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

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1) Media

ISP2 liquid medium: Yeast extract (0.4 %), Malt extract (1%), Glucose monohydrate (0.4%).

Lactose Minimal Media (Lac MM): NH₄Cl (40 mM), MgSO₄ (2.4 mM), K₂HPO₄ (25 mM), MOPS (100 mM), FeSO₄·7H₂O (10 mg L⁻¹), MnCl₂·4H₂O (10 mg L⁻¹), ZnSO₄·7H₂O (10 mg L⁻¹), CaCl₂ (10 mg L⁻¹), lactose (1% w/v).

2) Strains

Streptomyces coeruleorubidus AB1183F-64 (producer of pacidamycins) and Pseudomonas aeruginosa ATCC 15442 were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Escherichia coli DSM 1103 was purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, D).

3) Experimental details

Feeding protocol: Starter cultures in ISP2 medium were inoculated with S. coeruleorubidus spores to 10⁶ cfu ml⁻¹ and incubated at 28°C, 180 rpm for 48 hours. Production medium (Lac MM) was inoculated with 5% starter culture and incubated at 28°C, 180 rpm for 72 hours. A sterile, pH-neutral aqueous solution of the phenylalanine derivative of interest was added to the main culture to a final concentration of 1 mM. Incubation was continued for another 48 hours. Pacidamycins were extracted from the cell-free broth using XAD-16 resin (5% v/v). The resin was washed with 20 volumes of water and the extract was eluted with 10 volumes of methanol. The solvent was removed in vacuo.

Isolation and analysis of pacidamycins: For small scale (10 ml) main cultures, the residue was redissoled in 100 μl water/methanol 1:1 prior to LC-MS analysis. LC-MS analysis showed that feeding of phenylalanine shifts the pacidamycin production profile towards pacidamycin 5, which was dominant, pacidamycin 4 and 5T were also detected. Pacidamycins produced by large scale (2 L) cultures were purified and isolated by RP-HPLC using a C₁₈(2) Luna column (Phenomenex, Macclesfield, UK; 250×21.2 mm, 5μm) with a gradient of 35-90% solvent B over 30 min at a flow rate of 4ml/min (buffer A: 0.1 M ammonium acetate, pH 7.9; buffer B: methanol). The fractions of interest were desalted on the same column using a water/methanol gradient. The yield was 1 mg L⁻¹ and 1.2 mg L⁻¹ for the purified difluoro- and monochloropacidamycin analogues, respectively.

Bioassay: The compounds were bioassayed using broth microdilution protocol. Bacterial inoculum was made by 100 fold dilution of the overnight culture of the test organism in TSB broth. 100 μl aliquots were dispensed in each well of a 96-well plate. Each row of the plate was used for one of the compounds to be tested. To the first well in the row, added 100 μl of 2.048 mg/ml test compound in bacterial inoculum, mixed and 2 fold dilution was made across the wells in the same row. The plates were incubated at the specific temperatures for 18 hrs. Nalidixic acid was used as a positive control with an MIC value of 128 μg/ml. The negative control was water plus the test organism.
4) LC-MS/MS analysis

Extracted ion chromatograms

Supplementation with 2-fluorophenylalanine:
Supplementation with 3-fluorophenylalanine:

Supplementation with 4-fluorophenylalanine:
Comparison of incorporation of fluorophenylalanine derivatives at C-terminus:

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RT: 0.00 - 31.53

2-Fluoro

NL: 1.01E8
m/z: 782.5-783.5
F: + c ESI Full ms
[100.00-2000.00] MS 03c03

3-Fluoro

NL: 9.56E7
m/z: 782.5-783.5
F: + c ESI Full ms
[100.00-2000.00] MS 03c04

4-Fluoro

NL: 1.04E8
m/z: 782.5-783.5
F: + c ESI Full ms
[100.00-2000.00] MS 03c05

Comparison of incorporation of fluorophenylalanine derivatives at N- and C-termini:

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RT: 0.00 - 31.53

2-Fluoro

NL: 3.71E8
m/z: 784.5-785.5
F: + c ESI Full ms
[100.00-2000.00] MS 03c03

3-Fluoro

NL: 4.72E8
m/z: 784.5-785.5
F: + c ESI Full ms
[100.00-2000.00] MS 03c04

4-Fluoro

NL: 1.17E7
m/z: 784.5-785.5
F: + c ESI Full ms
[100.00-2000.00] MS 03c05
Supplementation of 2-chlorophenylalanine:

Pacidamycin 5

Incorporation at C terminus

Incorporation at C&N termini

Incorporation at N terminus
**MS/MS analysis**

Pacidamycin 5:

![Chemical structure of Pacidamycin 5](image)
Expected fragmentation pattern of N-termini only fluoro-substituted pacidamycins:

\[ [M^+ + 1] = 767 \]

From 2-fluorophenylalanine feeding

From 3-fluorophenylalanine feeding

From 4-fluorophenylalanine feeding
Expected fragmentation pattern of N- termini only chloro-substituted pacidamycins:

MS² data are not confirmative

From 2-chlorophenylalanine feeding

The key fragment 602 was not detected.
Expected fragmentation pattern of C-termini only fluo-substituted pacidamycins:
Expected fragmentation pattern of C- termini only chloro-substituted pacidamycins:

\[ [M^+ + 1] = 799 \]
Expected fragmentation patterns of N- and C-termini fluoro-substituted pacidamycins:
Expected fragmentation patterns of N- and C-termini chloro-substituted pacidamycins:

\[ [M^+ + 1] = 817 \]
Analysis

Key fragments of pacidamycin 5 were used for confirmation of the results. These fragments are: $m/z$ 600 for the loss of the C-terminal amino acid, $m/z$ 602 for the loss of the N-terminal amino acid, and $m/z$ 263 for the C-terminal pseudo-dipeptide fragment. The incorporation of fluorophenylalanine at the C-terminus shifts the $m/z$ 602 fragment to $m/z$ 620, and the $m/z$ 263 fragment to $m/z$ 281. The incorporation of chlorophenylalanine at the C-terminus causes a shift of the $m/z$ 602 fragment to $m/z$ 636, and a shift of the $m/z$ 263 fragment to $m/z$ 297. The $m/z$ 600 product ion remains unchanged in both cases. Double incorporation of halophenylalanine at both the C- and N-termini shifts all three selected key fragments. The double incorporation of fluorophenylalanine results in $m/z$ 602 and $m/z$ 620 product ions instead of $m/z$ 600 and $m/z$ 602 fragments, respectively. The $m/z$ 263 product ion is shifted to $m/z$ 281. The double incorporation of chlorophenylalanine shifts the $m/z$ 600 fragment to $m/z$ 618, and the $m/z$ 602 fragment to $m/z$ 636. The $m/z$ 263 product ion is shifted to 297.
4) NMR Analysis ((400 MHz))

$\textsuperscript{1}H$NMR spectrum of [3-F-Phe]-pacidamycin, freeze-dried from 10mM NaOH, redissolved in D$_2$O:

$\textsuperscript{1}$HNMR spectra of pacidamycins are complex due to the presence of several conformational states as reported by R. H. Chen, A. M. Buko, D. N. Whittern and J. B. McAlpine, J. Antibiot., 1989, 42, 512.
$^{19}$F-NMR spectrum of [3-F-Phe]$_2$-pacidamycin, freeze-dried from 10mM NaOH, redissolved in D$_2$O:

$^{19}$F-NMR reference spectrum of 3-F-phenylalanine, dissolved in D$_2$O:
The fluorine atom is represented by a triplet of doublets (-112.4 ppm) in the $^{19}$F-NMR spectrum of 3-fluorophenylalanine. The same pattern and a similar chemical shift were observed in the $^{19}$F-NMR spectrum of difluoropacidamycin. Other similar peaks in the same spectrum could be due to the different conformers originating from the restricted rotation around the amide bonds. The two fluorine atoms in the difluorinated pacidamycin are represented by one peak suggesting chemical and magnetic equivalence.