1:2. This is in contrast to the previously reported distribution, in which pacidamycins 1, 2 and 3 were the dominant compounds.[4] In our hands, these latter pacidamycins were typically only present in small amounts or not detectable.

A series of halogenated and methylated tryptophans were generated from serine and the corresponding indole utilizing a readily prepared cell-free extract containing tryptophan synthase as previously described (Scheme 2).[18] Halotryptophans with substituents at the 5-, 6-, or 7-position of the indole ring and 2,- 4-, 5-, 6- and 7-methyltryptophans were thus prepared. Incorporation of halogens into compounds is known to have significant effects on their physicochemical properties. We were particularly interested in chlorinated and brominated derivatives as these would enable selective chemical derivatisation.[19] Fluorination is used to block sites of metabolic modification and, hence, can increase the half-life of drugs.[20] In the context of this study, though, the fluorinated tryptophans serve as a control due the small degree of steric challenge they pose. The methylated derivatives were included to scan all possible indole substitutions for permissibility towards bulky substituents.

14 tryptophan analogues were added to individual cultures of *S. coeruleorubidus* in minimal medium at the onset of antibiotic production. The culture was harvested after



Scheme 2. Precursor-directed biosynthesis of pacidamycin 4 derivatives.

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a further two days of growth and the extracts were analysed by LC–MS. Chromatograms and spectra for the [7-R-Trp]-pacidamycin 4 series are given in Figure 2. Analytical data for all pacidamycin analogues are provided in the Supporting Information. Notably, eleven out of the 14 tryptophan analogues resulted in the production of new pacidamycin 4 derivatives with the expected molecular ion being detected (Figure 2B). Correct incorporation of the tryptophan analogues at the C-terminal position was confirmed through inspection of the MS–MS spectra (Figure 2C). Two product ions served particularly well as diagnostic peaks. The *m/z* 600 ion corresponds to loss of the C-terminal residue and remains the same for all pacidamycin 4 derivatives. Furthermore, a dipeptide fragment corresponding to the C-terminal pseudo-dipeptide (F1 in Figure 2C) was also observed. The mass of the latter fragment ion shifted, as expected, from *m/z* 302 for pacidamycin 4, to *m/z* 316, 320, 336 and 380 for the methylated, fluorinated, chlorinated and brominated pacidamycin 4 derivatives, respectively. Furthermore, the characteristic isotope pattern for chlorinated or brominated pacidamycins was evident (Figure 2B). These results clearly demonstrate that the supplemented tryptophan analogues were indeed incorporated at the C terminus. As mentioned above, the other two pacidamycins containing a C-terminal tryptophan residue, pacidamycins 1 and 6 (Figure 1), were only present in small amounts or below detectable levels. Only when 2-methyltryptophan was supplemented to the culture did we observe small amounts of the corresponding analogue [2-Me-Trp]-pacidamycin 1 (see the Supporting Information for analytical data). No pacidamycin 6 derivatives were detected. Pacidamycins in which the tryptophan analogue had been incorporated at a different position were also not detected (for example, no substitution for the N-terminal *meta*-tyrosine was observed).

Levels of incorporation were determined by analysing the extracted ion peak areas. Quantification based on UV absorption was not feasible in this case as many of the pacidamycins exhibit similar retention times (for example, pacidamycin 5: 14.9 min; pacidamycin 4: 15.2 min; see also Figure 2A). Furthermore, the indole substituents alter the absorption characteristics of tryptophans and hence those of the pacidamycins. Conversely, the indole substituents should not significantly affect the ionisation efficiency of the new pacidamycins compared to their parent compound. Relative quantification carried out by mass spectrometry was in excellent agreement with results based on bioactivity against *P. aeruginosa* (data not shown). Hence, for each sample the extracted ion peak area of the pacidamycin analogue was compared to that of its parent compound, pacidamycin 4, which served as an internal standard. The results proved striking with regards to the relative amount of analogue that was formed (Figure 3). The pacidamycin 4 derivatives obtained from feeding 2-methyl, 7-methyl-, 7-chloro- or 7-bromotryptophan were all present in larger amounts than pacidamycin 4 itself. This outcome was particularly surprising as, typically, the supplementation with isotopically labeled biosynthetic precursors results in maximal incorporation levels of 10% when supplied at similar concentrations.[21] Precursor-directed biosynthesis of other natural products has, to date, not yielded comparable levels of incorporation.[17] The only reported instances where the analogue is produced in higher amounts than the parent compound involve the employment of auxotrophic strains or inhibitors.[17,22] These results demonstrate that substitution of tryptophan at the 2- and 7-positions, even with sterically bulky groups, is very well tolerated, whereas low levels or no incorporation are observed for tryptophans substituted at positions 4, 5 and 6 (Figure 3).

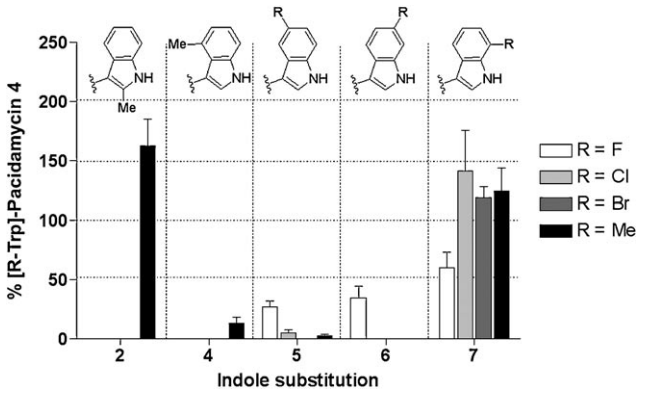

Figure 3. Levels of [R-Trp]-pacidamycin 4 formation relative to pacidamycin 4 (set to 100%). Experiments were performed in triplicate with the standard deviation indicated by error bars. The indole structure of the supplemented tryptophan and the resulting pacidamycin 4 derivative is indicated.

Interestingly, fluorinated tryptophans follow a different pattern. Fluorine is the smallest substituent tested, and hence, would impose the least amount of steric constraint on the enzyme responsible for attachment of the C-terminal amino acid. Not surprisingly, the relative amount of pacidamycin 4 analogue upon feeding of 5-fluorotryptophan was therefore significantly higher compared to the other 5-substituted tryptophan derivatives. Unexpectedly, more fluoropacidamycin 4 was produced upon supplementation with 6-fluorotryptophan than upon supplementation with the 5-fluoro compound. This is in contrast to the trend observed for methyl-, chloro- and bromotryptophans. Furthermore, the level of incorporation for 7-fluorotryptophan would have been expected to be equal to or better than the level observed for the sterically more demanding methyl, chloro and bromo substituents. However, the plasticity of the enzyme active site is only one parameter that affects levels of incorporation. Another crucial factor is the intracellular metabolite concentration, which is determined by active and passive cell uptake and export, as well as by loss of the compound to other metabolic pathways. By administering 7-chlorotryptophan concentrations from 125 μM to 1 mM to *S. coeruleorubidus*, it became apparent that the relative amount of chloropacidamycin produced increased in a nonlinear fashion and reached saturation towards 1 mM of the tryptophan analogue (data not shown). Furthermore, it was observed that the addition of tryptophan or tryptophan analogue induced the production of a new set of as yet uncharacterised metabolites that appear unrelated to the pacidamycin family. These two findings indirectly support the hypothesis that the lower

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than expected levels of [7-F-Trp]-pacidamycin 4 could be due to a diminished availability of 7-fluorotryptophan.

It has been postulated that the highly polar nature of the uridyl peptide antibiotics inhibits uptake of the compounds by whole cells and leads to this focused spectrum of activity, and that increasing the lipophilicity might confer a broader spectrum of activity.[15] Some support for this theory is given by the fact that a synthetic analogue of pacidamycin 4 in which the third residue was replaced by 4-fluorophenylalanine exhibited an increased spectrum of activity against *E. coli*, *Salmonella* and *Mycobacterium tuberculosis*.[15] In order to test for antibacterial activity, pacidamycin 1, pacidamycin 4, [7-Cl-Trp]-pacidamycin 1 and [7-Cl-Trp]-pacidamycin 4 were isolated from cultures supplemented with 0.5 mM 7-chlorotryptophan as described in the Experimental Section. The isolated yields for pacidamycin 4 and [7-Cl-Trp]-pacidamycin 4 from the same culture were 3.5 mg L^{-1} and 2 mg L^{-1}, respectively. Administration of tryptophan analogues does not appear to adversely affect the overall level of productivity. Cultures supplemented with tryptophan typically produced pacidamycin 4 at 4–5 mg L^{-1}. Pacidamycin 1 and its chlorotryptophan analogue were isolated from a separate culture in 2 mg L^{-1} and 2.5 mg L^{-1}, respectively. These yields were unexpected as typically only small amounts of pacidamycin 1 are produced by *S. coeruleorubidus*. In this particular instance, however, the ratio of pacidamycin 1 and 4 was reversed. The factors governing the profile of pacidamycin production are still unknown. Insights into the biosynthesis of these compounds may provide answers, and studies towards the elucidation of the pathway are ongoing in our laboratory. The purity of the pacidamycins was estimated to be $>80\%$ as judged by LC–MS and NMR analysis (Supporting Information).

The four isolated compounds allowed, for the first time, the effects on antibiotic activity of the same modification in different pacidamycin backbones to be investigated. Whilst the spectrum of activity of the chlorinated pacidamycins remained the same as for their parent compounds, interesting differences in the compounds' activities against *P. aeruginosa* were observed. The minimum inhibitory concentration (MIC) against *P. aeruginosa* of [7-Cl-Trp]-pacidamycin 1 (MIC 32 μg mL^{-1}) proved to be four-fold lower than for the parent compound pacidamycin 1 (MIC 128 μg mL^{-1}). Conversely, [7-Cl-Trp]-pacidamycin 4 (MIC 128 μg mL^{-1}) was two-fold less active than pacidamycin 4 (MIC 64 μg mL^{-1}). A possible explanation for these observations may lie in the different distances of the nucleoside portion to the amino terminus in pacidamycin 1 and 4. It is conceivable that the C-terminal region of the molecule reorients itself in the translocase I active site in order to achieve binding of both features. The C-terminal residue would thus be exposed to different environments and thus substitution of this residue would affect different binding interactions. Whilst MIC values may provide an indicator as to the levels of inhibition, in vitro studies with translocase I will provide a more complete picture.

Conclusions

We have demonstrated the first manipulation of a uridyl peptide antibiotic biosynthetic pathway, and that pacidamycin analogues are easily and efficiently produced through precursor-directed biosynthesis without the need to employ an auxotrophic strain or to genetically modify *S. coeruleorubidus*. Tryptophan derivatives with substituents at the 2- and 7-positions are incorporated with astonishing ease to give the corresponding pacidamycin analogues in higher amounts than the parent compound. Furthermore, it has become apparent that the same modification will have different physiological and biological effects in pacidamycins varying in the nature of their N termini. With the ability to generate bromo- and chloropacidamycins the opportunity to further functionalise these compounds through chemical cross-coupling, and thus to access a wider chemical space is presented.

Experimental Section

Material, bacterial strains: Microbiological media, buffer components, and reagents were purchased from BD Biosciences (Oxford, UK), Melford (Chelsworth, UK, Sigma–Aldrich, Alfa Aesar (Hyesham, UK), Fluorochem (Old Glossop, UK) and used without further purification. *Streptomyces coeruleorubidus* AB1183F-64 was obtained from the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research (NRRL, Peoria, USA), *Pseudomonas aeruginosa* ATCC 15442 from the American Type Culture Collection (ATCC, Middelsex, UK).

Precursor-directed biosynthesis: Starter cultures in ISP2 medium (1% malt extract, 0.4% yeast extract, 0.4% glucose, pH 7.2) were inoculated with *S. coeruleorubidus* spores to 10^6 cfu mL^{-1} and incubated at 28 °C, 180 rpm for 48 h. For small scale feeding, production medium (10 mL; 20.99 g L^{-1} MOPS, 10 g L^{-1} lactose, 4.41 g L^{-1} K_2HPO_4, 2.14 g L^{-1} NH_4Cl, 0.6 g L^{-1} $MgSO_4$, 10 mg L^{-1} $FeSO_4 \cdot 2H_2O$, 10 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 10 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 10 mg L^{-1} $CaCl_2$, pH 7.0) was inoculated with starter culture (0.5 mL) and incubated at 28 °C, 180 rpm for 72 h. A sterile, pH-neutral aqueous solution of the tryptophan derivative of interest was added to the main culture to a final concentration of 1 mM. Incubation was continued for another 48 h. Pacidamycins were extracted from the cell-free broth using XAD-2 resin (5% *v/v*). The resin was washed with water (20 volumes) and the extract was eluted with methanol (10 volumes). The solvent was removed in vacuo and the residue redissolved in water/methanol 1:1 (100 μL) prior to LC–MS analysis.

The extract components were separated by RP-HPLC on a 150× 2 mm 4 μm Synergi™ Polar-RP column (Phenomenex, Macclesfield, UK) using a gradient of 3–80% buffer B over 20 min at a flow rate of 260 μL min^{-1} (buffer A: 0.1% formic acid in water; buffer B: methanol). The elution of compounds was monitored using a Thermo Finnigan LCQ DecaXP^{plus} ion trap LC–MS system equipped with photodiode array detector. For relative quantification of individual pacidamycins, the peak area of the corresponding extracted ion chromatograms was used.

Isolation of pacidamycins: The culture conditions were as described above with the following exceptions: an aqueous solution of tryptophan derivative and phenylalanine were added to give final concentrations of 0.5 mM and 1 mM, respectively, and the main culture was incubated for a total of 7–8 days. The purification

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protocol was adapted from US Patent 6228842.[23] Solid-phase extraction was performed as described above. The extract was then purified by ion-exchange chromatography using a HiTrap™ SP-FF column (GE Healthcare). After loading, the column was washed with 50 mM sodium acetate, pH 3.6 (6 volumes). Pacidamycins were eluted with 50 mM sodium acetate in a stepwise gradient from pH 3.6 to pH 5.6. Pacidamycin-containing fractions were combined and further purified on a 150×2 mm 4 μm Synergi™ Polar-RP column (Phenomenex) using a gradient of 35–90%B over 30 min with a flow rate of 4 mL min^{-1} (buffer A: 0.1 M ammonium acetate, pH 7.9; buffer B: methanol). Desalting was achieved by passing fractions of interest through the same column using a water/methanol gradient. Elution was monitored at 280 nm.

Antibiotic activity assay: Minimum inhibitory concentrations were determined by the broth microdilution assay. The assay was carried out in a 96-well microtiter plate using trypticase soy broth with a final volume of 100 μL per well. Antibiotic concentrations in the range of 256 to 0.125 μg mL^{-1} were tested. Assays were performed in duplicate.

Acknowledgements

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Keywords: antibiotics · biosynthesis · pacidamycins · *Pseudomonas aeruginosa* · translocase I · uridyl peptide antibiotic

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