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

Christopher Cartmell, Antoine Abou Fayad, Rosemary Lynch, Sunil V. Sharma, Nils Hauck, Bertolt Gust, and Rebecca J. M. Goss*
Author Contributions

C.C. Investigation: Lead; Methodology: Supporting; Writing – review & editing: Supporting
Supporting information

1) General information and materials  pages 1-2
2) Experimental procedures  pages 2-6
3) Spectroscopic data  pages 6-47

I. General Information and Materials

Chemical and biological reagents and components for media, buffers and stock solutions were purchased from commercial suppliers, used without further purification and stored according to the suppliers’ instructions. Microorganisms were stored at -20 °C for frequent use and -80 °C for long-term storage. *Streptomyces* species were stored as spore suspensions in 20% (v/v) aqueous glycerol. Microorganisms were cultured under sterile conditions using a Faster BH-EN class II vertical laminar airflow cabinet and fermentation media was sterilised at 121 °C for 20 min at 1.3 bar in a Boxer Benchtop Denley autoclave prior to use. Alternatively, aqueous solutions of heat labile components were sterilised by passage through a 0.2 μm membrane. Culturing apparatus was sterilised by autoclaving as described above or alternatively, disposable pre-sterilised apparatus was used.

General apparatus: Pipetting of solutions and samples was done using LABNET Biopette autoclavable pipettes. Microbial cultures were incubated in a New Brunswick Scientific Innova 4300 incubator shaker, a New Brunswick Scientific I26 incubator shaker series, or Genlab incubator (static). pH measurements were taken using a Fisherbrand Hydrus 300 pH meter. Centrifugation was carried out using a Thermo Scientific IEC CL30R centrifuge or a Fisher Scientific accuSpin microcentrifuge. PCR reactions were carried out using a Techne TC-512 thermocycler. Mass spectrometry was carried out using a Shimadzu single quadrupole LC-mass spectrometer equipped with an electrospray ionisation source at the University of East Anglia, while at the University of St Andrews, LC-MS runs were performed using a Waters 2795 HPLC reflecting TOF LC-mass spectrometer equipped with a Z-flow atmospheric pressure ESI source coupled to a Waters 2996 photodiode array detector. Extracts from cultures and purified compounds were freeze-dried using an SCAVAC cool safe freeze-drier equipped with a vacuubrand chemistry hybrid pump RC6.

NMR measurements were recorded using an Ascend 500 or Ascend 700 spectrometer. Deuterated NMR solvents were purchased from Sigma Aldrich or Fluorochem. Chemical shifts (δ) are reported in ppm relative to internal reference peaks arising from methanol (δ = 3.31). Preparative HPLC was performed using a Gilson 322 pump, 151 UV/VIS detector and 233XL fraction collector.
2.1 Culture Media and Buffer Preparation

Components were purchased from BD Biosciences, Melford, ForMedium, Fisher Scientific, Sigma-Aldrich or Alfa-Aesar unless stated otherwise.

| Medium/Buffer   | Composition                                                                 |
|-----------------|----------------------------------------------------------------------------|
| ISP2            | 0.4% (w/v) yeast extract, 1% (w/v) malt extract, 0.4% (w/v) D-glucose monohydrate, pH 7.5 (2 M NaOH), autoclaved. |
| 2×YT            | 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% NaCl, autoclaved.         |
| SFM             | 2% (w/v) mannitol and tap water then heat at 50 °C for 2 h followed by 2% (w/v) soya flour, autoclaved twice. |
| Medium B        | 20% D-maltose (w/v), 5% (v/v) trace elements, tap water, autoclaved.        |
| Trace elements  | 1000x 2.86% H₃BO₄ (w/v), 1.81% MnCl₂ (w/v), 0.22% ZnSO₄ (w/v), 0.39% Na₂MoO₄ (w/v), 0.08% CuSO₄ (w/v), 0.05% Co(NO₃)₂ (w/v), before use solution was diluted 1000 fold. |

2.2 Spore stock preparation and storage

*Streptomyces* spores were harvested from *Streptomyces* SFM agar cultures after 10 – 14 days (at 28 °C) by suspension in sterile distilled water and then collected by centrifugation (1500 × g, 20 min, 4 °C) and re-suspended in 20% (v/v) aqueous glycerol before storage at -20 °C.

2.3 Production of *S. coeruleorubidus* NRRL18370/pNH07

*npsM*, encoding for a putative transcriptional regulator in the napsamycin gene cluster¹, was ordered as gBlock® (Integrated DNA Technologies, USA) and cloned into the EcoRV and SpeI sites of the integrative plasmid pSET152² under control of the strong constitutive promotor *PermE* to generate plasmid pNH07. Clones were verified by restriction analysis and sequencing with primers npsMF (ACGCCTGGTCGATGTCGGA) and npsMR (GAGCGAGGAAGCGGAAGAGC). DNA of plasmid pNH07 was passed through the non-methylating host *E. coli* ET12567/pUZ8002³ and conjugated into *S. coeruleorubidus* NRRL18370 using mycelia of a culture grown for three days in TSB media. *S. coeruleorubidus* NRRL18370/pNH07 exconjugants were confirmed via colony-PCR with
primers NpsMCF (ATCGCGTCCATGACCTTCAGGC) and NpsMCR (ATCTGTACGCGCCGCTAGC).

2.4 Culturing *S. coeruleorubidus* NRRL18370/pNH07 for pacidamycin production

Seed cultures of *S. coeruleorubidus* were prepared by inoculating medium 2 x YT (4 mL) in sterilised glass 10 mL vials fitted with a stainless steel spring for aeration and to avoid mycelial clumping with 1% (v/v) of a spore stock stored at -20 °C containing >10⁹ cfu/mL or 2-5% (v/v) of glycerol mycelium stock. Cultures were incubated in a shaking incubator (28 °C, 180-210 rpm, 2.54 cm incubator throw) for 2-3 days. Starter cultures were prepared by inoculating medium 2 x YT (50 mL) in 250 mL baffled flask with 5-10% (v/v) of disperse, well-grown seed culture and incubated as above, under the same conditions as above for 2-3 days. Large scale fermentation cultures were prepared in 2 L baffled flask and the fermentation media (500 mL ISP2 or medium B) inoculated with 5-10% (v/v) starter culture, and incubated as above for 8 days. LC-HRMS was used to confirm the presence of the natural product pacidamycin 4 (1).

2.5 Purification of Pacidamycin

After fermentation the supernatant was collected by centrifugation (JLA 8.1000 rotor, 6800 g, 30 min, 4 °C) and incubated with 2% (v/v) XAD 16N resin overnight in a shaking incubator (200 rpm, 2.54 cm incubator throw, ambient temperature). The resin was collected by filtration and washed thoroughly with distilled water until washings were colourless. The resin was transferred to a glass column and the adsorbed compounds were eluted using 100% methanol until resin and washing was clear. Solvent was removed under reduced pressure (<40 °C). The crude extract obtained from XAD 16N resin was then purified by ion-exchange chromatography using a 5 mL HiTrap™ SP-XL column (GE Healthcare). After loading, the column was washed with 6 column volumes (CV) (30 mL) of ion exchange buffer A (50 mM sodium acetate, pH 3.6). Pacidamycins were eluted using ion exchange buffer A (50 mM sodium acetate, pH 3.6) and ion exchange buffer B (50 mM sodium acetate, pH 5.6) stepwise gradient (0, 20, 40, 60, 80 100% ion exchange buffer B in ion exchange buffer A) collecting 10 mL fractions. Pacidamycin 4 containing fractions (eluted between 40 and 60% ion exchange buffer B) were combined and further purified by preparative HPLC using Luna C18(2) RP column (250 × 21.2 mm 4 µm, Phenomenex) using a gradient of 15% Solvent B to 90% solvent B over 60 min with a flow rate of 15 mL/min (Solvent A: 0.1 M ammonium acetate, pH 7.9; Solvent B: methanol) with UV monitoring at 280 nm. Desalting was achieved by passing fractions of interest through the same column using a water / methanol gradient. Pacidamycin 4 eluted after approximately 21 min with a final yield of ~1.2 mg/L.
2.6 General method for LC-HRMS analysis

For LC-HRMS analysis of the cross-coupling reactions, the HPLC separation was conducted on a Thermo Scientific™ Dionex™ Ultimate™ 3000 Rapid Separation LC system using Kinetix evo C18 column (130Å, 3.5 µm, 2.1 × 100 mm). The flow rate was set to 0.35 mL/min and the column temperature was maintained at 40 °C. A generic binary gradient elution was carried out using different ratios of eluents A (water containing 0.1% formic acid) and B (acetonitrile). Following gradient was used: 0-0.5 min (5% B), 0.5-9.5 min (5% to 95% B), 9.5-11.5 min (95% B), 11.5-12.0 min (95% to 5% B) and 12.0-15.0 min (5% B). The total LC run time was 15 min. The effluent from the HPLC was directly electrosprayed into the mass spectrometer. The mass spectrometric analysis was performed on an Orbitrap Velos Pro™ mass spectrometer system equipped with a Thermo Scientific Ion MAX API source housing. The MS conditions were as follows: heated electrospray ionization (HESI-II) probe, positive ionization mode, spray voltage 3.5 kV, capillary temperature 350 °C, normalized collision energy 35% for collision induced dissociation (CID), and sheath gas and auxiliary gas flow rates of 35 and 10 arbitrary units, respectively. Survey full scan MS spectra (from m/z 150-1500) were acquired in the orbitrap with resolution R=60K.

2.7 General Pictet-Spengler reaction on L-meta-tyrosine

In a sealed glass vial equipped with a stirrer bar, L-meta-tyrosine (20 mg, 0.11 mmol, 1 equiv) was suspended in phosphate buffer (1 mL, 100 mM, pH 6). The desired benzaldehyde (0.13 mmol, 1.2 equiv) was added in acetonitrile (1 mL) and the reaction left to stir overnight at 50 °C. Solvent was evaporated under reduced pressure (Genevac) and the resultant product re-suspended in 50% aqueous methanol (1 mL) before loading onto a Snap C18 12g Biotage cartridge. Purification was achieved, eluting at 12 mL/min using an initial composition of 95% Solvent A (water): 5% Solvent B (methanol) for 3 column volumes (CV) followed by a gradient to 95% solvent B over 15 CV. This composition was held for 3 CV before returning to initial conditions.

2.8 General Pictet-Spengler reaction on DL-meta-tyrosine

In a sealed glass vial fitted with a stirrer bar, DL-meta-tyrosine (20 mg, 0.11 mmol, 1 equiv) was suspended in phosphate buffer (1 mL, 100 mM, pH 6). The desired benzaldehyde (0.13 mmol, 1.2 equiv) was added in acetonitrile (1 mL) and the reaction was left to stir overnight at 50 °C. Reverse phase purification was achieved as described in section 2.7.

2.9 LC-HRMS analysis of Pictet-Spengler Diastereomer separation

LC-HRMS was performed using the Thermo Orbitrap Velos Pro system equipped with a phenomnex kinetix evo C18 held at 40 °C. An injection volume of 10 µL was used for all samples. Analytes were eluted using a gradient of 5% acetonitrile: 95%, 0.1% formic acid MQ water to 95% acetonitrile: 5%,
0.1% formic acid MQ water over 15 min. Eluent from the column was passed through a PDA monitoring absorbance from 220-800 nm (2 nm resolution, 10 Hz) along with a single channel UV measuring at 254 nm.

2.10 Chiral LC-HRMS of Pictet-Spengler Diastereomer separation

Chiral LC-HRMS was performed using the Thermo Orbitrap Velos Pro system equipped with a phenomenex lux 3 µm Cellulose-4 LC column 150 × 3.0 mm held at 40 °C. An injection volume of 10 µL was used for all samples. Analytes were eluted using an isocratic gradient of 20% acetonitrile: 80%, 0.1% formic acid MQ water over 15 min.

2.11 General Pictet-Spengler reaction on pacidamycin

In a sealed glass vial equipped with a stirrer bar, purified pacidamycin 4 (~1 mg, 1.2 µmol, 1 equiv) was suspended in phosphate buffer (1 mL, 100 mM, pH 6). The desired benzaldehyde (50 µmol in 1 mL acetonitrile) was added. The mixture was heated to 50 °C overnight, solvent was evaporated under reduced pressure (Genevac) and the resultant product resuspended in 50% aqueous methanol before being subjected to reverse phase HPLC purification as described in section 2.12.

2.12 HPLC method for purification of Pictet-Spengler modified pacidamycins

The Pictet-Spengler modified pacidamycin mixture was suspended in 50% aqueous methanol (1 mL) and sonicated for 30 min. The suspension was centrifuged (16060 g, 5 min) and the supernatant was taken for HPLC purification. Purification was achieved using a 250 × 21.2 mm 4 µm Luna C18(2) RP column (Phenomenex) with a gradient of 15% solvent B to 90% solvent B over 60 min with a flow rate of 15 mL/min (buffer A: 0.1 M ammonium acetate, pH 7.9; buffer B: methanol) with UV monitoring at 280 nm. Desalting was achieved by passing fractions of interest through the same column using a water/ methanol gradient. Elution was monitored at 280 nm.

2.13 General NMR technique for pacidamycin 4 (1) and pacidamycin derivatives (15-17b)

NMR analysis for pacidamycin 4 (1) and pacidamycin Pictet-Spengler derivatives (15-17b) was carried on a Bruker Ascend 700 MHz NMR. Due to small amounts of isolated material, all samples were dissolved in 100 µL of CD3OD and transferred to a 1.7 mm capillary NMR tube. Analysis of which presented two HOD peaks located at 4.6 and 4.9 ppm as well as a methanol solvent peak at 3.3 ppm. Impurities from formic acid and acetonitrile present from HPLC purification were observed at 7.9 and 1.95 ppm respectively.
3.0 Characterisation Pacidamycin 4 (1)

\(^1\)H NMR (500 MHz, CD\(_3\)OD) of 1

\(^1\)H-NMR of pacidamycin 4 (1) isolated from *S. coeruleorubidus* NRRL18370/pNH07. \(^1\)H-NMR analysis of a series of natural pacidamycin have been previously reported. It should be noted that NMR spectra of pacidamycins are complicated by the peptic nature of the compounds. Pacidamycins exist as conformers in solution that exchange slowly on the NMR time scale.\(^5\) The clearly visible singlets for the \(N\)-methyl group (2.6 and 2.64 ppm) and the doublets for one of the uracil protons (5.6 and 5.77 ppm) are good diagnostic peaks. The ratio and rate of exchange between conformers are dependent on salt concentration and pH of the sample.\(^4,5\)
COSY NMR (500 MHz, CD$_3$OD) of 1

HSQC NMR (500 MHz, CD$_3$OD) of 1
3.1 Characterisation of 5-bromosalicylaldehyde derivative of pacidamycin 4 (15)

Following the general Pictet-Spengler reaction on pacidamycin 4 and purification the desired product was isolated in a 73% yield, 0.9 mg.

$^1$H NMR (700 MHz, CD$_3$OD) $\delta$ 7.66 (d, $J = 7.9$ Hz, 1H), 7.63 (d, $J = 8.0$ Hz, 1H), 7.42 – 7.41 (m, 2H), 7.39 (d, $J = 2.5$ Hz, 1H), 7.35 (d, $J = 2.5$ Hz, 1H), 7.27 (s, 1H), 7.12 (d, $J = 10.7$ Hz, 1H), 7.02 (t, $J = 7.5$ Hz, 1H), 6.75 (d, $J = 8.6$ Hz, 1H), 6.72 (d, $J = 8.6$ Hz, 1H), 6.62 (d, $J = 2.4$ Hz, 1H), 6.06 (d, $J = 2.9$ Hz, 1H), 6.04 (s, 1H), 5.96 (s, 1H), 5.80 (d, $J = 8.1$ Hz, 1H), 5.59 (d, $J = 8.1$ Hz, 1H), 4.54 (dd, $J = 4.5$, 1.8 Hz, 1H), 4.42 – 4.34 (m, 1H), 4.09 (broad m, 1H), 4.05 (broad m, 1H), 3.02 (dd, $J = 15.7$, 5.9 Hz, 2H), 2.94 – 2.91 (m, 1H), 2.85 (s, 3H), 2.77 (dd, $J = 6.5$, 1.8 Hz, 1H), 2.70 – 2.63 (m, 1H), 2.56 (dd, $J = 4.3$, 1.5 Hz, 1H), 1.28 (d, $J = 6.6$ Hz, 3H), 1.18 (d, $J = 7.1$ Hz, 3H).
LC-HRMS results

(A) LC trace depicting TIC, EIC for pacidamycin 4 (I) and EIC for compound 15. (B) Accurate mass spectra for compound 15 with retention time 4.24 min. (C) HCD MS² fragmentation spectra for compound 15 using C₄₅H₄₇Br₉N₉O₁₂⁺ isotope.
Fragmentation

Observed fragments from HCD MS² spectra for compound 15.
Expansion showing the aromatic region, the inserted stereogenic centre (A), sugar doublet (B), and uracil doublet (C)
Comparison of the key region of interest in $^1$H-NMR of the 5-bromosalicylaldehyde derivative of pacidamycin 4 15 with the small molecule 5-bromosalicylaldehyde diastereomers 19a-19b.

**Graph:**

- **Compounds 19a-19b**
- **Compound 15**

![Graph showing NMR spectroscopy comparison](image)
3.2 Characterisation of the 4-bromobenzaldehyde derivative of pacidamycin 4 16a-16b

Following the General Pictet-Spengler reaction on pacidamycin 4 procedure and purification the desired product was isolated in a 49% yield, 0.6 mg.

Major Diastereomer: $^1$H NMR (700 MHz, CD$_3$OD) $\delta$ 7.67 (d, $J = 7.8$ Hz, 1H), 7.50 (dd, $J = 8.3$, 5.1 Hz, 2H), 7.42 – 7.38 (m, 2H), 7.28 (d, $J = 8.4$ Hz, 2H), 7.10 (s, 1H), 6.70 (d, $J = 8.4$ Hz, 2H), 6.60 (d, $J = 2.5$ Hz, 1H), 6.55 – 6.50 (m, 2H), 6.03 (s, 1H), 5.74 (d, $J = 8.1$ Hz, 1H), 5.67 (s, 1H), 5.62 (d, $J = 8.1$ Hz, 1H), 4.55 – 4.51 (m, 1H), 4.45 – 4.38 (m, 2H), 4.17 (broad m, 1H), 4.08 (broad m, 1H), 3.18 – 3.12 (broad m, 1H), 3.02 – 2.98 (m, 1H), 2.90 – 2.86 (m, 1H), 2.83 (s, 3H), 2.66 – 2.62 (m, 1H), 2.56 (dd, $J = 12.9$, 1.2 Hz, 1H), 1.17 (d, $J = 7.1$ Hz, 3H), 1.11 (d, $J = 7.3$ Hz, 3H).

Minor Diastereomer: $^1$H NMR (700 MHz, CD$_3$OD) 7.65 (d, $J = 7.7$ Hz, 1H), 7.45 (dd, $J = 8.1$, 5.2 Hz, 2H), 7.42 – 7.38 (m, 2H) 7.21 (d, $J = 8.1$ Hz, 2H), 7.13 (s, 1H), 6.65 (d, $J = 8.4$ Hz, 2H), 6.59 (d, $J = 2.5$ Hz, 1H), 6.45 (dd, $J = 8.5$, 4.8 Hz, 2H), 5.96 (s, 1H), 5.78 (d, $J = 8.1$ Hz, 1H), 5.59 (d, $J = 8.1$ Hz, 1H), 4.05 - 4.00 (m, 1H) 3.77 (m, 1H) 3.17-3.15 (m, 2H), 2.92-2.90 (m, 2H) 2.79 – 2.77 (m, 1H) 2.76 (s, 3H), 2.54 (dd, $J = 12.9$, 1.2 Hz, 1H) 1.26 (d, $J = 6.7$ Hz, 3H), 1.15 (d, $J = 6.2$ Hz, 3H)
LC-HRMS results

(A) LC trace depicting TIC, EIC for pacidamycin 4 starting material and EIC for compound 16a-16b. (B) Accurate mass spectra for compound 16a-16b with retention time 4.32 min. (C) HCD MS² fragmentation spectra for compound 16a-16b using C_{45}H_{49}^{79}BrN_{9}O_{11} + isotope.
Fragmentation

Observed fragments from HCD MS² spectra for compound 16a-16b.
Comparison of the key region of interest in $^1$H-NMR of the 4-bromobenzaldehyde derivative of pacidamycin 4 16a-16b with the small molecule 4-bromobenzaldehyde diastereomers 20a-20b.
3.3 Characterisation of 4-bromo-3-nitrobenzaldehyde derivative of pacidamycin 4 **17a-17b**

Following the general Pictet-Spengler reaction on pacidamycin 4 procedure and purification the desired product was isolated in a 61% yield, 0.77 mg.

Major Diastereomer: $^1$H NMR (700 MHz, CD$_3$OD) $\delta$ 7.90 (d, $J = 2.0$ Hz, 1H), 7.77 (d, $J = 8.3$ Hz, 1H), 7.65 (d, $J = 7.9$ Hz, 1H), 7.51 (dd, $J = 8.4$, 2.1 Hz, 1H), 7.39 (d, $J = 8.0$ Hz, 1H), 7.25 (d, $J = 8.1$ Hz, 1H), 7.12 (s, 1H), 7.10 (s, 1H), 6.64 (d, $J = 2.5$ Hz, 1H), 6.55 (d, $J = 2.5$ Hz, 1H), 6.46 (d, $J = 3.2$ Hz, 1H), 6.07 – 6.01 (m, 2H), 5.93 (s, 1H), 5.74 (d, $J = 8.1$ Hz, 1H), 5.59 (d, $J = 8.1$ Hz, 1H), 4.54 – 4.51 (m, 2H) 4.30 (dd, $J = 11.6$, 3.5 Hz, 1H), 3.97 (d, $J = 10.9$ Hz, 1H), 3.15 – 3.12 (m, 1H), 3.06 – 2.98 (m, 2H), 2.83 (s, 3H), 2.65 – 2.59 (m, 1H) 2.59 – 2.50 (m, 1H), 1.25 (d, $J = 6.6$ Hz, 3H), 1.16 (d, $J = 7.0$ Hz, 3H)

Minor Diastereomer: $^1$H NMR (700 MHz, CD$_3$OD) $\delta$ 7.83 (d, $J = 2.1$ Hz, 1H), 7.75 (d, $J = 8.2$ Hz, 1H), 7.72 (d, $J = 8.3$ Hz, 1H), 7.62 (d, $J = 7.8$ Hz, 1H), 7.44 (d, $J = 9.5$ Hz, 1H), 7.23 (d, $J = 8.1$ Hz, 2H), 7.11 (s, 1H), 7.01 – 6.92 (m, 2H) 6.62 (d, $J = 2.5$ Hz, 1H), 6.54 (d, $J = 2.5$ Hz, 1H), 6.45 (d, $J = 3.2$ Hz, 1H), 5.99 (s, 1H), 5.79 (d, $J = 7.9$ Hz, 1H), 5.69 (s, 1H), 5.62 (d, $J = 8.1$ Hz, 1H), 4.10–4.02 (m, 2H), 3.97 (d, $J = 10.9$ Hz, 1H), 3.18 – 3.16 (m, 2H), 3.15 – 3.12 (m, 1H), 2.89 (s, 3H), 2.64 – 2.62 (m, 1H), 1.23 (overlapping doublet, 3H), 1.17 (d, $J = 7.0$ Hz, 3H).
LC-HRMS results

(A) LC trace depicting TIC, EIC for pacidamycin 4 starting material and EIC for compound 17a-17b.
(B) Accurate mass spectra for compound 17a-17b with retention time 4.41 min. (C) HCD MS² fragmentation spectra for compound 17a-17b using C_{45}H_{48}^{79}BrN_{16}O_{13}⁺ isotope.
Fragmentation

Observed fragments from HCD MS² spectra for compound 17a-17b
$^1$H NMR (500 MHz, CD$_3$OD) of 17a–17b

Expansion showing the aromatic region, the inserted stereogenic centre (A, a), sugar doublet (B, b), and uracil doublet (C, c) (Major, minor diastereomers)
Comparison of the key region of interest in $^1$H-NMR of the 4-bromo-3-nitrobenzaldehyde derivative of pacidamycin 4 17a-17b with the small molecule 4-bromo-3-nitrobenzaldehyde diastereomers 21a-21b.
3.4 Characterisation of the 4-chlorobenzaldehyde derivative of pacidamycin 4 18a-18b

Following the General Pictet-Spengler reaction on pacidamycin 4 procedure and purification the desired product was not isolatable.

LC-HRMS results

Exact Mass: 926.3235
(A) LC trace depicting TIC, EIC for pacidamycin 4 starting material and EIC for compound 18a-18b.  
(B) Accurate mass spectra for compound 18a-18b with retention time 4.26 min. (C) HCD MS² fragmentation spectra for compound 18a-18b using C_{45}H_{49}^{35}ClN_{9}O_{11}⁺ isotope.

Fragmentation

![Fragmentation Diagram](image)

Exact Mass: 722.2336

Exact Mass: 696.2543

Exact Mass: 625.2172

Exact Mass: 302.1135

Observed fragments from HCD MS² spectra for compound 18a-18b.
3.5 Characterisation of reaction between DL-meta-tyrosine and 5-bromosalicylaldehyde to yield diastereomers 19a-19d

Following the general Pictet-Spengler reaction procedure and purification the desired diastereomic mixture was isolated in a 70% yield, 28 mg.

Major $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.44 (dd, $J$= 8.6, 2.5 Hz, 1H; Ar-H), 7.42 – 7.37 (m, 1H; Ar-H), 6.90 (d, $J$= 8.6 Hz, 1H; Ar-H), 6.72 (d, $J$= 2.5 Hz, 1H; Ar-H), 6.62 (dd, $J$= 8.5, 2.5 Hz, 1H; Ar-H), 6.58 (d, $J$= 8.5 Hz, 1H Ar-H), 5.68 (s, 1H; CH), 4.03 (dd, $J$= 10.0, 5.8 Hz, 1H; CH), 3.34 (dd, $J$= 16.8, 5.8 Hz, 1H; CH$_2$), 3.22 (dd, $J$= 16.8, 10.0 Hz, 1H; CH$_2$). $^{13}$C NMR (126 MHz CD$_3$OD) $\delta$ 173.4 (CO$_2$H), 158.8 (C), 156.4 (C), 135.4 (C), 134.7 (CH), 134.0 (CH), 129.0 (CH), 125.5 (C), 123.6 (C), 118.9 (CH), 115.9 (CH), 115.6 (CH), 112.7 (C), 58.2 (CH), 55.0 (CH), 31.1 (CH$_2$).

Minor $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.42 – 7.37 (m, 1H; Ar-H), 6.89 (d, $J$= 8.6, 2.5 Hz, 1H; Ar-H), 6.84 (d, $J$= 8.4 Hz, 1H; Ar-H), 6.80 (d, $J$= 2.4 Hz, 1H; Ar-H), 6.77 (d, $J$= 2.5 Hz, 1H; Ar-H), 6.75-6.71 (m, 1H; Ar-H), 5.99 (s, 1H; CH), 3.72 (dd, $J$= 10.3, 5.5 Hz, 1H; CH), 3.34 (dd, $J$= 17.3, 5.5 Hz, 1H; CH$_2$), 3.15 (dd, $J$= 17.3, 10.3 Hz, 1H CH$_2$). $^{13}$C NMR (126 MHz CD$_3$OD) $\delta$ 173.1 (CO$_2$H), 158.9 (C), 156.0 (C), 135.2 (C), 134.8 (CH), 134.4 (CH), 130.2 (CH), 126.6 (C), 121.3 (C), 118.5 (CH), 116.1 (CH), 116.1 (CH), 112.5 (C), 57.5 (CH), 54.1 (CH), 30.5 (CH$_2$).

HRMS (ESI +ve): Calculated for C$_{16}$H$_{15}$BrNO$_4^+$ = m/z 364.0179 Observed = m/z 364.0171.
Chiral HR-LCMS for compounds 19a-19d

(A) LC TIC trace for the chiral HR-LCMS for compounds 19a-19d. (B) Accurate mass spectra for compound 19a with retention time 2.40 min. (C) Accurate mass spectra for compound 19b with retention time 2.85 min. (D) Accurate mass spectra for compounds 19c-19d with retention time 3.40-3.90 min.
$^{1}$H NMR (500 MHz, CD$_3$OD) of 19a-19d

$^{13}$C NMR (126 MHz, CD$_3$OD) of 19a-19d
COSY NMR (500 MHz, CD$_3$OD) of 19a-19d

HSQC NMR (500 MHz, CD$_3$OD) of 19a-19d
HMBC NMR (500 MHz, CD$_3$OD) of 19a-19d

3.6 Characterisation of reaction between L-meta-tyrosine and 5-bromosalicylaldehyde to yield diastereomers 19a-19b

Following the general Pietet-Spengler reaction procedure and purification the expected diastereomic mixture was isolated in a 70% yield, 28 mg.

Major $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.39 (d, $J= 8.6$, 2.5 Hz, 1H; Ar-H), 6.88 (d, $J= 8.6$, Hz, 1H; Ar-H), 6.84 (d, $J= 8.5$ Hz, 1H; Ar-H), 6.79 (d, $J= 2.5$ Hz, 1H; Ar-H), 6.76 (d, $J= 2.5$ Hz, 1H; Ar-H), 6.73 (dd, $J= 8.5$, 2.5 Hz, 1H; Ar-H), 5.96 (s, 1H; CH), 3.71 (dd, $J= 10.3$, 5.5 Hz, 1H; CH), 3.40-3.32 (m, under solvent peak, 1H; CH$_2$), 3.15 (dd, $J= 17.3$, 10.3 Hz, 1H CH$_2$).

$^{13}$C NMR (126 MHz, CD$_3$OD) $\delta$ 177.3 (CO$_2$H), 158.9 (C), 154.3 (C), 135.4 (C), 134.6 (CH), 134.4
(CH), 130.2 (CH), 126.9 (C), 121.6 (C), 118.5 (CH), 116.1 (CH), 116.0 (CH), 112.4 (C), 55.0 (CH), 54.1 (CH), 30.6 (CH₂).

Minor ¹H NMR (500 MHz, CD₃OD) δ 7.43 (dd, J= 8.6, 2.5 Hz, 1H; Ar-H), 7.40-7.37 (m, 1H; Ar-H), 6.89 (d, J= 8.6 Hz, 1H; Ar-H), 6.72 (d, J= 2.5 Hz, 1H; Ar-H), 6.62 (dd, J= 8.5, 2.5 Hz, 1H; Ar-H), 6.58 (d, J= 8.5 Hz, 1H Ar-H), 5.66 (s, 1H; CH), 4.01 (dd, J= 10.0, 5.8 Hz, 1H; CH), 3.40-3.32 (m, under solvent peak, 1H; CH₂), 3.27-3.22 (m, 1H; CH₂)

¹³C NMR (126 MHz CD₃OD) δ 177.3 (CO₂H), 158.9 (C), 154.3 (C), 135.4 (C), 134.6 (CH), 134.4 (CH), 130.2 (CH), 126.9 (C), 121.6 (C), 118.5 (CH), 116.1 (CH), 116.0 (CH), 112.4 (C), 55.0 (CH), 54.1 (CH), 30.6 (CH₂).

HRMS (ESI +ve): Calculated for C₁₆H₁₅BrNO₄⁺ = m/z 364.0179 Observed = m/z 364.0170.

Chiral HR-LCMS for compounds 19a-19b

(A) LC trace for the chiral HR-LCMS for compounds 19a-19b. (B) Accurate mass spectra for compound 19a with retention time 2.39 min. (C) Accurate mass spectra for compound 19b with retention time 2.83-3.03 min.
$^{1}$H NMR (500 MHz, CD$_3$OD) of 19a-19b

$^{13}$C NMR (126 MHz, CD$_3$OD) of 19a-19b
COSY NMR (500 MHz, CD$_3$OD) of 19a-19b

HSQC NMR (500 MHz, CD$_3$OD) of 19a-19b
3.7 Characterisation of reaction between L-meta-tyrosine and 4-bromobenzaldehyde to yield diastereomers 20a-20b

Following the General Pictet-Spengler reaction procedure and purification the desired product was isolated in a 52% yield, 20 mg.

Major $^1$H NMR (500 MHz CD$_3$OD): $\delta$ 7.60 (d, $J= 8.5$ Hz, 2H; 2 × Ar-H), 7.23 (d, $J= 8.5$ Hz, 2H; 2 × Ar-H), 6.75 (d, $J= 8.6$ Hz, 1H; Ar-H), 6.74 (s, 1H; Ar-H), 6.67 (dd, $J= 8.6$, 2.6 Hz, 1H; Ar-H), 5.76 (s, 1H; CH), 3.83 (dd, $J= 9.7$, 5.6 Hz, 1H; CH) 3.38 (dd, $J= 17.4$, 5.6 Hz, 1H; CH$_2$), 3.19 (dd, $J= 17.4$, 9.7 Hz, 1H; CH$_2$).

$^{13}$C NMR (126 MHz, CD$_3$OD): $\delta$ 173.3 (CO$_2$H), 158.82 (C), 137.7 (C), 134.9 (C), 133.2 (CH), 133.1 (CH), 130.2 (CH), 124.7 (C), 122.6 (C), 116.2 (C), 115.8 (C), 58.7 (CH), 53.6 (CH), 30.6 (CH$_2$).

Minor $^1$H NMR (500 MHz CD$_3$OD): $\delta$ 7.65 (d, $J= 8.5$ Hz, 2H; 2 × Ar-H), 7.38 (d, $J= 8.5$ Hz, 2H; 2 × Ar-H), 6.72 (d, $J= 2.5$ Hz, 1H; Ar-H), 6.60 (dd, $J= 8.6$, 2.5 Hz, 1H; Ar-H), 6.48 (d, $J= 8.6$ Hz, 1H; Ar-H), 5.54 (s, 1H, CH), 4.02 (dd, $J= 12.2$, 5.1 Hz, 1H; CH) 3.41-3.35 (m, 1H; CH$_2$), 3.27-3.21 (m, 1H; CH$_2$).

$^{13}$C NMR (126 MHz, CD$_3$OD): $\delta$ 173.2 (CO$_2$H), 158.76 (C), 137.4 (C), 135.7 (C), 133.4 (CH), 132.9 (CH), 130.0 (CH), 124.8 (C), 123.9 (C), 115.9 (C), 115.7 (C), 62.0 (CH), 59.0 (CH), 31.3 (CH$_2$).

HRMS (ESI +ve): Calculated for C$_{16}$H$_{15}$BrNO$_3$ $^+$ = m/z 348.0230, Observed = m/z 348.0221.
Chiral HR-LCMS for compounds 20a-20b

(A) TIC LC trace for the chiral HR-LCMS for compound 20a-20b. (B) Accurate mass spectra for compound 20a-20b with retention time 3.67 min. (C) Accurate mass spectra for compound 20a-20b with retention time 5.15 min.
$^1$H NMR (500 MHz, CD$_3$OD) of 20a-20b

$^{13}$C NMR (126 MHz, CD$_3$OD) of 20a-20b
COSY NMR (500 MHz, CD$_3$OD) of 20a-20b

HSQC NMR (500 MHz, CD$_3$OD) of 20a-20b
HMBC NMR (500 MHz, CD$_3$OD) of 20a-20b
3.8 Characterisation of reaction between L-meta-tyrosine and 4-bromo-3-nitrobenzaldehyde to yield diastereomers 21a-21b

Following the General Pictet-Spengler reaction procedure and purification the desired product was isolated in a 65% yield, 28 mg.

Major $^1$H NMR (500 MHz, CD$_3$OD) δ 8.02 (d, $J$= 2.2 Hz, 1H; Ar-H), 8.00 (d, $J$= 8.3, 1H; Ar-H), 7.61 (dd, $J$= 8.3, 2.2, 1H; Ar-H), 6.77 (d, $J$= 2.6, 1H; Ar-H), 6.67 (dd, $J$= 8.6, 2.6, 1H; Ar-H), 6.54 (d, $J$= 8.6, 1H; Ar-H), 5.79 (s, 1H; CH), 4.53 (dd, $J$= 12.6, 5.2, 1H; CH), 3.50 (dd, $J$= 17.4, 5.2, 1H; CH$_2$), 3.36 (dd, under solvent peak, 1H; CH$_2$).

$^{13}$C NMR (126 MHz, CD$_3$OD) δ 170.8 (CO$_2$H), 159.3 (C), 151.8 (C), 138.8 (C), 137.1 (CH), 135.9 (CH), 134.4 (C), 130.1 (CH), 128.2 (CH), 122.2 (C), 116.62 (C), 116.3 (CH), 116.1 (CH), 61.4 (CH), 57.0 (CH), 30.3 (CH$_2$).

Minor $^1$H NMR (500 MHz, CD$_3$OD) δ 7.94 (d, $J$= 2.2 Hz, 1H; Ar-H), 7.90 (d, $J$= 8.3 Hz, 1H; Ar-H), 7.41 (dd, $J$= 8.3, 2.2 Hz, 1H; Ar-H), 6.83 (d, $J$= 8.5 Hz, 1H; Ar-H), 6.79 (d, $J$= 2.6 Hz, 1H; Ar-H), 6.74 (dd, $J$= 8.5, 2.6 Hz, 1H; Ar-H), 5.99 (s, 1H; CH), 4.24 (dd, $J$= 10.0, 5.6 Hz, 1H; CH), 3.51 (dd, $J$= 17.3, 5.6, 1H; CH$_2$), 3.26 (dd, $J$= 17.3, 10.0 Hz, 1H; CH$_2$). $^{13}$C NMR (126 MHz, CD$_3$OD) δ 171.0 (CO$_2$H), 159.4 (C), 151.7 (C), 139.1 (C), 136.8 (CH), 136.0 (CH), 133.6 (C), 130.4 (CH), 128.5 (CH), 120.9 (C), 116.9 (CH), 116.55 (C), 115.9 (CH), 57.8 (CH), 52.0 (CH), 29.7 (CH$_2$).

HRMS (ESI +ve): Calculated for C$_{16}$H$_{14}$BrN$_2$O$_5$ = m/z 393.0081, Observed = m/z 393.0079.
Chiral HR-LCMS for compounds 21a-21b

(A) Chiral TIC-LC trace for compound 21a-21b. (B) Chiral EIC-LC trace for compound 21a-21b. (C) Accurate mass spectra for compound 21a-21b with retention time 4.21 min.

$^1$H NMR (500 MHz, CD$_3$OD) of 21a-21b

---

39
$^{13}$C NMR (126 MHz, CD$_3$OD) of 21a-21b

COSY NMR (500 MHz, CD$_3$OD) of 21a-21b
HSQC NMR (500 MHz, CD$_3$OD) of 21a-21b

HMBC NMR (500 MHz, CD$_3$OD) of 21a-21b
3.9 Characterisation of reaction between L-meta -tyrosine and 4-chlorobenzaldehyde to yield diastereomers 22a-22b

Following the General Pictet-Spengler reaction procedure and purification the desired product was isolated in a 48% yield, 16 mg.

Major $^1$H NMR (500 MHz, CD$_3$OD) δ 7.49 (d, $J= 8.6$ Hz, 2H; 2 × Ar-H), 7.44 (d, $J= 8.6$ Hz, 2H; 2 × Ar-H), 6.72 (d, $J= 2.6$ Hz, 1H; Ar-H), 6.59 (dd, $J= 8.6$, 2.6 Hz, 1H; Ar-H), 6.48 (d, $J= 8.6$ Hz, 1H; Ar-H), 5.54 (s, 1H; CH), 4.02 (dd, $J= 12.2$, 5.0 Hz, 1H; CH), 3.38 (dd, $J= 17.2$, 5.0 Hz, 1H; CH), 3.24 (dd, $J= 17.2$, 5.0 Hz, 1H; CH$_2$).

$^{13}$C NMR (126 MHz, CD$_3$OD) δ 173.3 (CO$_2$H), 158.7 (C), 137.0 (C), 136.7 (C), 135.7 (C), 132.7 (CH), 130.30 (CH), 129.98 (CH), 124.1 (C), 115.9 (CH), 115.6 (CH), 62.0 (CH), 59.1 (CH), 31.3 (CH$_2$).

Minor $^1$H NMR (500 MHz, CD$_3$OD) δ 7.44 (d, $J= 8.6$ Hz, 2H; 2 × Ar-H), 7.29 (d, $J= 8.6$ Hz, 2H; 2 × Ar-H), 6.75 (d, $J= 8.5$ Hz, 1H; Ar-H), 6.74 (s, 1H; Ar-H), 6.67 (dd, $J= 8.5$, 2.6 Hz, 1H; Ar-H), 5.77 (s, 1H; CH), 3.85 (dd, $J= 9.1$, 4.2 Hz, 1H; CH), 3.49 (dd, $J= 15.3$, 4.2 Hz, 1H; CH$_2$), 3.17 (dd, $J= 15.3$, 9.1 Hz, 1H; CH$_2$).

$^{13}$C NMR (126 MHz, CD$_3$OD) δ 173.3 (CO$_2$H), 158.7 (C), 137.0 (C), 136.7 (C), 135.7 (C), 132.8 (CH), 130.22 (CH), 130.19 (CH), 124.1 (C), 116.1 (CH), 115.8 (CH), 58.6 (CH), 56.6 (CH), 28.4 (CH$_2$).

HRMS (ESI +ve): Calculated for C$_{16}$H$_{15}$ClNO$_3^+$ = m/z, 304.0735 Observed = m/z 304.0727.
Chiral HR-LCMS for compounds 22a-22b

(A) Chiral EIC-LC trace for compound 22a-22b.  (B) Chiral TIC-LC trace for compound 22a-22b.  (C) Accurate mass spectra for compound 22a-22b with retention time 3.18 min.  (D) Accurate mass spectra for compound 22a-22b with retention time 4.01 min.
$^1$H NMR (500 MHz, CD$_3$OD) of 22a-22b

$^{13}$C NMR (126 MHz, CD$_3$OD) of 22a-22b
COSY NMR (500 MHz, CD$_3$OD) of 22a-22b

HSQC NMR (500 MHz, CD$_3$OD) of 22a-22b
HMBC NMR (500 MHz, CD$_3$OD) of 22a-22b
NOESY analysis of compounds 19a-19b

(A) $^1$H NMR (CD$_3$OD) of compounds 19a-19b. (B) Spectrum with selective irradiation of H-1 at 5.66 ppm and observed correlation with H-3 protons at 4.01 ppm indicating cis-diastereomer. (C) Spectrum with selective irradiation of H-1 at 5.66 ppm.

References

1. Kaysser, L., Tang, X., Wemakor, E., Sedding, K., Hennig, S., Siebenberg, S. & Gust, B. Identification of a napsamycin biosynthesis gene cluster by genome mining. *Chembiochem*. 2011, 12, 477-487.
2. Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N. & Schoner, B.E. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene*, 1992, 116, 43-49.
3. MacNeil, D.J., Gewain, K.M., Ruby, C.L., Dezeny, G., Gibbons, P.H. & MacNeil, T. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene*. 1992, 111, 61-68.
4. Chen, R. H.; Buko, A. M.; Whittern, D. N.; McAlpine, J. B. *J. Antibiot.*, 1989, 42, 512.
5. A. Deb Roy, S. Gräuschow, N. Cairns, R. J. M. Goss, *J. Am. Chem. Soc.*, 2010, 132, 12243-12245.