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# New pacidamycins biosynthetically: probing N- and C-terminal substrate specificity†

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**Feeding phenylalanine analogues to *Streptomyces coeruleorubidus* reveals the remarkable steric and electronic flexibility of this biosynthetic pathway and leads to the generation of a series of new halopacidamycins.**

Pacidamycin (Fig. 1) is a member of the recently discovered family of uridyl peptide antibiotics.[1,2] The uridyl peptide antibiotics have attracted particular interest as they act on a cellular target that, so far, is clinically unexploited.[2] They inhibit the enzyme translocase I which is an essential transmembrane protein involved in bacterial cell wall biosynthesis.[2,3] The uridyl peptide antibiotics have a number of biosynthetically intriguing features that are unique to this class of compounds. The pacidamycins and mureidomycins are composed of a pseudo-penta- or tetrapeptide backbone which is inverted twice by the incorporation of a diamino acid and the insertion of a ureido moiety.[2–5] The choice of the C-terminus residue of pacidamycin is strikingly flexible and the suite of natural pacidamycins contains members in which phenylalanine is replaced with tryptophan or *m*-tyrosine.[6] We have previously exploited this natural biosynthetic promiscuity in order to generate a series of pacidamycins containing halotryptophan at the C-terminus.[7] On examination of the natural suite of pacidamycins, flexibility at the N-terminus is far less obvious as all natural pacidamycins, with one exception, contain a *m*-tyrosine residue

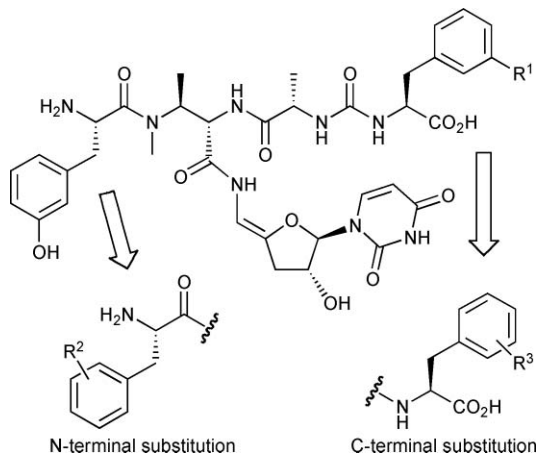


**Fig. 1** Structure of pacidamycin 5 ($R^1$=H) and pacidamycin 5T ($R^1$= OH).

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**Table 1** Percentages of halopacidamycins relative to pacidamycin 5 (set to 100%). The standard error is indicated. n.d.: not detected

| | % [R-Phe]-pacidamycin | | |
|---|---|---|---|
| X-Phe | 'Mono' C- terminus | 'Mono' N-terminus | 'Di' both C and N termini |
| X: | $R^2 = OH, R^3 = X$ | $R^2 = X, R^3 = H$ | $R^3 = R^2 = X$ |
| 2-Fluoro | 215 ± 53 | n.d. | 453 ± 153 |
| 3-Fluoro | 116 ± 27 | n.d. | 187 ± 88 |
| 4-Fluoro | 130 ± 20 | n.d. | 19 ± 4 |
| 2-Chloro | 152 ± 9 | n.d. | 46 ± 3 |
| 4-Chloro | n.d. | n.d. | n.d. |
| 2-Bromo | n.d. | n.d. | n.d. |

attached to the β-amino group of the branching diamino acid. We set out to explore the possibility of using precursor-directed biosynthesis in order to generate N-terminal analogues of pacidamycin.

Commercially available 2-, 3-, 4-fluoro-, 2-, 4-chloro- and 2-bromophenylalanine were used in our study. These compounds were fed in quadruplicate to cultures (10 ml) of *S. coeruleorubidus* at a final concentration of 1mM (see ESI†). Culture extracts were analysed by LC-MS for the presence of new pacidamycin analogues (Fig. 2). The position of incorporated fluoro- and chlorophenylalanine residues was confirmed by inspection of the MS/MS fragmentation patterns (Fig. 2C). Relative levels of incorporation were determined by comparing, for each sample, the peak area of the corresponding extracted ion chromatogram of the pacidamycin analogue to that of its parent compound pacidamycin 5 (Table 1).

Feeding studies with 2-fluoro-, 3-fluoro-, 4-fluoro-, and 2-chlorophenylalanine resulted in either double incorporation into the antibiotic at both the N- and C-termini or in single incorporation into the C-terminus alone (Table 1). Interestingly, we were unable to detect any analogues in which substitution occurred at the N-terminus alone. No new pacidamycin derivatives were detected when 4-chloro- or 2-bromophenylalanine were supplemented. Strikingly high levels of incorporation were observed in the case of the 2-fluoro, 3-fluoro and 2-chlorophenylalanines with a considerable proportion of material resulting from double incorporation; over four times more $[2\text{-F-Phe}]_2$-pacidamycin than pacidamycin 5 was formed, and almost twice as much $[3\text{-F-Phe}]_2$-pacidamycin compared to its parent compound. For the sterically more demanding 2-chlorophenylalanine analogue levels of double incorporation were considerably lower, and no incorporation was detected for the bromo derivative clearly indicating steric constraints. Furthermore, substituents in the *para* position were less well tolerated for double incorporation than substituents in

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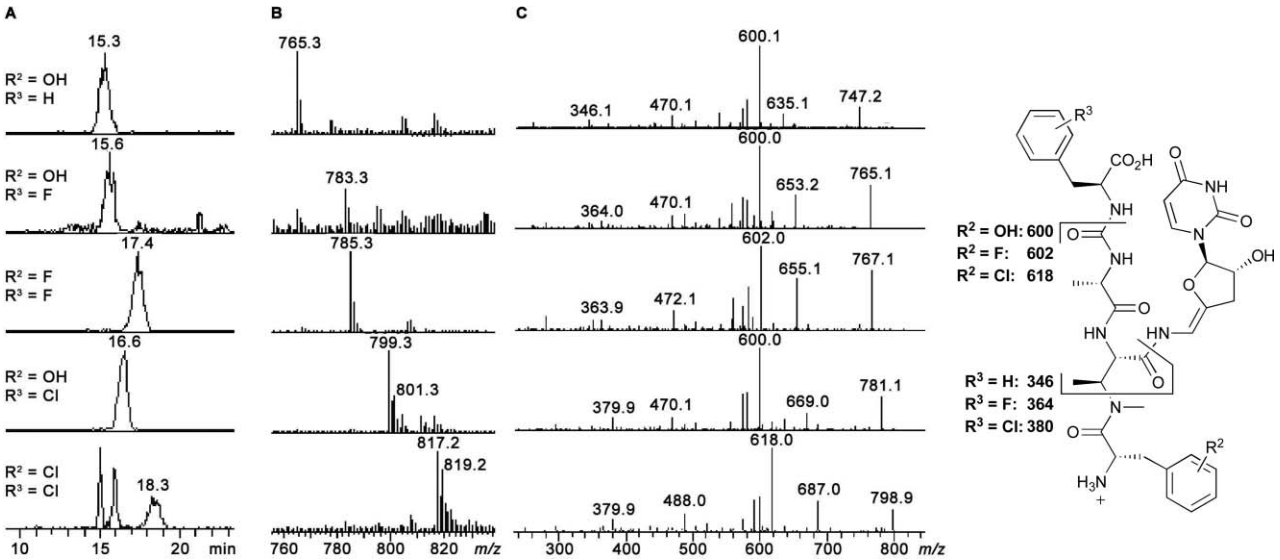


**Fig. 2** LC-MS/MS analysis of halophenylalanine-supplemented *S. coeruleorubidus* culture extracts. From top to bottom: pacidamycin 5, mono- and disubstituted pacidamycin 5 analogues generated from supplementation with 3-fluorophenylalanine and from supplementation with 2-chlorophenylalanine, respectively. A) Extracted ion chromatograms for the expected molecular ions. Retention times are indicated. B) MS spectra showing molecular ion peaks. Spectra were averaged over the relevant retention time. C) MS/MS analysis of pacidamycin 5 analogues. Expected diagnostic molecular fragments are indicated. The fragment ions with *m/z* 747, 765, 767, 781 and 799 correspond to the loss of water from the respective molecular ions.

the *ortho* and *meta* positions. Double incorporation was greatly affected by the size and position of the substituent as discussed above, whereas single incorporation of phenylalanine analogues at the C-terminus seems less sensitive to these factors (Table 1).

In order to gain a greater understanding of the impact on bioactivity and bioavailability of introducing halogen substituents, fermentations with 3-fluorophenylalanine and with 2-chlorophenylalanine were scaled up in order to obtain sufficient material for further testing. Only the disubstituted [3-F-Phe]$_2$-pacidamycin was isolated because the monosubstituted derivative co-eluted with pacidamycin 5 under all conditions tested. Insufficient [2-Cl-Phe]$_2$-pacidamycin was produced to warrant isolation of this compound. Purified [2-Cl-Phe]- and [3-F-Phe]$_2$-pacidamycin analogues as well as pacidamycin 5T [3-OH-Phe]$_2$ pacidamycin (Fig. 1) were bioassayed against *Pseudomonas aeruginosa* ATCC 15442 and *Escherichia coli* DSM 1103. Pacidamycin 5T was included as a reference as it is the closest structural homologue to [3-F-Phe]$_2$-pacidamycin with its *m*-tyrosine-derived hydroxyl groups being replaced by fluorine atoms in the latter.

Pacidamycin 5T, when tested against *P. aeruginosa*, displayed a minimum inhibitory concentration (MIC) of 32 μg ml$^{-1}$. [2-Cl-Phe]-pacidamycin exhibited a two-fold decrease in activity (MIC 64 μg ml$^{-1}$) whilst [3-F-Phe]$_2$-pacidamycin exhibited no antibiotic activity against the tested organisms (up to 2 mg ml$^{-1}$). All three compounds were inactive against *E. coli*.

The inactivity of the difluorinated pacidamycin analogue may be due to the significantly changed lipophilicity of this compound resulting in decreased penetration through the bacterial cell membrane. However, it can equally be argued that the presence of the substituents affects binding of the molecule to its cellular target, translocase I, or that the derivatives are more susceptible to cell metabolism or efflux. It would be of interest to assay pacidamycin derivatives directly against translocase I in order to determine whether uptake or binding to the enzyme is the limiting factor in the activity of these compounds.

To conclude, it has been demonstrated that there is striking flexibility in the biosynthesis of pacidamycins, and that both the N- and C-termini of the pseudotetrapeptide tolerate incorporation of more sterically challenging analogues. Strikingly high levels of double incorporation are observed. Through precursor-directed biosynthesis with a series of halophenylalanine, we have demonstrated the generation of four new halopacidamycin analogues. Larger scale fermentations enabled the preparation of 2 mg quantities of material for NMR analysis and for the assessment of antibiotic activity against *E. coli* and *P. aeruginosa*.

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