First Ring-Expanded Maytansin Lactone Accessed by a New Mutasynthetic Variant

Friederike Wesemann*, Anja Heutling*, Paul Wienecke, and Andreas Kirschning*
Table of Contents

1. Materials and methods

1.1 General information
1.2 Chromatography
1.3 Preparation of mutasynths
1.4 Microbiological methods

2. Mutasynthetic experiments

3. Copies of NMR spectra

4. References

1. Materials and methods

1.1 General Information

$^1$H-NMR spectra were recorded on Ultrashield-400 and Ascend-400 (400 MHz) and Ultrashield 500 (500 MHz) by Bruker at ambient temperature if not mentioned otherwise. Chemical shifts $\delta$ are reported in ppm and relative to the shift of the residual solvents (CD$_2$OD = 3.31 ppm, C$_6$D$_5$ = 7.16 ppm, (CD$_2$)$_2$SO = 2.50). Coupling constants were reported in Hz. Signals in NMR are described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) or a combination of these, which refers to the spin-spin coupling pattern observed. $^{13}$C-NMR spectra were recorded on Ultrashield-400 and Ascend-400 (100 MHz) and Ultrashield 500 (125 MHz) by Bruker at ambient temperature. Chemical shifts $\delta$ are reported in ppm and relative to the shift of the residual solvents (CHD$_2$OD = 49.0 ppm, C$_6$H$_5$ = 128.0 ppm, (CHD)$_2$SO = 39.5). Two dimensional NMR spectroscopy (COSY, HSQC and HMBC) was used to assist the assignment of signals in $^1$H- and $^{13}$C-NMR spectra of novel compounds. High resolution mass spectra (HRMS), characteristic retention times ($t_r$ [min]) and mass spectra for quantification were recorded with a Micromass’ type Q-Tof Premier™ spectrometer in lock spray mode with Electrospray ionization (ESI) combined with a Waters ACQUITY UPLC™ system equipped with a Waters’ ACQUITY UPLC™ BEH C18 1.7 (SN 01473711315545) column [solvent A: water + 0-1% (v/v) FA, solvent B: MeOH + 0.1% (v/v) FA; flow rate=0.4 mL/min; gradient (t [min] / solvent B [%]): 0/5, 2.5/95, 6.5/95, 6.6/5, 8/5]. Ion mass signals ($m/z$) are reported as values in atomic mass units. Mass spectra were visualized and analyzed with the software MassLynx™ from Waters. Mutasynths provided by former laboratory members were checked for purity by NMR- and HRMS-analysis prior to fermentation.
1.2 Chromatography

Flash column chromatography was performed on MACHERY-NAGEL silica gel (particle size = 40-63 μm). Size exclusion chromatography was performed using Sephadex® LH-20 from SIGMA ALDRICH (500 mm x ø 20 mm) with MeOH as eluent. Collected fractions were analyzed by LC-MS. Semi preparative HPLC (< 20 mg) was performed with a MERK HITACHI LaCrome HPLC containing a L-7150 pump, D-7000 interface and a L-7450 diode array detector [λ = 220-400 nm]. Separation was conducted through a RP-C18 (Nucleodur®, 5 μm) and RP-CN (Nucleodur® 100-5, 5 μm) column by MACHERY NAGEL at 3.0 mL/min flow rate. Preparative HPLC (< 400 mg) was performed with a HPLC containing a fraction collector (Varian pro Star, Modell 701) and pumps (Varian preStar, Modell 218) by ALPHA CHROM and a variable UV detector (proStar [λ = 248 nm]) along with a mass detector (MICROMASS type LCT) by WATERS. Separation was conducted with a RPC18 (Nucleodur®, 5 μm) and RP-CN (Nucleodur® 100-5, 5 μm) column by MACHERY NAGEL at 15.0 mL/min flow rate. Analytical grade solvents were supplied by FISHER CHEMICALS and used as received. Water used in analytical LC-MS and preparative and semi-preparative HPLC was bidistilled and membrane filtered and supplemented with 0.1% formic acid (FA) prior to use. For solutions in EtOAc or MeOH the solvent was removed at 40 °C and 35 °C, respectively, using a conventional rotary evaporator. Solvent mixtures of H₂O and MeOH were removed at ambient temperature and under high vacuum. Solvent mixtures of H₂O and MeCN were removed at ambient temperature either under high vacuum or in a vacuum centrifuge from CHRIST type Alpha RVC connected to a freeze dryer from CHRIST type Alpha 24.

1.3 Preparation of Mutasynthon

The preparation of benzoic acids were achieved according to literature procedures: 1 (ref. S1), 12, 24 (ref. S2), 13, 18, 21 (ref. S3), 20 (ref. S4), 22 (ref. S5), 23 (ref. S6), 25, 28 (ref. S7) and 30 (ref. S8). Aminobenzoic acids 14, 15, 17, 19 and 27 are commercially available. Mutasynths 1, 12, 18, 21, 23, 24 and 26 were prepared and fed as their respective hydrochloride salts.

3-Amino-4-chloro-5-hydroxybenzoic acid (16)

For preparation of S1 see ref. S9. Nitromethyl vanilate S1 (40 mmol, 1.0 eq.), was dissolved in DMF (100 mL). Oxalylchloride (11.3 mL, 120 mmol, 3.0 eq.) was added at -20 °C under nitrogen atmosphere. The mixture was heated to 80 °C and stirred for 3 h before slow cooling. When room temperature was reached the mixture was poured on vigorously stirred ice., filtered and washed with ice cold water until the precipitate was colorless. The residue was
dissolved in CH_2Cl_2, washed with a sat. aq. NaH_2CO_3 solution and with brine. The organic layer was dried over MgSO_4 filtered and the solvent was removed \textit{in vacuo} which gave 9.46 g of crude methyl-4-chloro-3-methoxy-5-nitrobenzoate.

Half of the amount of the resulting solid (4.94 g, 20 mmol, 1.0 eq) was dissolved in CH_2Cl_2 (60 mL) and the mixture was cooled to -78 °C. A solution of borontribromide\textsuperscript{S10} in CH_2Cl_2 (1 M, 60 mmol, 3.0 eq.) was added dropwise and the mixture was stirred for 48 h at rt. The reaction was terminated by addition of H_2O. The aqueous phase was extracted with EtOAc (3 x 30 mL). The combined organic layers were dried over MgSO_4 filtered and concentrated \textit{in vacuo} which gave 4.34 g of crude 4-chloro-3-hydroxy-5-nitrobenzoic acid.

The resulting solid (20 mmol, 1.0 eq) was dissolved in EtOAc (45 mL). Glacial acetic acid (1.5 mL) and ZnCl_2\cdot2H_2O\textsuperscript{S11} (22.5 g, 100 mmol, 5 eq.) was added. The reaction mixture was stirred for 5 h under refluxing conditions, cooled and poured onto ice. The pH value was adjusted to 4-5 by addition of a sat. aq. NaHCO_3 solution and extracted with EtOAc. The organic layer was dried over MgSO_4 filtered and the solvent was removed under reduced pressure. The crude product was suspended in half concentrated HCl and heated under refluxing conditions for 1 h. After cooling to rt, the hydrochloride salt \textbf{16} (3.9 g, 17.4 mmol, 79% over 3 steps) was filtered and dried \textit{in vacuo}.

\textbf{1H-NMR} (400 MHz, DMSO-d_6): \(\delta\) 10.37 (bs, 1H, COOH), 8.21 (bs, 2H, NH_2), 7.09 (s, 1H, Ar-H), 7.00 (s, 1H, Ar-H) ppm; \textbf{13C-NMR} (100 MHz, CDCl_3): \(\delta\) 167.0, 153.8, 142.1, 129.7, 111.5, 109.1, 107.2 ppm; \textbf{HRMS} (ESI) \(m/z\) for C_7H_5O_3ClN \[\text{[M-H]}\]: calcd. 185.9958, found 185.9959; \textbf{Mp.}: 233.2 °C (decomposition)

\textbf{2-(3-Amino-5-hydroxyphenyl)acetic acid (26)}

\begin{center}
\textbf{S2} \quad \textbf{S3} \quad \textbf{26}
\end{center}

Benzyl bromide \textbf{S2} was prepared as described previously (ref. S12). \textbf{S1} (1.5 g, 2.78 mmol, 1.0 eq.) was diluted with DMF (150 mL) and sodium cyanide (0.2 mg, 4.17 mmol, 1.5 eq.) was added. The mixture was stirred for 1.5 h at rt before it was terminated by addition of distilled water (150 mL). The aqueous phase was extracted with EtOAc (4 x 200 mL). The combined organic layers were dried over Na_2SO_4 filtered and the solvent was removed \textit{in vacuo}. Column chromatography (silica; PE:EE = 10:1) gave benzyl nitrile \textbf{S3} (1.09 g, 2.23 mmol, 80%).

\textbf{1H-NMR} (400 MHz, CDCl_3): \(\delta\) 7.73 - 7.68 (m, 4H, TBDPS), 7.46 - 7.36 (m, 6H, TBDPS), 7.08 (m, 1H, Ar-H), 6.57 (m, 1H, Ar-H), 6.37 (m, 1H, Ar-H), 6.26 (bs, 1H, NH), 3.49 (s, 2H, CH_2CN), 1.47 (s, 9H, Boc), 1.08 (s, 9H, TBDPS) ppm; \textbf{13C-NMR} (100 MHz, CDCl_3): \(\delta\) 156.7 (C-Ar), 152.5 (Boc), 140.0 (C-Ar), 135.6 (TPDPS), 132.5 (TBDPS), 131.7 (C-Ar), 130.2 (TBDPS), 128.0 (TBDPS), 117.7 (CN), 114.1 (C-Ar), 110.8 (C-Ar), 109.4 (C.Ar), 80.9
**Boc**, 28.4 (Boc), 26.7 (TBDPS), 23.6 (CH$_2$CN), 19.6 (TBDPS) ppm; **HRMS** (ESI) $m/z$ for C$_{29}$H$_{34}$NO$_3$Si [M+Na]$^+$: calcd. 509.2236, found 509.2231.

Benzyl nitrile S3 (1.34 g, 2.76 mmol, 1.0 eq.) was dissolved in MeOH/H$_2$O (10:1, 110 mL). NaOH (5.51 g, 137.85 mmol, 50.0 eq.) was added and stirring was continued for 48 h at 80 °C. Methanol was removed in vacuo and the residue was extracted with EtOAc (1 x 50 mL). The pH value of the aqueous phase was adjusted to 1 and extracted again with EtOAc (3 x 50 mL). The combined organic layers were dried over MgSO$_4$, filtered and the solvent was removed under reduced pressure. The resulting solid was mixed with half conc. HCl (100 mL) and stirring was continued for 30 min under refluxing conditions. The solvent was removed under reduced pressure and the acid 26 (342 mg, 2.05 mmol, 74%) was recrystallized from half conc. HCl.

**$^1$H-NMR** (400 MHz, DMSO-d$_6$): $\delta$ 10.04 (bs, 3H, NH$_3^+$), 6.69 (m, 2H, Ar-H), 6.65 (m, 1H, Ar-H), 3.53 (s, 2H, CH$_2$COOH) ppm; **$^{13}$C-NMR** (100 MHz, DMSO-d$_6$): $\delta$ 172.3, (COOH), 158.3 (C-Ar), 137.8 (C-Ar), 132.9 (C-Ar), 115.9 (C-Ar), 114.2 (C-Ar), 108.3 (C-Ar), 40.4 (CH$_2$) ppm; **HRMS** (ESI) $m/z$ for C$_8$H$_8$NO$_3$ [M-H]$^-$: calcd. 166.0504, found 166.0505; **Mp.:** 185 °C (decomposition).

**3-Chloro-5-(hydroxymethyl)benzoic acid (29)**

![Chemical Structure](image)

For the preparation of aminobenzoic acid S4 see ref. S2.

Methyl 3-(hydroxymethyl)-3-aminobenzoate (S4) (6.20 g, 34.2 mmol, 1 eq.) was dissolved in half conc. HCl (100 mL). The solution was cooled to 0 °C, NaNO$_2$ (2.91 g, 42.2 mmol, 1.2 eq.) was added successively and the solution was stirred for 10 min. CuCl (21.2 g, 210 mmol, 6.2 eq.) was added to the reaction mixture and the whole stirred for 5 h at 0 °C, then for 18 h at rt. The suspension was diluted with EtOAc and filtered through Celite®. The aqueous layer was extracted twice with EtOAc (2 x 30 mL). The combined organic layers were washed with sat. aq. NH$_4$Cl (3 x 30 mL), dried over MgSO$_4$ filtered and the solvent was removed in vacuo to yield compound S5 (5.85 g, 29.2 mmol, 85 %) as a red oil which was employed in the next step without further purification.

**$^1$H-NMR** (400 MHz, methanol-d$_4$): $\delta$ 7.92 (s, 1 H, H-Ar), 7.85 (s, 1 H, H-Ar), 7.60 (s, 1 H, H-Ar), 4.64 (s, 2 H, -CH$_2$-Ar), 3.91 (s, 3 H, COOME) ppm.

A solution of methyl-3-(hydroxymethyl)-5-chlorobenzoate (S5) (5.82 g, 29.0 mmol, 1.0 eq.) in EtOH (30 mL) and NaOH (4 M, 30 mL, 120 mmol, 4.1 eq.) was stirred for 18 h at rt. EtOH was removed in vacuo. The pH value of the aqueous layer was adjusted to 2 ba addition of 1N HCl (2 M). The resulting precipitate was filtered, dissolved in EtOAc, dried over MgSO$_4$ and recrystallized from MTBE to afford compound 29 (3.55 g, 19.0 mmol, 66 %) as a red solid.
1H-NMR (400 MHz, DMSO-d6) δ 7.86 (s, 1 H, H-Ar), 7.75 (s, 1 H, H-Ar), 7.60 (s, 1 H, H-Ar), 5.45 (s, 1 H, HO-CH2-), 4.56 (s, 2 H, -CH2-Ar) ppm; 13C-NMR (100MHz, DMSO-d6) δ 166.2, 145.8, 133.1, 132.7, 130.2,127.0, 125.7, 61.8 ppm; HRMS [ESI] m/z for C9H7ClO3 [M-H] calc. 185.0005, found 185.0005; Mp.: 155-159 °C (decomposition).

3-(Hydroxymethyl)-5-methoxybenzoic acid (31)

Dimethyl-5-methoxyisophtalate (S6) (3.91 g, 17.4 mmol, 1 eq.) was dissolved in dry THF (50 mL) and cooled to 0 °C. DIBAL-H (2 M in hexane, 35 mL, 35.0 mmol, 2.0 eq.) was added dropwise and the solution was allowed to stir overnight at rt. The reaction was stopped with Na-K-tartrate (50 mL) solution and stirred for another 3 h. EtOAc (50 mL) and sat. aq. NaCl (50 mL) were added and the organic layer was separated. The organic layer was dried over MgSO4 and the solvent was removed under reduced pressure. Silica column chromatography (PE:EA = 10:1 - 5:1) gave compound S7 (1.3 g, 6.6 mmol, 48 %) as an oil.

1H-NMR (200 MHz, CDCl3) δ 7.61-7.58 (m, 1 H, H-Ar), 7.47-7.44 (m, 1 H, H-Ar), 7.14-7.10 (m, 1 H, H-Ar), 4.69 (s, 2 H, -CH2-Ar), 3.90 (s, 3 H, MeO-Ar), 3.84 (s, 3 H, COOMe) ppm; the recorded data are consistent with those previously reported.

A solution of methyl-3-(hydroxymethyl)-5-methoxybenzoate (S7) (1.30 g, 6.6 mmol, 1 eq.) in EtOH (6 mL) and NaOH (4 M, 6 mL, 24 mmol, 3.6 eq.) was stirred for 18 h at rt. Ethanol was removed in vacuo. The pH value of the aqueous layer was adjusted to 2 with HCl (2 M), the precipitated solid was filtered and dried to give compound 31 (0.86 g, 4.7 mmol, 71 %) as a colorless solid.

1H-NMR (400 MHz, methanol-d4) δ 7.61 (s, 1 H, H-Ar), 7.44 (s, 1 H, H-Ar), 7.16 (s, 1 H, H-Ar), 4.91 (s,1 H, HO-CH2-), 4.62 (s, 2 H, -CH2-Ar), 3.84 (s, 3 H, MeO-Ar) ppm; 13C-NMR (100MHz, Methanol-d4) δ 169.7, 161.3, 144.9, 133.2, 121.3, 118.2, 114.3, 64.6, 55.9 ppm; HRMS [ESI] m/z for C9H9O4 [M-H] calcd. 181.0501, found 181.0501; Mp: 134 °C.

1.4 Microbiological methods

All protocols containing living microorganisms were conducted in the appropriate S1 or S2 facilities. Inoculation, feeding and sampling was executed under a type safe 2020 biological safety cabinet from THERMO SCIENTIFIC. The A. pretiosum strain AH1 was generated from A. pretiosum Δasm12/21 HGF073 and plasmid pHGF9029 through E. coli conjugation following the protocol of KIESER et al. and is insufficient for AHBA production. A. pretiosum HGF073 was provided by the FLOSS group. All strains were stored at -80°C in 50 % (v/v) kryo media and destroyed with conc. KOH after fermentation. All flasks and cannula/tube-systems of
syringe pumps were autoclaved at 121 °C and 2 bar for 15 min in a Systec V-150 prior to fermentation. Media was prepared in membrane filtered water and autoclaved. Heat sensible compounds were filtered through 2 µm filters. Agar plates were incubated at 30 °C in an Heratherm Incubator from Thermo Scientific. Liquid cultures were incubated at 28 °C and 180 rpm in an Innova 44 shaker by New Brunswick Scientific Co. If not mentioned otherwise, liquid cultures were fermented carried out in Erlenmeyer flasks (250 mL for 50 mL culture, 500 mL for 100 mL culture) containing a steel spring. One drop of SAG 471-antifoam from GE Bayer Silicones was added to all liquid media containing flasks.

**Media**

All media were prepared in distilled water.

| kryo medium | KOREA medium |
|-------------|--------------|
| 20 % | glycerol | 60 g/L | dextrin |
| 10% | sacharose | 30 g/L | D(+)−maltose · H₂O |
| 5.25 g/L | cottonseed fluor | 5.0 g/L | Ca₂CO₃ |
| 4.5 g/L | yeast extract | 0.3 g/L | K₂HPO₄ |
| 2 mg/L | FeSO₄ · 7H₂O |

| YMG Agar | YMG medium |
|----------|------------|
| 4 g/L | D(+)−glucose |
| 10 g/L | malt extract |
| 4 g/L | yeast extract |
| 2 g/L | Ca₂CO₃ |
| 12 g/L | Agar |

| 4 g/L | D(+)−glucose |
| 10 g/L | malt extract |
| 4 g/L | yeast extract |

**Precultures**

Kryo cultures of *A. pretiosum* AH1 were plated on yeast/malt/glucose YMG-agar. Plates were incubated 3-5 days until sporulation was clearly visible. Five to eight well sporulating colonies were picked and mixed with 1 mL autoclaved, distilled water. Sterile glass marbles were used to assist mixing. This suspension was then used to inoculate 50 mL of YMG media, hereafter called preculture, in 250 mL Erlenmeyer flasks containing a steel spring. One drop of SAG 471-antifoam from GE Bayer Silicones was added to each flask. The preculture was incubated for two days.

**Main culture**

A: If not mentioned otherwise 2 mL of preculture was used to inoculate 50 mL of media in 250 mL Erlenmeyer flasks containing a steel spring. One drop of SAG 471-antifoam from
GE BAYER SILICONES was added to each flask. The cultures were incubated for 12 days. A solution of the mutasynthon (0.5 mL: 42 mM in H<sub>2</sub>O : DMSO = 1:1) was added on days 3, 4 and 5 p.i. Every test fermentation was carried out in three separate flasks along with two positive controls (AHBA addition) and one negative control (addition of H<sub>2</sub>O : DMSO = 1:1 solution without the mutasynthon).

**B**: If not mentioned otherwise 4 mL of preculture was used to inoculate 100 mL of media in 500 mL Erlenmeyer flasks containing a steel spring. One drop of SAG 471-antifoam from GE BAYER SILICONES was added to each flask. The cultures were incubated for 10 days. The mutasython solution (3.0 mL: 21 mM in H<sub>2</sub>O : DMSO = 1:1) was added continuously from days 3 to 5 using BS-9000-8 syringe pumps from BRAIN TREE SCIENTIFIC connected to TEFZEL-capillaries with luer-lock connection at a flow rate of 83.3 µL/h.

**Extraction**

If not mentioned otherwise, fermentation broths were extracted with EtOAc (3 x 50 ml per 100 ml culture) 10 days after inoculation. Phase separation was achieved by centrifugation at 5000 rpm for 10 min in a Sorvall™ LYNX™ 6000 centrifuge from THERMO FISHER SCIENTIFIC. For fermentation over 5 L cells were separated through centrifugation at 5000 rpm for 10 min prior to extraction with EtOAc. After removal of the cell pellet, phase separation could be achieved without centrifugation. Combined organic layers were concentrated in vacuo at 40 °C, washed with a sat., aq. NaCl solution (3 x 50 ml per 50 ml organic fraction), dried over MgSO<sub>4</sub> and filtered. The solvent was removed in vacuo at 40 °C before analysis or product isolation was carried out.

**Qualitative and Quantitative analysis of proansamitocin**

The fermentation broth (250 µL) was diluted with an equal volume of methanol in an 1.5 mL EPPENDORF tube. After mixing, the suspension was centrifuged at 5000 rpm for 5 min in the Centrifuge 5427 R from EPPENDORF. The supernatant was prepared for qualitative and quantitative analysis. Quantification of proansamitocin during fermentation was performed by comparison of peak area (A) of PA (m/z = 466.2 [M+Na]) to the internal standard Nimodipin (is1) (m/z = 441.1 [M+Na]) in mass spectra of the UPLC in positive mode as is described below. The prepared sample was diluted with 1:10 MeOH : H<sub>2</sub>O = 1:1 (v/v) and mixed with 300 µL of MeOH : H<sub>2</sub>O = 1:1 (v/v) containing 1 ppm or 2 ppm is1. Then, the sample was diluted with MeOH : H<sub>2</sub>O = 1:1 (v/v) up to 900 µL so that the integral of PA in UPLC was between 50 and 210. Integration was performed with MassLynx™ employing ApexTrack Peak Integration using the following parameters:

| Parameter                         | Value   |
|-----------------------------------|---------|
| Peak-to-Peak Baseline Noise       | Automatic |
| Peak Width at 5% Height (Mins)    | Automatic |
| Baseline Start Threshold%         | 0       |
| Baseline End Threshold            | 10      |
| Detect Shoulders                  | No      |
| Response Threshold                | Relative Area |

Calibration was performed in matrix samples of AH1 culture in KOREA media without any additives and without AHBA addition. Matrix samples were taken on day 10 p.i. and prepared
as described above. 300 µL of 1:10 diluted matrix sample was mixed with 300 µL of MeOH : H₂O = 1:1 (v/v) containing 1 ppm or 2 ppm is, and 300 µL of MeOH : H₂O = 1:1 (v/v) containing different concentrations of proansamitocin. Total concentration of PA in the fermentation cₜ was calculated according to formula (1) with x being the analyte, Aₓ and Aᵢₛ being the peak area of the analyte and internal standard, respectively, a being the y-axis intercept of the calibration curve, b being the slope of the calibration curve and d the total dilution. All calculated concentrations < 0 were set to 0. For quantitative analysis of PA derivatives calibration was performed with PA.

\[
c(x) = \frac{A_x}{A_{is}} \cdot a - \frac{A_{is}}{b} \cdot d
\]

(1)

**Figure S.1.** A: Calibration of proansamitocin concentration with Nimodipin as internal standard. I = Integral, PA = Proansamitocin, IS = Internal standard. B: Proansamitocin concentration during fermentation in 50 mL media with addition of AHBA on days 3, 4 and 5. Four independent experiments are compared. Shown are the standard deviations from the mean (n = 3).
**Figure S.2.** Comparison of proansamitocin \(2\) concentrations on day 10 after inoculation in 50 mL fermentation media and addition of AHBA on days 3, 4 and 5. A control with no additives was compared to additional 3 g/L L-valine and 3 g/L L-valine together with addition of 2.5 g/L fructose on days 3, 4 and 5 after inoculation. Shown are the deviations from the mean (control and L-valine: \(n = 6\), L-valine + fructose \(n = 3\)).

![Graph showing comparison of proansamitocin concentrations](image)

---

### 2. Mutasynthetic experiments

**Proansamitocin derivatives from AHBA fermentation**

**Fermentation protocol:** B, 37 x 100 mL

**Culture media:** KOREA containing 0.1 g/L L-val, 0.3 g/L L-thr, 3% (v/v) coconut water

**Mutasynthon:** 3-Amino-5-hydroxybenzoic acid (804 mg, 4.4 mmol)

**Extraction day:** 10 days post inoculation

Fractions containing \(m/z = 400 – 500\) were collected after size exclusion chromatography (Sephadex LH-20, \(\sim 1.5\) mL/min, MeOH) of crude extracts and the solvent was removed *in vacuo* at 35 °C which resulted in a brown, viscous oil. The residue was further separated into 6 different fractions by preparative HPLC (RP-C18, MeOH : H\(_2\)O + 0.1% FA = 35% - 80% over 90 min).

**Proansamitocin (2)**

![Chemical structure of proansamitocin 2](image)

Fractions collected between 23-30 min were combined and the solvent was removed *in vacuo*. The residue was further purified by preparative HPLC (RP-CN, MeCN : H\(_2\)O + 0.1% FA = 10% - 25% over 80 min). Collection of fractions between 47 – 57 min gave proansamitocin \(2\) as a colorless solid (48.8 mg, 0.11 mmol, 2.5 %).

**\(^1H\)-NMR** (400 MHz, methanol-d\(_4\)): \(\delta\) 6.82 (dd, \(J = 15.2, 11.0\) Hz, 1H, 12-H), 6.77 (s, 1H, 19-H), 6.76 (s, 1H, 17-H), 6.42 (1, 1H, 21-H), 6.04 (d, \(J = 11.0\) Hz, 1H, 13-H), 5.33 (dd, \(J = 15.2\), \(J = 11.0\) Hz, 1H, 13-H), 5.14 (s, 1H, 9-H), 4.93 (m, 1H, 11-H), 4.87 (s, 1H, 19-H), 4.79 (s, 1H, 14-H), 3.14 (dd, \(J = 15.2, 11.0\) Hz, 1H, 12-H), 2.04 (s, 3H, 22-H), 1.80 (s, 3H, 22-H), 1.15 (s, 3H, 7-H), 0.93 (s, 3H, 8-H), 0.80 (s, 3H, 16-H), 0.01 (s, 3H, 16-H).
8.6 Hz, 1H, 11-H), 5.27 (d, J = 9.3 Hz, 1H, 5-H), 4.49 (d, J = 8.6 Hz, 1H, 10-H), 4.33 (dd, J = 10.0, 4.5 Hz, 1H, 3-H), 4.04 (ddd, J = 10.0, 7.4, 5.8 Hz, 1H, 7-H), 3.32 (s, 3H, 10-OMe), 3.31 (d, J = 13.3 Hz, 1H, 15-Ha), 3.10 (d, J = 13.3 Hz, 1H, 15-Hb), 2.88 (dd, J = 16.1, 7.4 Hz, 1H, 8-Ha), 2.64 (dd, J = 12.6, 4.5 Hz, 1H, 2-Ha), 2.54 (dd, J = 12.6, 10.0 Hz, 1H, 2-Hb), 2.38 (dd, J = 16.1, 5.8 Hz, 1H, 8-Hb), 2.19 (ddq, J = 10.4, 9.3, 6.6 Hz, 1H, 6-H), 1.72 (s, 3H, 14-Me), 1.61 (s, 3H, 4-Me), 0.58 (d, J = 6.6 Hz, 3H, 6-Me) ppm; UPLC-MS (grad) t_R = 2.54 min; HRMS (ESI) m/z for C_{25}H_{33}NO_{6}[M+Na]^+: calcd. 466.2206, found 466.2202.

Recorded data are in accordance with those previously reported.\textsuperscript{S14}

10-S-proansamitocin (4)

Fractons collected between 36-41 min were combined and the solvent was removed \textit{in vacuo}. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H₂O + 0.1% FA = 15% - 30% over 60 min). Collection of the fraction after 30 min gave 10-S-proansamitocin 4 as a colorless semisolid (1 mg, 0.002 mmol, 0.05 %).

\textsuperscript{1}H-NMR (500 MHz, methanol-d₄): \(\delta\) 8.55 (s, 1H, N-H), 6.78 (m, 3H, 19-H), 6.68 (ddd, J = 15.3, 12.4, 1.5 Hz, 1H, 12-H), 6.60 (m, 3H, 17-H/21-H), 6.41 (m, 3H, 17-H/21-H), 6.03 (d, J = 11.0 Hz, 1H, 13-H), 5.46 (dd, J = 15.3, 5.3 Hz, 1H, 11-H), 5.22 (d, J = 9.3 Hz, 1H, 5-H), 4.35 (dd, J = 9.0, 5.7 Hz, 1H, 3-H), 4.24 (dd, J = 5.3, 1.5 Hz, 1H, 10-H), 4.06 (ddd, J = 8.4, 5.3, 3.4 Hz, 1H, 7-H), 3.39 (s, 3H, 10-OMe), 3.25 (d, J = 13.3 Hz, 1H, 15-Ha), 3.13 (d, J = 13.3 Hz, 1H, 15-Hb), 3.12 (dd, J = 16.4, 8.4 Hz, 1H, 8-Ha), 2.59 (d, J = 5.7 Hz, 1H, 2-Ha), 2.59 (d, J = 9.0 Hz, 1H, 2-Hb), 2.26 (dd, J = 16.4, 5.3 Hz, 1H, 8-Hb), 2.18 (ddq, J = 9.3, 3.4, 6.7 Hz, 1H, 6-H), 1.68 (s, 3H, 14-Me), 1.65 (s, 3H, 14-Me), 0.52 (d, J = 6.7 Hz, 3H, 6-Me) ppm; UPLC-MS (grad) t_R = 2.11 min; HRMS (ESI) m/z for C_{25}H_{33}NO_{6}[M+Na]^+: calcd. 466.2206, found 466.2206.

Recorded data are in accordance with those previously reported.\textsuperscript{S14}

20-O-Methyl-proansamitocin (5)

Fractons collected between 44-48 min were combined and the solvent was removed \textit{in vacuo}. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H₂O + 0.1% FA = 20% - 35% over 60 min). Collection of the fraction after 25 min gave 20-O-methyl-proansamitocin 5 as a white semisolid (< 1 mg, < 0.002 mmol < 0.05%).
1H-NMR (400 MHz, methanol-d₄): δ 8.55 (s, 1H, N-H), 6.89 (s, 1H, 17-H), 6.86 (s, 1H, 19-H), 6.82 (dd, J = 10.5 Hz, 1H, 12-H), 6.55 (s, 1H, 21-H), 6.06 (d, J = 10.5 Hz, 1H, 13-H), 5.35 (dd, J = 15.0, 8.5 Hz, 1H, 11-H), 5.27 (d, J = 9.4 Hz, 1H, 5-H), 4.50 (d, J = 8.5 Hz, 1H, 10-H), 4.35 (dd, J = 10.0, 4.4 Hz, 1H, 3-H), 4.04 (ddd, J = 9.4, 7.4, 5.8 Hz, 1H, 7-H), 3.77 (s, 3H, 20-OMe), 3.36 (d, J = 13.4 Hz, 1H, 15-Ha), 3.33 (s, 3H, 10-OMe), 3.17 (d, J = 13.4 Hz, 1H, 15-Hb), 2.89 (dd, J = 16.0, 7.4 Hz, 1H, 8-Ha), 2.66 (dd, J = 12.6, 4.4 Hz, 1H, 2-Ha), 2.55 (dd, J = 12.6, 10.0 Hz, 1H, 2-Hb), 2.39 (dd, J = 16.0, 5.8 Hz, 1H, 8-Hb), 2.25 – 2.16 (m, 3H, 6-H), 1.73 (s, 3H, 14-Me), 1.62 (s, 3H, 4-Me), 0.58 (d, J = 6.6 Hz, 6-Me) ppm; UPLC-MS tr = 2.70 min; HRMS (ESI) m/z for C₂₆H₄₄NO₆ [M+Na]+: calcd. 481.2202, found 481.2200.

Recorded data are in accordance with those previously reported. S14

14-Hydroxy-∆¹⁰,¹²-proansamitocin (6a, diastereomer #1)

Fractions collected between 14-17 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H₂O + 0.1% FA = 5% - 15% over 60 min). Collection of the fraction between 33-34 min gave 14-(dia-#1)-hydroxy-∆¹⁰,¹²-proansamitocin 6a as a white semisolid (2 mg, 0.004 mmol 0.1 %).

1H-NMR (500 MHz, methanol-d₄): δ 7.98 (s, 1H, N-H), 6.98 (dd, J = 1.5, 1.5 Hz, 1H, 17-H), 6.80 (d, J = 10.6 Hz, 1H, 11-H), 6.70 (dd, J = 2.0, 2.0 Hz, 1H, 19-H), 6.47 (dd, J = 15.6, 10.6 Hz, 1H, 12-H), 6.44 (dd, J = 2.0, 1.5 Hz, 1H, 21-H), 6.22 (d, J = 15.6 Hz, 1H, 13-H), 5.47 (d, J = 9.8 Hz, 1H, 5-H), 4.32 (dd, J = 8.5, 3.5 Hz, 1H, 3-H), 3.82 (ddd, J = 8.6, 6.6, 4.3 Hz, 1H, 7-H), 3.58 (s, 3H, 10-OMe), 2.97 (dd, J = 15.1, 4.4 Hz, 1H, 8-Ha), 2.77 (d, J = 12.4 Hz, 1H, 15-Ha), 2.76 (dd, J = 13.7, 3.2 Hz, 1H, 2-Ha), 2.75 (dd, J = 15.2, 8.6 Hz, 1H, 8-Hb), 2.72 (d, J = 12.4 Hz, 1H, 15-Hb), 2.72 (dd, J = 13.7, 8.8 Hz, 1H, 2-Hb), 2.52 (ddq, J = 9.8, 6.6, 6.6 Hz, 1H, 6-H), 1.67 (s, 3H, 4-Me), 1.38 (s, 3H, 14-Me), 1.03 (d, J = 6.6 Hz, 3H, 6-Me) ppm; UPLC-MS (grad) tr = 2.31 min; HRMS (ESI) m/z for C₂₅H₃₄O₇ [M+Na]+: calcd. 482.2155, found 482.2154.

Recorded data are in accordance with those previously reported. S14

14-Hydroxy-∆¹⁰,¹²-proansamitocin (6b, diastereomer #2):
Fractions collected between 14-17 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H₂O + 0.1% FA = 5% - 15% over 60 min). Collection of the fraction between 37-39 min gave 14-(dia-#1)-hydroxy-Δ¹⁰,¹²-proansamitocin 6b as a white semisolid (3 mg, 0.007 mmol, 0.15%).

¹H-NMR (500 MHz, methanol-d₄): δ 7.98 (s, 1H, N-H), 6.94 (dd, J = 1.5, 1.5 Hz, 1H, 17-H), 6.75 (d, J = 10.9 Hz, 1H, 11-H), 6.69 (dd, J = 2.0, 2.0 Hz, 1H, 19-H), 6.53 (dd, J = 15.5, 10.9 Hz, 1H, 12-H), 6.45 (dd, J = 2.0, 1.5 Hz, 1H, 21-H), 6.14 (d, J = 15.5 Hz, 1H, 13-H). 5.49 (d, J = 9.8 Hz, 1H, 5-H), 4.33 (dd, J = 9.0, 3.2 Hz, 1H, 3-H), 3.84 (ddd, J = 9.7, 6.5, 3.2 Hz, 1H, 7-H), 3.59 (s, 3H, 10-OMe), 3.09 (dd, J = 16.0, 3.2 Hz, 1H, 8-Ha), 2.80 (dd, J = 14.0, 3.2 Hz, 1H, 2-Ha), 2.75 (s, 2H, 15-H), 2.62 (dd, J = 16.0, 9.7 Hz, 1H, 8-Hb), 2.59 (dd, J = 14.0, 9.0 Hz, 1H, 2-Hb). 2.39 (ddq, J = 9.8, 6.5, 6.7 Hz, 1H, 6-H), 2.17 (s, 3H, 14-Me), 1.71 (s, 3H, 4-Me), 1.02 (d, J = 6.7 Hz, 3H, 6-Me) ppm; HRMS (ESI) m/z for C₂₅H₃₃O₇[M+Na]⁺: calcd. 482.2155, found 482.2154.

Recorded data are in accordance with those previously reported.

**19-Hydroxy-proansamitocin (7)**

Fractions collected between 17-21 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H₂O + 0.1% FA = 10% - 20% over 60 min). Collection of the fraction after 29 min gave a mixture of three proansamitocin derivatives (1.5 mg) as a colorless semisolid. The main product was identified as 19-hydroxy-proansamitocin 7.

¹H-NMR (500 MHz, methanol-d₄): δ 6.85 (dd, J = 15.5, 11.0 Hz, 1H, 12-H), 6.80 (d, J = 2.5 Hz, 1H, 21-H/17-H), 6.53 (d, J = 2.5 Hz, 1H, 21-H/17-H), 6.03 (d, J = 11.0 Hz, 1H, 13-H), 5.32 (dd, J = 15.5, 8.5 Hz, 1H, 11-H), 5.27 (d, J = 9.4 Hz, 1H, 19-H), 4.51 (d, J = 8.5 Hz, 1H, 10-H), 4.34 (dd, J = 10.0, 4.5 Hz, 1H, 3-H), 4.04 (ddd, J = 7.6, 5.3, 4.4 Hz, 1H, 7-H), 3.52 (d, J = 14.0 Hz, 1H, 15-Ha), 3.33 (s, 3H, 10-OMe), 3.13 (d, J = 14.0 Hz, 1H, 15-Hb), 2.82 (dd, J = 16.0, 7.6 Hz, 1H, 8-Ha), 2.60 (dd, J = 12.5, 4.5 Hz, 1H, 2-Ha), 2.51 (dd, J = 12.5, 10.0 Hz, 1H, 2-Hb), 2.38 (dd, J = 16.0, 5.3 Hz, 1H, 8-Hb), 2.21 (ddq, J = 9.4, 4.4, 6.6 Hz, 1H, 6-H). 1.77 (s, 3H, 14-Me), 1.62 (s, 3H, 4-Me), 0.60 (d, J = 6.6 Hz, 3H, 6-Me) ppm; ¹³C-NMR/HSQC/HMBC (125 MHz, methanol-d₄): δ 207.2 (C-9), 170.5 (C-1), 142.5 (C-14), 141.2 (C-16), 134.6 (C-12), 131.0 (C-5), 126.4 (C-13), 125.2 (C-11), 89.3 (C-10), 75.9 (C-3), 72.4 (C-7), 56.8 (10-OMe), 45.6 (C-8), 44.4 (C-2), 39.2 (C-15), 37.9 (C-6), 17.09 (14-Me), 14.6 (6-Me), 11.8 (4-Me) ppm; The ¹³C-signals for C-18-20 and C-4 could not unequivocally be determined; UPLC-MS (grad) tᵣ = 2.39 min; HRMS (ESI) m/z for C₂₅H₃₃O₇[M+Na]⁺: calcd. 482.2155, found 482.2155.
10-Desmethoxy-proansamitocin (8)

Fractions collected between 29-35 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H2O + 0.1% FA = 15% - 25% over 60 min). Collection of fractions between 28-29 min gave a mixture of derivatives as a yellow semisolid (1 mg). The major product was identified to be 10-desmethoxy-proansamitocin 8.

\[ ^1H-NMR \text{ (500 MHz, methanol-d}_4 \text{): } \delta \, 6.90 \, (s, \, 1H, \, 21-H), \, 6.64 \, (s, \, 1H, \, 17-H), \, 6.51 \, (dd, \, J = 15.0, \, 10.0 \, Hz, \, 1H, \, 12-H), \, 6.41 \, (s, \, 1H, \, 19-H), \, 6.03 \, (d, \, J = 10.0 \, Hz, \, 1H, \, 13-H), \, 5.63 \, (ddd, \, J = 15.0, 7.5, 7.5 \, Hz, \, 1H, \, 11-H), \, 5.28 \, (d, \, J = 9.0 \, Hz, \, 1H, \, 5-H), \, 4.38 \, (dd, \, J = 9.0, 4.1 \, Hz, \, 1H, \, 3-H), \, 4.01 \, (ddd, \, J = 12.0, 6.4, 6.0 \, Hz, \, 1H, \, 7-H), \, 3.22 \, (dd, \, J = 12.8, 7.5 \, Hz, \, 1H, \, 10-Ha), \, 3.19 \, (bs, \, 2H, \, 15-H), \, 3.06 \, (dd, \, J = 12.8, 7.5 \, Hz, \, 1H, \, 10-Hb), \, 2.95 \, (dd, \, J = 16.0, 6.0 \, Hz, \, 1H, \, 8-Ha), \, 2.63 \, (dd, \, J = 13.0, 4.1 \, Hz, \, 1H, \, 2-Ha), \, 2.57 \, (dd, \, J = 13.0, 9.0 \, Hz, \, 1H, \, 12-Hb), \, 2.38 \, (dd, \, J = 16.5, 6.4 \, Hz, \, 1H, \, 8-Hb), \, 2.35 \, (ddq, \, J = 12.0, 9.0, 6.6 \, Hz, \, 1H, \, 6-H), \, 1.67 \, (s, \, 3H, \, 14-Me), \, 1.65 \, (s, \, 3H, \, 4-Me), \, 0.65 \, (d, \, J = 6.6 \, Hz, \, 3H, \, 6-Me) \text{ ppm}; \]

\[ ^{13}C-NMR/HSQC/HMBC \text{ (125 MHz, methanol-d}_4 \text{): } \delta \, 209.6 \, (C-9), \, 170.2 \, (C-1), \, 158.5 \, (C-20), \, 143.4 \, (C-20), \, 139.4 \, (C-14), \, 137.3 \, (C-14), \, 133.7 \, (C-12), \, 131.0 \, (C-5), \, 127.3 \, (C-13), \, 124.1 \, (C-11), \, 113.5 \, (C-21), \, 113.2 \, (C-17), \, 106.6 \, (C-19), \, 75.9 \, (C-3), \, 72.2 \, (C-7), \, 49.9 \, (C-10), \, 47.1 \, (C-8), \, 46.9 \, (C-15), \, 44.2 \, (C-2), \, 30.8 \, (C-6), \, 16.6 \, (14-Me), \, 15.6 \, (6-Me), \, 11.9 \, (4-Me) \text{ ppm}; \]

\[ \text{UPLC-MS } tr = 2.66 \, \text{min; HRMS (ESI) for } C_{24}H_{31}NO_5 \, [M+Na]^+: \text{ calcld. 436.2089, found 436.2089.} \]

\[ \Delta^{10,11} \text{-Proansamitocin (9)} \]

Fractions between 36-41 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H2O + 0.1% FA = 15% - 30% over 60 min). Collection of the fractions after 31-32 min gave proansamitocin isomer 9 as a colorless semisolid (3 mg, 0.007 mmol, 0.15 %).

\[ ^1H-NMR \text{ (500 MHz, methanol-d}_4 \text{): } \delta \, 8.55 \, (s, \, 1H, \, N-H), \, 7.21-7.19 \, (m, \, 1H, \, 17-H/21-H), \, 6.61-6.60 \, (m, \, 1H, \, 19-H), \, 6.39-6.37 \, (m, \, 1H, \, 17-H/21-H), \, 6.12 \, (dd, \, J = 8.8, \, 6.1 \, Hz, \, 1H, \, 11-H), \, 5.37 \, (d, \, J = 9.5 \, Hz, \, 1H, \, 5-H), \, 5.35 \, (ddd, \, J = 9.0, 7.8, 0.8 \, Hz, \, 1H, \, 13-H), \, 4.32 \, (dd, \, J = 9.1, 3.4 \, Hz,} \]
1H, 3-H), 3.86 (ddd, \( J = 9.5, 7.8, 3.0 \) Hz, 1H, 7-H), 3.60 (s, 3H, 10-OMe), 3.20-3.12 (m, 3H, 12-Ha, 15-H), 2.99 (dd, \( J = 15.8, 3.0 \) Hz, 1H, 8-Ha), 2.88 (ddd, \( J = 14.6, 7.8, 6.1 \) Hz, 1H, 12-Hb), 2.75 (dd, \( J = 14.4, 3.9 \) Hz, 1H, 2-Ha), 2.65-2.56 (m, 2H, 2-Hb, 8-Hb), 2.50-2.42 (m, 1H, 6-H), 1.69 (s, 3H, 4-Me), 1.56 (d, \( J = 0.8 \) Hz, 3H, 14-Me), 0.96 (d, \( J = 6.6 \) Hz, 3H, 6-Me) ppm; \(^{13}\text{C}-\text{NMR} \) (125 MHz, methanol-d4): \( \delta \) 198.6 (C-9), 171.0 (C-1), 158.5 (C-18), 154.3 (C-10), 143.5 (C-16), 138.9 (C-14), 138.4 (C-4), 130.8 (C-11), 127.9 (C-5), 123.2 (C-13), 113.4 (C-17/C-21), 111.8 (C-19), 105.3 (C-17/C-21), 73.8 (C-7), 73.6 (C-3), 60.3 (10-OMe), 46.9 (C-15), 44.6 (C-8), 44.2 (C-2), 39.8 (C-6), 25.6 (C-12), 17.3 (6-Me), 16.0 (14-Me), 15.3 (4-Me) ppm; two-dimensional NMR spectroscopy (COSY, HSQC, HMBC) assisted assignment of \(^1\text{H}-\) and \(^{13}\text{C}-\) signals. Arrows highlight relevant NOESY correlations.

11,12-Z-Proansamitocin (10)

Fractions collected between 29-35 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H₂O + 0.1% FA = 15% - 25% over 60 min). Collection of fractions between 20-21 min gave 13-Z-proansamitocin 10 as a colorless solid (1 mg, 0.002 mmol, 0.05 %).

\(^1\text{H}-\text{NMR} \) (500 MHz, methanol-d4): \( \delta \) 7.04 (dd, \( J = 1.5, 1.5 \) Hz, 1H, 17-H), 6.60 (dd, \( J = 11.4, 10.0, 0.8 \) Hz, 1H, 12-H), 6.44 (dd, \( J = 2.0, 1.5 \) Hz, 1H, 21-H), 6.22 (d, \( J = 11.4 \) Hz, 1H, 13-H), 5.38 (dq, \( J = 9.4, 1.2 \) Hz, 1H, 5-H), 5.10 (dd, \( J = 10.0, 9.7 \) Hz, 1H, 11-H), 4.80 (dd, \( J = 9.7, 0.8 \) Hz, 1H, 10-H), 4.43 (dd, \( J = 8.6, 4.6 \) Hz, 1H, 3-H), 3.65 (ddd, \( J = 8.2, 6.4, 2.7 \) Hz, 1H, 7-H), 3.42 (d, \( J = 14.6 \) Hz, 1H, 14-Me), 3.29 (s, 3H, 10-OMe), 3.21 (d, \( J = 14.6 \) Hz, 1H, 15-Me), 2.70 (ddq, \( J = 9.4, 6.4, 6.8 \) Hz, 1H, 6-H), 2.65 (dd, \( J = 15.2, 8.6 \) Hz, 1H, 2-Ha), 2.63 (dd, \( J = 15.2, 4.6 \) Hz, 1H, 2-Hb), 2.63 (dd, \( J = 16.7, 2.7 \) Hz, 1H, 8-Ha), 2.40 (dd, \( J = 16.7, 8.2 \) Hz, 1H, 8-Hb), 1.79 (s, 3H, 14-Me), 1.68 (d, \( J = 1.2 \) Hz, 3H, 4-Me), 0.79 (d, \( J = 6.8 \) Hz, 3H, 6-Me) ppm; \(^{13}\text{C}-\text{NMR} \) (125 MHz, methanol-d4): \( \delta \) 208.8 (C-9), 171.3 (C-1), 158.8 (C-20), 144.8 (C-14), 142.5 (C-16), 140.4 (C-18), 138.4 (C-4), 132.1 (C-12), 127.8 (C-5), 124.0 (C-11), 121.7 (C-13), 114.3 (C-17), 113.8 (C-21), 106.6 (C-19), 83.9 (C-10), 74.4 (C-3), 73.2 (C-7), 56.9 (10-OMe), 47.1 (C-15), 43.7 (C-2), 42.7 (C-8), 38.9 (C-6), 17.2 (6-Me), 17.1 (14-Me), 13.45 (4-Me) ppm; two-dimensional NMR spectroscopy (COSY, HSQC, HMBC) assisted assignment of \(^1\text{H}-\) and \(^{13}\text{C}-\) signals. UPLC-MS \( t_R = 2.62 \) min; HRMS (ESI) \( m/z \) for C\(_{25}\)H\(_{33}\)NO\(_6\) [M+Na\(^+\)]: calcd. 466.2206, found 466.2202.
13,14-Z-Proansamitocin (11)

Fractions collected between 29-35 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H$_2$O + 0.1% FA = 15% - 25% over 60 min). Collection of fractions between 23-25 min gave, amongst other unknown derivatives, 13, 14-Z-proansamitocin.

$^1$H-NMR (500 MHz, methanol-d$_4$): $\delta$ 8.55 (s, 1H, N-H), 7.01 (dd, $J$ = 1.5, 1.5 Hz, 1H, 17-H), 6.72 (dd, $J$ = 15.3, 11.0 Hz, 1H, 12-H), 6.68 (dd, $J$ = 2.0, 1.5 Hz, 1H, 19-H), 6.44 (dd, $J$ = 2.0, 1.5 Hz, 1H, 21-H), 6.00 (d, $J$ = 11.0 Hz, 1H, 13-H), 5.48 (dd, $J$ = 15.3, 8.0 Hz, 1H, 11-H), 5.38 (dd, $J$ = 9.5, 1.0 Hz, 1H, 5-H), 4.46 (d, $J$ = 8.0 Hz, 1H, 10-H), 4.33 (dd, $J$ = 8.9, 4.0 Hz, 1H, 3-H), 3.79 (ddd, $J$ = 9.5, 8.2, 2.6 Hz, 1H, 7-H), 3.55 (d, $J$ = 14.5 Hz, 1H, 15-Ha), 3.33 (s, 3H, 10-OMe), 3.25 (d, $J$ = 14.5 Hz, 1H, 15-Hb), 2.70 (dd, $J$ = 13.8, 4.0 Hz, 1H, 2-Ha), 2.66-2.60 (m, 1H, 2-Hb), 2.56 (dd, $J$ = 15.3, 2.6 Hz, 1H, 8-Ha), 2.50 (ddq, $J$ = 9.5, 8.2, 6.8 Hz, 1H, 6-H), 2.48 (dd, $J$ = 15.3, 9.5 Hz, 1H, 8-Hb), 1.79 (s, 3H, 14-Me), 1.66 (d, $J$ = 1.0 Hz, 3H, 4-Me), 0.95 (d, $J$ = 6.8 Hz, 3H, 6-Me) ppm; $^{13}$C-NMR (125 MHz, methanol-d$_4$): $\delta$ 210.9 (C-9), 171.1 (C-1), 158.73 (C-arom), 141.9 (C-14), 140.4 (C-18), 138.5 (C-4), 134.5 (C-12), 127.2 (C-5), 126.6 (C-13), 125.4 (C-11), 113.2 (C-arom), 112.0 (C-arom), 105.7 (C-arom), 88.8 (C-10), 74.9 (C-7), 73.4 (C-3), 57.2 (10-OMe), 44.9 (C-8), 44.0 (C-2), 40.4 (C-6), 39.4 (C-15), 24.4 (14-Me), 17.8 (6-Me), 15.2 (4-Me) ppm. The $^{13}$C-signals for C-16, C-18 and C-20 could not unequivocally be determined; Noe experiments provided the following corelations: 13-H→14-Me and 11-H; 14-Me 13-H and 21-H; 12-H→ 17-H, 10-H, and 15-a (all weak);

UPLC-MS (grad) $t_R$ = 2.65 min; HRMS (ESI) $m/z$ for C$_{25}$H$_{33}$NO$_6$ [M+Na]$^+$: calcd. 466.2206, found 466.2207.

20-Chloro-proansamitocin (32)

Fermentation protocol: B, 20 x 100 mL

Culture media: KOREA containing 3.0 g/L L-val

Mutasython: 5-Chloro-3-aminobenzoic acid (390.0 mg, 2.5 mmol)

Extraction day: 8 days post inoculation

After extraction the residue was filtered over a pad of silica gel with 1.5 L EtOAc. The residue was purified by preparative HPLC (RP-18, MeOH : H$_2$O + 0.1% FA = 5% - 10% over 10 min, 10%-80% over 70 min, 80% - 100% min over 10 min). Fractions containing the new product were combined and the solvent was removed in vacuo. The residue was further purified by preparative HPLC (RP-C18, MeOH : H$_2$O + 0.1% FA = 10% over 10 min, 10%-50% over 10 min, 50% over 65 min, 50% - 100% min over 5 min). Fractions collected between 47-56 min were combined and the solvent was removed in vacuo. Collection of
fractions from a second preparative HPLC purification (RP-C18, MeOH : H₂O + 0.1% FA = 20% over 5 min, 20%-40% over 5 min, 60% min over 70 min, 40%-100% over 6 min) gave 20-chloroproansamitocin 32 (1.1 mg, 0.002 mmol, 0.10%) as a colorless semisolid.

\[
{^1}^H-\text{NMR} \ (500 \text{ MHz, methanol-d}_4): \delta 7.31 \ (s, 1H, 17-H), 7.27 \ (s, 1H, 19-H), 6.89 \ (s, 1H, 21-H), \ 6.82 \ (dd, J = 15.3, 11.0 \text{ Hz}, 1H, 12-H), 6.09 \ (d, J = 11.0 \text{ Hz}, 1H, 13-H), 5.38 \ (dd, J = 15.3, 8.6 \text{ Hz}, 1H, 11-H), 5.35 \ (d, J = 9.4 \text{ Hz}, 1H, 5-H), 4.50 \ (d, J = 8.6 \text{ Hz}, 1H, 10-H), 4.33 \ (dd, J = 9.8, 4.3 \text{ Hz}, 1H, 3-H), 4.02 \ (ddd, J = 7.0, 6.3, 5.5 \text{ Hz}, 1H, 7-H), 3.38 \ (d, J = 13.5 \text{ Hz}, 1H, 15-Ha), 3.33 \ (s, 3H, 10-OMe), 3.21 \ (d, J = 13.5 \text{ Hz}, 1H, 15-Hb), 2.86 \ (dd, J = 16.1, 7.0 \text{ Hz}, 1H, 8-Ha), 2.69 \ (dd, J = 12.9, 4.3 \text{ Hz}, 1H, 2-Ha), 2.55 \ (dd, J = 12.9, 9.8 \text{ Hz}, 1H, 2-Hb), 2.40 \ (dd, J = 16.1, 6.3 \text{ Hz}, 1H, 8-Hb), 2.21 \ (ddq, J = 9.4, 5.5, 6.7 \text{ Hz}, 1H, 6-H), 1.72 \ (s, 3H, 14-Me), 1.61 \ (s, 3H, 4-Me), 0.59 \ (d, J = 6.7 \text{ Hz}, 3H, 6-Me) \text{ ppm}; \ ^{13}C-\text{NMR} \ (125 \text{ MHz, methanol-d}_4): \delta 207.7 \ (C-9), 171.3 \ (C-1), 144.01 \ (C-16), 141.4 \ (C-14), 137.1 \ (C-4), 134.7 \ (C-20), 134.5 \ (C-12), 130.6 \ (C-5), 127.5 \ (C-13), 126.1 \ (C-11), 125.6 \ (C-21), 119.5 \ (C-17), 119.2 \ (C-19), 89.4 \ (C-10), 75.7 \ (C-3), 72.6 \ (C-7), 57.0 \ (10-OMe), 46.2 \ (C-15), 45.5 \ (C-8), 44.4 \ (C-2), 38.2 \ (C-6), 16.8 \ (14-Me), 14.8 \ (6-Me), 12.4 \ (4-Me) \text{ ppm.}
\]

19-Chloro-proansamitocin (33)

**Fermentation protocol:** B, 20 x 100 mL

**Culture media:** KOREA containing 3.0 g/L L-val

**Mutansyphon:** 4-Chloro-3-aminobenzoic acid (389.0 mg, 2.5 mmol)

**Extraction day:** 8 days post inoculation

After extraction the residue was filtered over a pad of silica gel with 1.5 L EtOAc. The residue was purified by preparative HPLC (RP-18, MeOH : H₂O + 0.1% FA = 5% - 10% over 10 min, 10%-80% over 70 min, 80% - 100% min over 10 min). Fractions containing the product were combined and the solvent was removed in vacuo. The residue was further purified by preparative HPLC (RP-C18, MeOH : H₂O + 0.1% FA = 10% over 10 min, 10%-50% over 10 min, 50% over 65 min, 50% - 100% min over 5 min). Fractions collected between 48-54 min were combined and the solvent was removed in vacuo. Collection of fractions from a second preparative HPLC purification (RP-C18, MeOH : H₂O + 0.1% FA = 20% over 5 min, 20%-40% over 5 min, 60% min over 70 min, 40%-100% over 6 min) gave 19-chloroproansamitocin 33 (1.5 mg, 0.003 mmol, 0.14%) as a colorless solid.
\[\text{H-NMR (500 MHz, methanol-d}_4\]: } \delta 7.51 (s, 1H, 17-H), 7.32 (d, \text{J} = 7.7 \text{ Hz}, 1H, 21-H), 7.01 (d, \text{J} = 7.7 \text{ Hz}, 1H, 20-H), 6.82 (dd, \text{J} = 14.3, 11.0 \text{ Hz}, 1H, 12-H), 6.02 (d, \text{J} = 11.0 \text{ Hz}, 1H, 13-H), 5.46 (dd, \text{J} = 14.6, 8.3 \text{ Hz}, 1H, 11-H), 5.37 (d, \text{J} = 9.0 \text{ Hz}, 1H, 5-H), 4.48 (d, \text{J} = 8.3 \text{ Hz}, 1H, 10-H), 4.42-4.36 (m, 1H, 3-H), 4.08 - 4.01 (m, 1H, 7-H), 3.41 (d, \text{J} = 14.6 \text{ Hz}, 1H, 15-Ha), 3.34 (s, 3H, 10-OMe), 3.32 (d, \text{J} = 14.6 \text{ Hz}, 1H, 15-Hb), 2.86 (dd, \text{J} = 15.6, 5.0 \text{ Hz}, 1H, 8-Ha), 2.81 (dd, \text{J} = 13.5, 3.0 \text{ Hz}, 1H, 2-Ha), 2.72-2.65 (m, 1H, 2-Hb), 2.50-2.36 (m, 2H, 6-H, 8-Hb), 1.76 (s, 3H, 14-Me), 1.66 (s, 3H, 4-Me), 0.83 (d, \text{J} = 6.4 \text{ Hz}, 3H, 6-Me) ppm; \]

\[\text{C-NMR (125 MHz, methanol-d}_4\]: } \delta 207.7 (C-9), 171.7 (C-1), 144.1, 140.9, 140.5, 137.8, 134.2 (C-12), 130.3 (C-21), 129.7 (C-5), 128.3 (C-20), 128.1 (C-13), 126.4 (C-11), 126.0 (C-17), 89.1 (C-10), 74.9 (C-3), 72.3 (C-7), 56.9 (10-OMe), 45.8 (C-15), 45.3 (C-8), 43.2 (C-2), 38.8 (C-6), 17.1 (14-Me), 16.1 (6-Me), 13.0 (4-Me) ppm; due to low yields not all \text{C-signal} could unequivocally be assigned.

19-Bromo-proansamitocin (34)

Fermentation protocol: B, 20 x 100 mL

Culture media: KOREA containing 3.0 g/L L-val

Mutasython: 4-Bromo-3-aminobenzoic acid (545.0 mg, 2.5 mmol)

Extraction day: 8 days post inoculation

After extraction the residue was filtered over a pad of silica gel with 1.5 L EtOAc. The residue was purified by preparative HPLC (RP-18, MeOH : H\text{2O} + 0.1% FA = 5% - 10% over 10 min, 10%-80% over 70 min, 80% - 100% min over 10 min). Fractions collected between 62-69 min were combined and the solvent was removed in vacuo. The residue was further purified by preparative HPLC (RP-C18, MeOH : H\text{2O} + 0.1% FA = 10% over 10 min, 10%-50% over 10 min, 50% over 65 min, 50% - 100% min over 5 min). Fractions collected between 45-51 min were combined and the solvent was removed in vacuo. Collection of fractions 72 – 29 from a second preparative HPLC purification (RP-C18, MeOH : H\text{2O} + 0.1% FA = 20% over 5 min, 20%-40% over 5 min, 60% min over 70 min, 40%-100% over 6 min) gave 19-bromoproansamitocin 34 (2.2 mg, 0.004 mmol, 0.17%) as a colorless solid.

\[\text{H-NMR (500 MHz, methanol-d}_4\]: } \delta 7.50 (d, \text{J} = 8.0 \text{ Hz}, 1H, 21-H), 7.40 (s, 1H, 17-H), 6.67 (d, \text{J} = 8.0 \text{ Hz}, 1H, 20-H), 6.82 (dd, \text{J} = 14.5, 10.1 \text{ Hz}, 1H, 12-H), 6.02 (d, \text{J} = 10.1 \text{ Hz}, 1H, 13-H), 5.47 (dd, \text{J} = 14.5, 8.2 \text{ Hz}, 1H, 11-H), 5.38 (d, \text{J} = 8.6 \text{ Hz}, 1H, 5-H), 4.49 (d, \text{J} = 8.2 \text{ Hz}, 1H, 10-H), 4.37 (d, \text{J} = 8.2 \text{ Hz}, 1H, 9-H), 3.38 (s, 3H, 10-OMe), 3.32 (d, \text{J} = 14.6 \text{ Hz}, 1H, 15-Hb), 2.85 (dd, \text{J} = 13.5, 3.0 \text{ Hz}, 1H, 2-Ha), 2.72-2.65 (m, 1H, 2-Hb), 2.50-2.36 (m, 2H, 6-H, 8-Hb), 1.76 (s, 3H, 14-Me), 1.66 (s, 3H, 4-Me), 0.83 (d, \text{J} = 6.4 \text{ Hz}, 3H, 6-Me) ppm;
Hz, 1H, 10-H), 4.40 (bs, 1H, 3-H), 4.06 (bs, 1H, 7-H), 3.39 (d, J = 15.0 Hz, 1H, 15-Ha), 3.36-3.32 (m, 4H, 10-OMe, 15-Hb), 2.87 (dd, J = 15.6, 4.5 Hz, 1H, 8-Ha), 2.80 (dd, J = 12.5, 2.0 Hz, 1H, 2-Hb), 2.69 (dd, J = 12.5, 8.0 Hz, 1H, 2-Hb), 2.49-2.39 (m, 2H, 6-H, 8-Hb), 1.76 (s, 3H, 14-Me), 1.68 (s, 3H, 4-Me), 0.83 (d, J = 5.7 Hz, 3H, 6-Me) ppm; $^{13}$C-NMR (125 MHz, methanol-d$_4$): $\delta$ 207.8 (C-9), 171.4 (C-1), 141.2 (C-16 or C-14), 140.7 (C-16 or C-14), 137.7 (C-4), 137.2 (C-19), 134.0 (C-12), 133.6 (C-20), 129.8 (C-5), 129.1 (C-20), 128.2 (C-13), 126.7 (C-11), 126.5 (C-17), 89.0 (C-10), 74.9 (C-3), 72.3 (C-7), 55.4 (10-OMe), 45.7 (C-12), 45.3 (C-8), 43.2 (C-3), 16.9 (6-Me), 16.0 (14-Me), 12.9 (4-Me) ppm. The $^{13}$C-signal C-18 could not unequivocally be determined; UPLC-MS $t_R = 2.92$ min; HRMS (ESI) $m/z$ for C$_{25}$H$_{32}$NO$_5$Br [M+Na]$^+$: calcd. 528.1362, found 528.1362.

Proansamitocin derivatives from CHBA fermentations

**Fermentation protocol:** B, 24 x 100 mL

**Culture media:** KOREA containing 3.0 g/L L-val

**Mutasython:** 3-Amino-4-chloro-5-hydroxybenzoic (677.5 mg, 3.0 mmol)

**Extraction day:** 10 days post inoculation

After extraction the residue was purified by MPLC (NP, MeOH : CH$_2$Cl$_2$ = 0% for 5min, 0% - 20% over 30 min, 20% over 10 min). UV active peaks were combined and the solvent was removed in vacuo. The residue was separated into two fractions by preparative HPLC (RP-CN, MeCN : H$_2$O + 0.1% FA = 10% for 5 min, 10% - 20% over 80 min, 20% - 90% over 15 min).

**19-Chloro-proansamitocin (35)**

Fractions collected between 21-51 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-18, MeOH : H$_2$O + 0.1% FA = 10% - 70% over 70 min, 60% - 80% over 30 min). Collection of the fractions between 56-70 min gave 35 in <1 mg yield.

$^{1}$H-NMR (600 MHz, methanol-d$_4$, 330 K): $\delta$ 8.56 (bs, 1H, N-H), 7.03 (bs, 1H, 21-H) 6.75 (dd, J = 15.4, 11.0 Hz, 1H, 12-H), 6.66 (bs, 1H, 17-H), 5.95 (d, J = 11.1 Hz, 1H, 13-H), 5.46 (dd, J = 15.4, 7.8 Hz, 1H, 11-H), 5.35 (bs, 1H, 5-H), 4.40 (d, J = 7.8 Hz, 1H, 10-H), 4.35-4.30 (m, 1H, 3-H), 3.94 (bs, 1H, 7-H), 3.35-3.30 (m, 1H, 15-Ha), 3.33 (s, 3H, 10-OMe), 3.22 (d, J = 14.2 Hz, 1H, 15-Hb), 2.78-2.67 (m, 1H, 8-Ha), 2.78-2.67 (m, 1H, 2-Ha), 2.67-2.56 (m, 1H, 2-Hb), 2.46 (dd, J = 16.4, 7.6 Hz, 1H, 8-Hb), 2.48-2.40 (m, 1H, 6-H), 1.74 (s, 3H, 14-Me), 1.60 (bs, 3H, 4-Me), 0.93-0.85 (m, 3H, 6-Me) ppm; $^{13}$C-NMR (150 MHz, methanol-d$_4$, 330 K): $\delta$ 133.8 (C-12), 129.1 (C-5), 127.7 (C-13), 126.4 (C-11), 89.2 (C-10), 74.2 (C-3), 72.5 (C-7), 57.0 (10-OMe), 46.1 (C-15), 45.2 (C-8), 39.1 (C-6), 16.9 (14-Me), 16.3 (6-Me), 13.3 (4-
Me) ppm; due to low yields not all $^{13}$C-signal could unequivocally be assigned; UPLC-MS $t_R$ = 2.48 min; HRMS (ESI) $m/z$ for C$_{25}$H$_{32}$NO$_6$Cl [M+Na]$^+$: calcd. 500.1816, found 500.1817.

**19-Chloro-methoxy-proansamitocin (36)**

Fractions collected between 83-95 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-18, MeOH : H$_2$O + 0.1% FA = 40% - 90% over 60 min, 100% over 20 min). Collection of the fractions between 25-28 min gave 36 as a white semisolid (3.0 mg, 0.008 mmol, 0.3%).

$^1$H-NMR (600 MHz, methanol-d$_4$, 330 K): $\delta$ 8.51 (s, 1H, N-H), 7.19 (s, 1H, 21-H), 6.80 (s, 1H, 17-H), 6.77 (dd, $J$ = 15.4, 11.0 Hz, 1H, 12-H), 5.97 (d, $J$ = 11.0 Hz, 1H, 13-H), 5.47 (dd, $J$ = 15.4, 8.1 Hz, 1H, 11-H), 5.36 (d, $J$ = 9.0 Hz, 1H, 5-H), 4.40 (d, $J$ = 8.1 Hz, 1H, 10-H), 4.34 (bs, 1H, 3-H), 3.98 - 3.92 (m, 1H, 7-H), 3.89 (s, 3H, 22-OMe), 3.40 (d, $J$ = 15.5 Hz, 1H, 15-Ha), 3.36-3.32 (m, 4H, 10-OMe, 15-Hb), 2.75 (dd, $J$ = 17.0, 4.6 Hz, 1H, 8-Ha), 2.70 - 2.59 (m, 2H, 2-H), 2.46 (dd, $J$ = 17.0, 8.9 Hz, 1H, 8-Hb), 2.51-2.41 (m, 1H, 6-H), 1.77 (s, 1H, 14-Me), 1.60 (s, 3H, 4-Me), 0.90 (d, $J$ = 6.6 Hz, 3H, 6-Me) ppm; $^{13}$C-NMR (150 MHz, methanol-d$_4$, 330 K): $\delta$ 207.8 (C-9), 156.7 (C-20), 140.7 (C-14), 133.7 (C-12), 128.9 (C-5), 127.7 (C-13), 126.4 (C-11), 89.0 (C-10), 74.2 (C-3), 72.4 (C-7), 56.9 (10-OMe, 20-OMe), 46.3 (C-15), 45.0 (C-8), 39.1 (C-6), 16.8 (14-Me), 16.2 (6-Me), 13.3 (4-Me) ppm. The $^{13}$C-signals for C1, C2, C4 and aromatic carbons could not unequivocally be determined; UPLC-MS $t_R$ = 2.74 min; HRMS (ESI) $m/z$ for C$_{26}$H$_{34}$NO$_6$Cl [M+Na]$^+$: calcd. 514.1972, found 514.1970.

**Proansamitocin derivatives from HHBA fermentation**

**Fermentation protocol:** B, 24 x 100 mL

**Culture media:** KOREA containing 0.1 g/L l-val, 0.3 g/L l-thr, 3% (v/v) coconut water

**Mutasynthon:** 3-Hydroxy-5-(hydroxymethyl)benzoic acid (490 mg, 3.2 mmol)

**Extraction day:** 10 days post inoculation

Fractions containing $m/z$ = 400 – 500 were collected after size exclusion chromatography (Sephadex LH-20, ~ 1.5 mL/min, MeOH) of the crude extract and the solvent was removed in vacuo at 35 °C resulting in a brown, viscous liquid. The residue was separated into two fractions by preparative HPLC (RP-C18, MeOH : H$_2$O + 0.1% FA = 65% - 100% over 80 min).

**10-R-Macrolacton 37a**
Fractions collected between 29-36 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H\textsubscript{2}O = 20\% - 30\% over 60 min). Collection of the fractions between 26-27 min gave 37a as a white semisolid (2.0 mg, 0.004 mmol, 0.14\%).

\textsuperscript{1}H-NMR (500 MHz, C\textsubscript{6}D\textsubscript{6}): \(\delta\) 6.90 (dd, \(J = 1.5\), 0.5 Hz, 1H, 17-H), 6.59 (ddd, \(J = 15.2\), 11.0, 0.9 Hz, 1H, 12-H), 6.28 (dd, \(J = 2.0\), 0.5 Hz, 1H, 21-H), 6.11 (dd, \(J = 2.0\), 1.5, 1H, 19-H), 5.75 (d, \(J = 11.0\) Hz, 1H, 13-H), 5.37 (dd, \(J = 15.2\), 7.0 Hz, 1H, 11-H), 5.05 (d, \(J = 13.0\) Hz, 1H, 22-Ha), 4.98-4.96 (m, 1H, 5-H), 4.97 (d, \(J = 13.0\), 1H, 22-Hb), 4.30 (dd, \(J = 10.4\), 3.0 Hz, 1H, 3-H), 3.89 (dd, \(J = 7.0\), 0.9 Hz, 1H, 10-H), 3.06 (s, 3H, 10-OMe), 3.04 (s, 2H, 15-H), 2.77 (dd, \(J = 18.0\), 3.0 Hz, 1H, 8-Ha), 2.58 (ddq, \(J = 10.0\), 8.0, 6.6 Hz, 1H, 6-H), 2.45 (dd, \(J = 15.0\), 10.4 Hz, 1H, 2-Ha), 2.31 (dd, \(J = 15.0\), 3.0 Hz, 1H, 2-Hb), 2.31 (dd, \(J = 18.0\), 8.0 Hz, 1H, 8-Hb), 1.55 (d, \(J = 1.4\) Hz, 3H, 4-Me), 1.46 (s, 3H, 14-Me), 1.05 (d, \(J = 6.6\) Hz, 2H, 6-Me) ppm; \textsuperscript{13}C-NMR (125 MHz, C\textsubscript{6}D\textsubscript{6}): \(\delta\) 209.2 (C-9), 171.2 (C-1), 156.2 (C-20), 141.8 (C-16), 140.4 (C-14), 138.9 (C-18), 137.5 (C-4), 131.0 (C-12), 128.3 (C-5), 126.4 (C-11), 125.7 (C-13), 118.5 (C-17), 116.3 (C-21), 112.0 (C-19), 88.3 (C-10), 73.7 (C-3), 72.7 (C-7), 65.2 (C-22), 56.9 (10-Me), 46.3 (C-15), 42.1 (C-8), 41.4 (C-2), 38.2 (C-6-Me), 16.4 (14-Me), 12.5 (4-Me) ppm; UPLC-MS \(t_{R} = 2.78\) min; HRMS (ESI) \(m/z\) for C\textsubscript{26}H\textsubscript{34}O\textsubscript{7} \([M+Na]^{+}\) : calcd. 481.2202, found 481.2201. Two-dimensional NMR spectroscopy (COSY, HSQC, HMBC) was used to assist in assigning \textsuperscript{1}H- and \textsuperscript{13}C-signals.

10-\textit{S}-Macrolactam 37b

Fractions collected between 29-36 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H\textsubscript{2}O + 0.1\% FA = 20\% - 30\% over 60 min). Collection of the fractions between 33-34 min gave 37b as a white semisolid (0.2 mg, 0.4 µmol, 0.01\%).

\textsuperscript{1}H-NMR (500 MHz, C\textsubscript{6}H\textsubscript{5}D\textsubscript{6}): \(\delta\) 6.86 (dd, \(J = 1.5\), 1.5, 1H, 17-H), 6.71 (ddd, \(J = 15.0\), 11.0, 1.7 Hz, 1H, 12-H), 6.25 (dd, \(J = 2.0\), 1.5 Hz, 1H, 21-H), 6.10 (dd, \(J = 2.0\), 1.5 Hz, 1H, 19-H), 5.77 (d, \(J = 11.0\) Hz, 1H, 13-H), 5.41 (dd, \(J = 15.0\), 5.3 Hz, 1H, 11-H), 5.07-5.02 (m, 1H, 5-H), 5.05 (d, \(J = 13.0\) Hz, 1H, 22-Ha), 4.92 (d, \(J = 13.0\), 1H, 22-Hb), 4.25 (dd, \(J = 10.3\), 3.0 Hz, 1H, 3-H), 4.06 (d, \(J = 5.3\), 1.7 Hz, 1H, 10-H), 3.82 (dd, \(J = 13.0\), 9.0, 2.9 Hz, 1H, 7-H),...
3.10 (d, $J = 15.0$ Hz, 1H, 15-Ha), 3.00 (dd, $J = 15.0$ Hz, 1H, 15-Hb), 3.00 (s, 3H, 10-OMe), 2.88 (dd, $J = 18.6$, 2.9 Hz, 1H, 8-Ha), 2.57 (dd, $J = 18.6$, 9.0 Hz, 1H, 8-Hb), 2.41 (ddq, $J = 13.0$, 3.0, 6.6 Hz, 1H, 6-H), 2.37 (dd, $J = 15.3$, 10.3 Hz, 1H, 2-Ha), 2.28 (dd, $J = 15.3$, 3.0 Hz, 1H, 2-Hb), 1.47 (s, 3H, 14-Me), 1.39 (d, $J = 1.5$ Hz, 3H, 4-Me), 1.09 (d, $J = 6.6$ Hz, 2H, 6-Me) ppm; HSQC/HMBC (125 MHz, C$_6$D$_6$ = 7.16 ppm: δ 210.2 (C-9), 170.9 (C-1), 141.5 (C-16), 139.3 (C-14), 138.2 (C-18), 136.7 (C-5), 128.6 (C-4), 126.3 (C-11), 125.8 (C-13), 118.1 (C-17), 115.6 (C-21), 111.6 (C-19), 88.4 (C-10), 72.7 (C-3), 71.6 (C-7), 64.9 (C-22), 56.9 (10-OMe), 46.0 (C-15), 42.0 (C-8), 40.0 (C-2), 38.4 (C-6), 17.1 (6-Me), 16.3 (14-Me), 12.8 (4-Me) ppm; UPLC-MS $t_R = 2.86$ min; HRMS (ESI) $m/z$ for C$_{26}$H$_{34}$O$_7$ [M+Na]$^+$: calcd. 481.2202, found 481.2201.

Seco-acid 38

Fraction between 21-28 min were combined and the solvent was removed in vacuo. The residue was further purified by semi-preparative HPLC (RP-CN, MeCN : H$_2$O + 0.1% FA = 15% - 25% over 60 min). Collection of the fractions between 29-30 min gave 38 as a white semisolid (2 mg, 0.004 mmol, 0.13%).

$^1$H-NMR (500 MHz, methanol-$d_4$): δ 8.52 (s, 1H, O-H), 6.70 (dd, $J = 15.1$, 11.0 Hz, 1H, 12-H), 6.65 (s, 2H, 21-H, 19-H), 6.52 (s, 1H, 17-H), 5.96 (d, $J = 11.0$ Hz, 1H, 13-H), 5.44 (dd, $J = 15.1$, 7.7 Hz, 1H, 11-H), 5.26 (d, $J = 9.9$ Hz, 1H, 5-H), 4.50 (s, 2H, 22-H), 4.34 (t, $J = 6.6$ Hz, 1H, 3-H), 4.31 (d, $J = 7.7$ Hz, 1H, 10-H), 3.81 (dt, $J = 7.4$, 6.3 Hz, 1H, 7-H), 3.35 (s, 3H, 10-OMe), 3.30 (s, 2H, 15-H), 2.63 (d, $J = 6.3$ Hz, 2H, 8-H), 2.42 (ddq, $J = 9.9$, 7.4, 6.7 Hz, 1H, 6-H), 2.40 (d, $J = 6.6$ Hz, 2H, 2-H), 1.73 (s, 3H, 14-Me), 1.65 (d, $J = 1.3$ Hz, 3H, 4-Me), 0.98 (d, $J = 6.7$ Hz, 3H, 6-Me) ppm;

$^{13}$C-NMR (125 MHz, methanol-$d_4$): δ 209.7 (C-9), 177.8 (C-1), 158.6 (C-20), 144.24 (C-18), 142.3 (C-16), 141.8 (C-14), 138.4 (C-4), 133.7 (C-12), 129.6 (C-5), 126.3 (C-13), 126.0 (C-11) 119.9 (C-17), 115.7 (C-21) 112.8 (C-19), 89.6 (C-10), 75.3 (C-3), 73.1 (C-7), 65.1 (C-22), 57.1 (10-OMe), 47.1 (C-15), 45.0 (C-8), 42.8 (C-2), 39.8 (C-6), 16.9 (6-Me), 16.7 (14-Me), 12.5 (4-Me) ppm; UPLC-MS $t_R = 2.60$ min; HRMS (ESI) $m/z$ for C$_{26}$H$_{36}$O$_8$ [M+Na]$^+$: calcd. 499.2308, found 499.2307.
3. Copies of NMR Spectra
4. References

(S1) A. M. Becker, R. W. Rickards, R. F. C. Brown, *Tetrahedron* 1983, 39, 4189-4192; A.
(S2) T. Knobloch, H. G. Floss, K. Harmrolfs, F. Taft, B. Thomaszewski, F. Sasse, A.
Kirschning, *ChemBioChem* 2011, 12, 540-547;
(S3) S. Eichner, H. G. Floss, F. Sasse, A. Kirschning, *ChemBioChem* 2009, 10, 1801-1805
(S4) Adams, E. S.; Rinehart, K. L. *J. Antibiot.* 1994, 47, 1456-1465
(S5) N. Fujii, J. J. Haresco, K. A. P. Novak, D. Stokoe, I. D. Kuntz, R. K. Guy, *J. Am. Chem.
Soc.* 2003, 125, 12074-12075
(S6) I. Bulyszko, G. Dräger, A. Klenge, A. Kirschning, *Chem. Eur. J.* 2015, 21, 19231-19242
(S7) S. Eichner, T. Eichner, H. G. Floss, J. Fohrer, E. Hofer, F. Sasse, C. Zeilinger, A.
Kirschning, *J. Am. Chem. Soc.* 2012, 134, 1673–1679.
(S8) M. Miura, S. Norio, T. Koike, T. Ishihara, T. Niimi, F. Hirayama, T. Shigenaga, Y.
Sakai-Moritani, T. Kawasaki, S. Sakamoto, M. Okada, M. Ohta, S. Tsukamoto, *Bioorg. Med.
Chem.* 2006, 14, 7688–7705.
(S9) A. I. Meyers, K. A. Babiak, A. L. Campbell, D. L. Comins, M. P. Fleming, R. Henning,
M. Heuschatmann, J. P. Hudspeth, J. M. Kane, P. J. Reider, D. M. Roland, K. Shimizu, K.
Tomoka, R. D. Walkup, *J. Am. Chem Soc.* 1983, 105, 5015-5024
(S10) McOmie, J. F. W.; Watts, M. L.; West, D. E. *Tetrahedron* 1968, 24, 2289-2292
(S11) F. D. Bellamy, K. Ou, *Tetrahedron Lett.* 1984, 25, 839-842
(S12) T. Kubota, M. Brünjes, T. Frenzel, J. Xu, A. Kirschning, H. G. Floss, *ChemBioChem*
2006, 7, 1221-1225
(S13) H. Zhao, A. Thurkauf, *Synth. Commun.* 2001, 31, 1921 – 1926
(S14) S. Eichner, T. Knobloch, H. G. Floss, J. Fohrer, K. Harmrolfs, J. Hermane, A. Schulz,
F. Sasse, P. Spiteller, F. Taft, A. Kirschning, *Angew. Chem.* 2012, 124, 776–781; *Angew.
Chem. Int. Ed.* 2012, 51, 752–757.