Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Supplementary information: Novel FK506 and FK520 analogues *via* mutasynthesis – mutasynthon scope and product characteristics

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Microbiology

Strains – storage and manipulation

The FK506 producer *Streptomyces tsukubaensis* no. 9993 (FERM BP-927) (International Patent Organism Depositary, Tsukuba, Japan) and its derivatives were maintained on MAM, ISP4, ISP3, or ISP2 (Shirling and Gottlieb, 1966) agar plates at 28 °C.

The FK520 producer, *Streptomyces hygroscopicus* subsp. *hygroscopicus* (DSM 40822, purchased from DSMZ, Braunschweig, Germany) (known to be equivalent to *Streptomyces hygroscopicus* subsp. *ascomyceticus* ATCC 14891) and its derivatives were maintained on MAM, ISP2, ISP3 or ISP4 agar plates at 28 °C.

Production of FK520 was carried out by fermentation of *Streptomyces hygroscopicus* subsp. *hygroscopicus* (DSM 40822), also termed BIOT-4081. *Streptomyces tsukubaensis* no. 9993 (FERM BP-927), also termed BIOT-3119 was used for producing FK506. Single spore isolates of both strains, termed BIOT-4168 (containing the genes for FK520 biosynthesis) and BIOT-4206 (containing the genes for FK506 biosynthesis), were used for strain construction.

Media Recipes

Water used for preparing media was prepared using Millipore Elix Analytical Grade Water Purification System.

FK-seed medium

component	Source	g/L
Soy peptone	Organo Technie	10
glucose	Sigma	20
Baker's yeast (fermipan red)	DSM	5
NaCl	Fisher	2

$ZnSO_4.7H_2O$	Sigma	0.05
$MgSO_4.7H_2O$	Sigma	0.125
$MnSO_4.4H_2O$	Sigma	0.01
$FeSO_4.7H_2O$	Sigma	0.02

The medium is adjusted to pH 7.0, with NaOH and then sterilised by autoclaving 121°C, 15 minutes.

FK production medium

component	**Source**	**g/L**
Nutrisoy Soy Bean Flour	ADM	20
glucose	Sigma	20
Baker's yeast (fermipan red)	DSM	6
K_2HPO_4	Sigma	2.5
KH_2PO_4	Sigma	2.5
NaCl	Fisher	5
glycerol	Fisher	30
soybean oil	Sigma	20
$ZnSO_4.7H_2O$	Sigma	0.05
$MgSO_4.7H_2O$	Sigma	0.125
$MnSO_4.4H_2O$	Sigma	0.01
$FeSO_4.7H_2O$	Sigma	0.02

The medium is adjusted to pH 6.4, and then sterilised by autoclaving 121°C, 15 minutes.

NGY

component	**Source**	**g/L**

Difco Nutrient Broth	Difco	8g
Glucose	Sigma	10g
Yeast Extract	Difco	5g

The medium is adjusted to pH 7.0, with NaOH and then sterilised by autoclaving 121°C, 15 minutes.

PYDG+MES

component	**Source**	**g/L**
Peptone from Milk Solids	Sigma	15g
Yeast Extract	Difco	1.5g
Dextrin	Avedex	45g
Glucose	Sigma	5g
MES	Acros	21.2

The medium is adjusted to pH 7.0 with NaOH, and then sterilised by autoclaving 121°C, 15 minutes.

MAM agar

component	**Source**	**g/L**
Wheat starch	Sigma	10g
Corn steep liquor	Sigma	5g
Yeast Extract	Difco	3g
$CaCO_3$	Sigma	3g
FeSO4	Sigma	300mg
Bacto agar	BD	20g

The medium is roughly pH 5.8 and is not adjusted, and then sterilised by autoclaving 121°C, 15 minutes.

Molecular Biology

Genomic DNA preparation, genome sequencing and cosmid library construction

Genomic DNA preparation

Strains were grown in shake flasks containing 25 mL TSB or ISP2 medium at 250 – 300 rpm and 28 °C and harvested after 2 to 3 days. Cell pellets were washed with 10.3% sucrose and frozen at -20 °C until used. The following method was most successful for genomic DNA isolation from *S. hygroscopicus*, *S. tsukubaensis* and *S. avermitilis*. A pellet originating from 12.5 mL of culture was resuspended in 1 mL STE buffer (100 mM NaCl, 10 mM Tris HCl pH8, 1 mM EDTA). 20 mL STE buffer supplemented with 2 mg/mL lysozyme were added and the resuspension incubated for 30 min at 37 °C. 20 µL of RNaseA (10 mg/mL) were added and the mixture incubated for another 30 min at 37 °C. 4.8 mL EDTA (0.1 M final concentration) were added to stop the reaction. 1.4 mL 20% SDS were added. After careful mixing the lysate was incubated on ice for 5 min, then extracted with one volume of phenol/chloroform/isoamylalcohol (25:24:1) and centrifuged at 2300 g and 4 °C for at least 15 min up to 1 h. Extractions were repeated until no more protein was visible at the interface, followed by a final chloroform/isoamylalcohol (49:1) extraction. The upper phase was precipitated with 1/10 vol. 5 M NaCl and 1 vol. cold isopropanol. After a few min, the DNA was spooled out with a glass rod and washed in ice cold 70% EtOH. After brief drying, the recovered DNA was dissolved in 0.5 - 1 mL TE 10:1. The proteinase K method (Kieser *et al*., 2000) was also applied successfully to recover genomic DNA from *S. tsukubaensis*.

DNA manipulation and sequencing

DNA manipulations, PCR and electroporation procedures were carried out as described in Sambrook *et al*. (1989). DNA sequencing was performed as described previously (Gaisser *et al*., 2000). Genome sequencing was carried out using 454 technology (Margulies *et al*., 2005) at Cogenics and the University of Cambridge.

Cosmid library preparation for *S. tsukubaensis*

High molecular weight DNA from several genomic DNA preps was partially digested with *Bfu*CI, an isoschizomer of *Sau*3A, to a mean size of 30 – 60 kb. The digested DNA was then dephosphorylated and ligated into the *Bam*HI site of SuperCos1 (Stratagene), previously linearized with *Xba*I. Gigapack® III XL Packaging Extract (Stratagene) was used for packaging and *Escherichia coli* VCS257 (Stratagene) for transfection. The titre was 6.7 x 10^5 cfu / µg vector. DNA of 10 cosmids was isolated and digested with *Eco*RI to check the insert size which was 40 kb on average. 2000 clones were grown in 96-well microtiter plates (150 µL LB Ampicillin (100 mg/L) and Kanamycin (50 mg/L) per well) at 37 °C and frozen at -80 °C after mixing wells with 50 µL LB/glycerol 1:1.

fkbO probe preparation

A DIG labeled *fkbO* probe was used to detect cosmids containing this region of the FK506 biosynthetic cluster. The probe was prepared by PCR using primers UES2for (5'-CACTCCTTCGATCTCCACGAGCAGGTCGCCACGGGC-3') and UES2rev (5'-

ACCCTGCCGTCCTCACGGCACACCACTACCCCACGG-3') and DIG labelled dNTP mix (Roche). It comprises 410 bp of 3'-terminal *fkbO* sequence. Sequence information for primer design had been obtained by 454 sequencing of BIOT-3119. Annealing temperatures between 66 and 71 °C and extension for 20 sec at 68 °C proved to be successful.

Colony hybridization

Thawed microplate-cultures were stamped onto positively charged filter membranes (Roche) which had been placed on LB Ampicillin (100 mg/L) and Kanamycin (50 mg/L) plates. After overnight growth at 37 °C membranes were taken off. Cells were lysed and cell debris removed according to the DIG Application Manual for Filter Hybridization (Roche). DNA was cross-linked by exposing membranes to UV (302 nm) for 5 min. Membranes were kept between two sheets of filter paper soaked with 2xSSC at 4 °C or used immediately for hybridization. Hybridization was carried out using standard hybridization buffer and DIG labeled *fkbO* probe (see above) at a hybridization temperature of 68 °C. Stringent washes were performed at 68 °C. The nonradioactive DIG Nucleic Acid Detection kit from Roche was used to identify 5 positive clones on 4 library plates. The procedure followed the instructions of the DIG Application Manual for Filter Hybridization (Roche).

End-sequencing and cosmid sequencing

The sequence information of FK506 *fkbP, fkbO and fkbB* is needed for the construction of *ave* load variants of BIOT-3119. 454 sequencing of BIOT-3119 gave two contigs covering part of the FK506 cluster including *fkbO* and *fkbP*, but neither of them covering *fkbB* sufficiently. The 5 positive cosmids obtained via hybridization of the BIOT-3119 cosmid library with an FK506 *fkbO* probe were end-sequenced. The alignment of end sequences with the FK520 cluster sequence (AF235504) showed that two of the cosmids contained *fkbP*, *O*, *B* completely. One of them, 3G9, was sequenced in full (Cambridge University DNA Sequencing Facility). The sequence of cosmid 3G9 has been deposited and has the accession code JQ044685.

Construction of BIOT-4254$_{506}$

Oligos AES43 (5'-CCAAGCTTGAGCGCCTCGTCCCAGAGCGCGGCCTGGTC-3') and AES40 (5'-CCATGCATCGGGACACCGTCCGTGAGCGACACCTCGGCATGACC-3') were used to amplify a 2057 bp region of DNA upstream of the planned small deletion to introduce a frame-shift in *fkbO* from *Streptomyces tsukubaensis* no. 9993 (FERM BP-927) in a standard PCR reaction (Sambrook and Russell, 2001) using genomic DNA (Kieser *et al*., 2000) as the template and hot start KOD DNA polymerase. A 5'-extension was designed in each oligo to introduce the restriction sites (*Hin*dIII/*Nsi*I, underlined in primer sequence) to aid cloning of the amplified fragment. This 2057 bp fragment was cloned into pUC19 that had been linearized with *Sma*I, resulting in plasmid pAES8 and the insert verified by sequencing.

Similarly, oligos AES41 (5'-CCATGCATCCGATGCCGTCGCGGCGCTCTACACGCGGG-3') and AES42 (5'-CCAGATCTGAAGGGCTCGGCGGTCACACCGGGCAGCGC-3'), with *Nsi*I and *Bgl*II restriction sites, were used to amplify a 1985 bp region of DNA downstream of the planned small deletion to introduce a

frame-shift in *fkbO*. This 1985 bp fragment was cloned into pUC19 that had been linearized with *Sma*I, resulting in plasmid pAES9 and the insert verified by sequencing.

The ~2.0 kb *Hin*dIII/*Nsi*I fragment from pAES8 and ~2.0 kb *Nsi*I/*Bgl*II I fragment from pAES9 were cloned into the ~6.0 kb *Hin*dIII/*Bgl*II fragment of pKC1139 (Bierman *et al*., 1992) to make pAES10. pAES10 therefore contained the upstream and downstream regions such that the double crossover event would result in the desired small deletion, introducing a frame-shift in the *fkbO* gene. (See Figure S1).

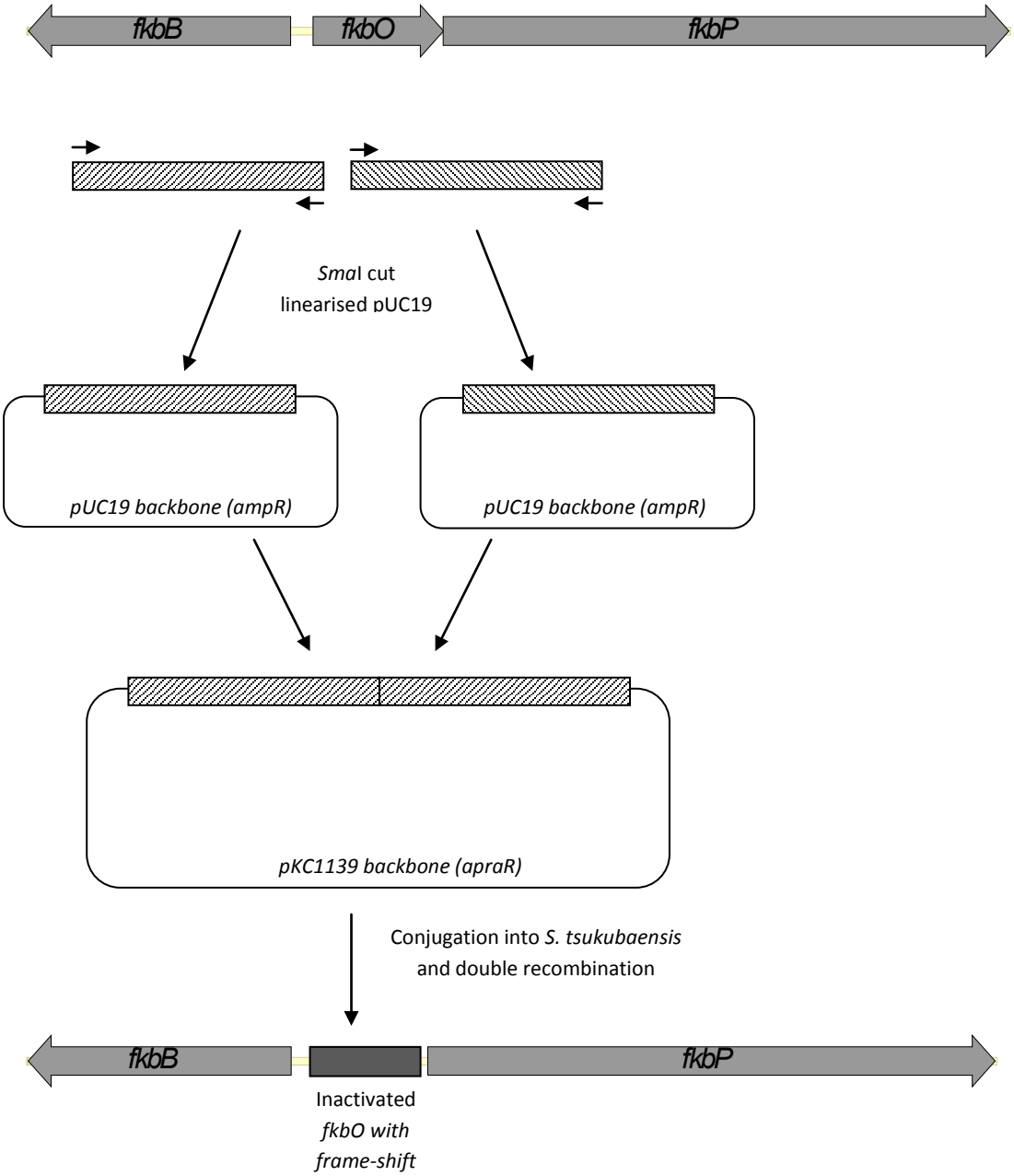


Figure S1: Strategy for inactivation of *fkbO* using a small deletion to induce a frame-shift.

Escherichia coli ET12567, harboring the plasmid pUZ8002, was transformed with pAES10 by electroporation to generate the *E. coli* donor strain for conjugation. Fresh spores were harvested in water from plates of *Streptomyces tsukubaensis* (BIOT-4206). Spore suspensions were heat-shocked at 50 °C for 10 min. They were then mixed with the *E. coli* donor strain, which had been washed twice with 2xTY, in a ratio of 3:1 streptomycete to *E. coli*, and the mixture shaken at 37 °C, 300 rpm, 2.5 cm throw for 1.5 - 2 h. The conjugation mixture was then plated on R6 medium and incubated at 37 °C. After ~20 hr, the plates were overlaid with water containing 2 mg of apramycin sulfate and 1 mg of nalidixic acid and incubation continued at 28 °C.

Exconjugants were plated on R6 agar and incubated at 37 °C. pAES10 is not able to self-replicate in *Streptomyces tsukubaensis* no. 9993 (FERM BP-927) at 37°C and must integrate into the genome. Transformants were subcultured onto MAM plates with apramycin (0.050 mg/mL) at 37°C two to three times, to ensure that the pAES10 plasmid with resistance marker had integrated. Subculturing to allow secondary recombination was carried out as follows: the transformants were subcultured for two subsequent rounds at 37°C on MAM plates with no antibiotic and then a final time at 28°C. The transformants from the fourth subculture on antibiotic free plates were plated for spore harvest on ISP4 medium at 28°C. Serial dilutions were made from the filtered collected spores and plated on ISP4 plates at 28°C to achieve single colonies.

Single colonies were patched in duplicate onto ISP4 supplemented with 0.050 mg/mL apramycin and ISP4 containing no antibiotics, and grown at 28 °C for 3-4 days. Patches that grew on the no antibiotic plate but did not grow on the apramycin plate were screened to test if the desired double recombination event had occurred. A 6 mm circular plug from each patch that had lost the marker was used to inoculate individual 50 mL falcon tubes containing 7 mL NGY (See Media Recipes) without antibiotics and grown for 2 days at 28°C, 300 rpm with a 2.5 cm throw. These were then used to inoculate (0.5 mL into 7 mL - 7% inoculum) PYDG+MES (See Media Recipes) in a 50 mL falcon tube at 28°C, 300 rpm with a 2.5 cm throw. After 24 hours, each falcon tube was fed with 0.050 mL 0.32 M 4-trans-hydroxy cyclohexane carboxylic acid (**12**) to give a final concentration of 2.12 mM acid and shaking incubation was continued as before. The cultures were sampled after 6 days growth and analyzed by LCMS by the methods described above. Of the 93 patches that were screened, 49 had undergone the desired double recombination event. These strains produced a metabolite that was 30 amu less than FK506 itself. This compound was shown to be **10** following isolation and characterisation of that compound. One of these strains was selected and designated BIOT-4254$_{506}$ and was then used exclusively for analytical and preparative biotransformations.

Construction of BIOT-4131$_{520}$

Oligos SG165 (5'-CCAGATCTCGTCGGGCACCTTGAAGTAGGCGAGCCG) and SG166 (5'-CCCTCGAGGTCCGGTGATCCGGTCTTCTCGAAGC-3') were used to amplify a 1727 bp region of DNA upstream of the planned small deletion to introduce a frame-shift in *fkbO* from *Streptomyces hygroscopicus* subsp. *hygroscopicus* (DSM 40822) in a standard PCR reaction (Sambrook and Russell, 2001) using genomic DNA (Kieser *et al*., 2000) as the template and hot start KOD DNA polymerase. A 5'-extension was designed in each oligo to include the restriction sites (*Bgl*II/*Xho*I, underlined in primer sequence) present in the genomic sequence to aid cloning of the amplified fragment. This

1727 bp upstream fragment was cloned into pUC19 that had been linearized with *Sma*I, resulting in plasmid pAESA5 and the insert verified by sequencing.

Similarly, Oligos SG167 (5'-CCCTCGAGCGCACAGCGCCCTGTCGAGTCCGGCATG-3') and SG168 (5'-CCGAATTCGAGGTCGAAGCGGGTGAACCAGCGTC-3'), with *Xho*I and *Eco*RI restriction sites, were used to amplify a 2034 bp region of DNA downstream of the planned small deletion to introduce a frameshift in *fkbO*. This 2034 bp downstream fragment was cloned into pUC19 that had been linearized with *Sma*I, resulting in plasmid pAESB1 and the insert verified by sequencing.

The ~1.7 kb *Bgl*II/*Xho*I fragment from pAESA5 and ~2.0 kb *Xho*I/*Eco*RI fragment from pAESB1 were cloned into the ~5.9 kb *Bgl*II/*Eco*RI fragment of pKC1139 (Bierman *et al*., 1992) by three-part ligation to make pAESAB1. pAESAB1 therefore contained the upstream and downstream regions such that the double crossover event would result in the desired small deletion, introducing a frame-shift in the *fkbO* gene. (See Figure S1)

Escherichia coli ET12567, harbouring the plasmid pUZ8002 (MacNeil *et al*., 1992, Paget *et al*., 1999) was transformed with pAESAB1 by electroporation to generate the *E. coli* donor strain for conjugation. This strain was used to transform *Streptomyces hygroscopicus* subsp. *hygroscopicus* (DSM 40822) by spore conjugation (Kieser *et al*. 2000).

Fresh spores were harvested in water from plates of *Streptomyces hygroscopicus* subsp. *hygroscopicus* (DSM 40822) and were heat-shocked at 50 °C for 10 minutes. These were then mixed with the *E. coli* donor strain, which had been washed twice with 2xTY, in a ratio of 1:3 streptomycete to *E. coli*, and plated on R6 medium, incubating at 28°C. After ~20 hr, the plates were overlaid with water containing 2 mg of apramycin sulphate and 1 mg of nalidixic acid and incubation continued at 28°C.

After 3-5 days, apramycin resistant colonies were seen, indicating integration of the pAESAB1 plasmid into the genome.

pAESAB1 is able to self-replicate in *Streptomyces hygroscopicus* subsp. *hygroscopicus* (DSM 40822) at 28°C. Transformants were subcultured onto MAM plates with apramycin (0.050 mg/mL) at 28°C to ensure the pAESAB1 plasmid with resistance marker was present. Subculturing to allow secondary recombination was carried out as follows: the transformants were subcultured again on to MAM plates with apramycin at 37 °C to induce the plasmid to integrate, as the plasmid cannot self-replicate at 37 °C. The transformants were then subcultured for four subsequent rounds at 37 °C on MAM plates with no antibiotic. The transformants from the fourth subculture on antibiotic free plates were plated for spore harvest on ISP3 medium at 28 °C. Serial dilutions were made from the filtered collected spores and were plated on MAM plates to achieve single colonies.

Single colonies were patched in duplicate onto MAM supplemented with 0.050 mg/mL apramycin and MAM containing no antibiotics, and grown at 28 °C for 3-4 days. Patches that grew in the absence of antibiotics but did not grow in the presence of apramycin were screened to test if the desired double recombination event had occurred. A 6 mm circular plug from each patch that had lost the marker was used to inoculate individual 50 mL falcon tubes containing 7 mL FK seed medium (See Media Recipes) without antibiotics and grown for 2 days at 28°C, 300 rpm with a 2.5 cm throw.

These were then used to inoculate (0.5 mL into 7 mL - 7% inoculum) FK production medium (See Media Recipes) in a 50 mL falcon tube at 28°C, 300 rpm with a 2.5 cm throw. After 24 hours, each falcon tube was fed with 0.050 mL 0.32 M 4-*trans*-hydroxycyclohexane carboxylic acid (**12**) to give a final concentration of 2.12 mM acid and shaking incubation was continued as before. The cultures were sampled after 6 days growth and analysed by LC-MS, using the methods described below.

14 out of 79 apramycin sensitive strains tested had undergone the desired recombination event to give the disruption of the *fkbO* gene. These strains produced a metabolite that was 30 amu less than FK520 itself. This compound was shown to be **11** following isolation and characterisation of that compound. One strain was selected and designated BIOT-4131$_{520}$ and was then used exclusively for analytical and preparative biotransformations.

Construction of BIOT-4276$_{506}$

Oligos UES4_For (5'-CGACTAGTGCAGCGCGAGCGTGTTGACGAACATGCCGATGAGG-3') and UES4_Rev (5'-GGCATATGAACGCCTTTCTCTCGGCTGACCGTACGGCAGCACG-3') were used to amplify a 2.27 kb region of DNA from *Streptomyces tsukubaensis* no. 9993 using cosmid 3G9 template and KOD polymerase. A 5' extension was designed for each oligo to introduce restriction sites (*Spe*I, *Nde*I) to aid cloning of the amplified fragment. The *Nde*I site of UES4_For comprises the loading module start codon (ATG), and the 3 non-coding bases upstream are mutated to CAT (replacing TCC). The 2.27 kb PCR fragment was ligated with *Sma*I digested and SAP-treated pUC19, resulting in plasmid 'pUC19 fkbP'O 506 left'. The insert sequence was verified by sequencing.

Oligos UES7_For (5'-TTCGATCGCCATCGTCGGCATGGCCTGCCGACTGCCGGGCGGCGT-3') and UES7_Rev (5'- GCTCTAGACGGATGGGCGCTGCACTCGACGAAGAGGGAGCCGT-3') were used to amplify a 2.29 kb region of DNA from *Streptomyces tsukubaensis* no. 9993 using 3G9 cosmid template. A 5' extension was designed for UES7_Rev to introduce an *Xba*I site. UES7_For comprises the internal *Pvu*I site at the 5' end of ave KS1 followed by the internal *Pvu*I site of FK506 KS1 which was mutated to 'CCATCG'. The 2.29 kb PCR fragment was ligated with *Sma*I digested and SAP-treated pUC19 resulting in plasmid 'pUC19 fkbB' 506 right'. The insert sequence was verified by sequencing.

Oligos PCR12F (5'- TTCATATGCAGAGGATGGACGGCGGGGAAGAACCCCGCCCTGCGG-3') and UES5_Rev (5'-TTCGATCGGTTCGTCCGCCGCTGCCGTGCCTCCGTGGCCGCTGGGTG-3') were used to amplify a 1.46 kb region of DNA from *Streptomyces avermitilis* using genomic DNA template (Kieser *et al*., 2000) and KOD polymerase. A 5' extension was designed for PCR12F to introduce an *Nde*I site, whereas UES5_Rev comprises the internal *Pvu*I site at the 5'-end of *ave* KS1. The 1.46 kb PCR fragment was ligated with *Sma*I digested and SAP-treated pUC19, resulting in plasmid 'pUC19 ave load middle' and the insert verified by sequencing. PCR12F comprises the 5' end of *ave* loading module and the choice of *Nde*I introduces a mutation resulting in the N-terminal sequence of the loading module being 'MQR' instead of 'VQR' in the avermectin PKS. UES5_Rev comprises the 5' end of *ave* KS1 and introduces a mutation resulting in the KS1 N-terminal sequence of 'EPIA' instead of 'DPIA' in the avermectin cluster and as 'EPIA' in the FK506 cluster.

The *Spe*I/*Nde*I fragment of 'pUC19 fkbP'O 506 left' was ligated with *Spe*I/*Nde*I cut pKC1139B01(a modified version of plasmid pKC1139, replacing the linker region between the *Bgl*II and *Pvu*II sites to include *Spe*I, *Nde*I, *Eco*RV and *Xba*I sites) resulting in pUS11. A 3-fragment ligation was performed with the 4.72 kb *Nde*I/*Xba*I fragment of pUS11, the 1.46 kb *Nde*I/*Pvu*I fragment of 'pUC19 ave load middle' and the 2.29 kb *Pvu*I/*Xba*I fragment of 'pUC19 fkbB' 506 right'. The resulting plasmid was named pUS14. (See Figure S2.)

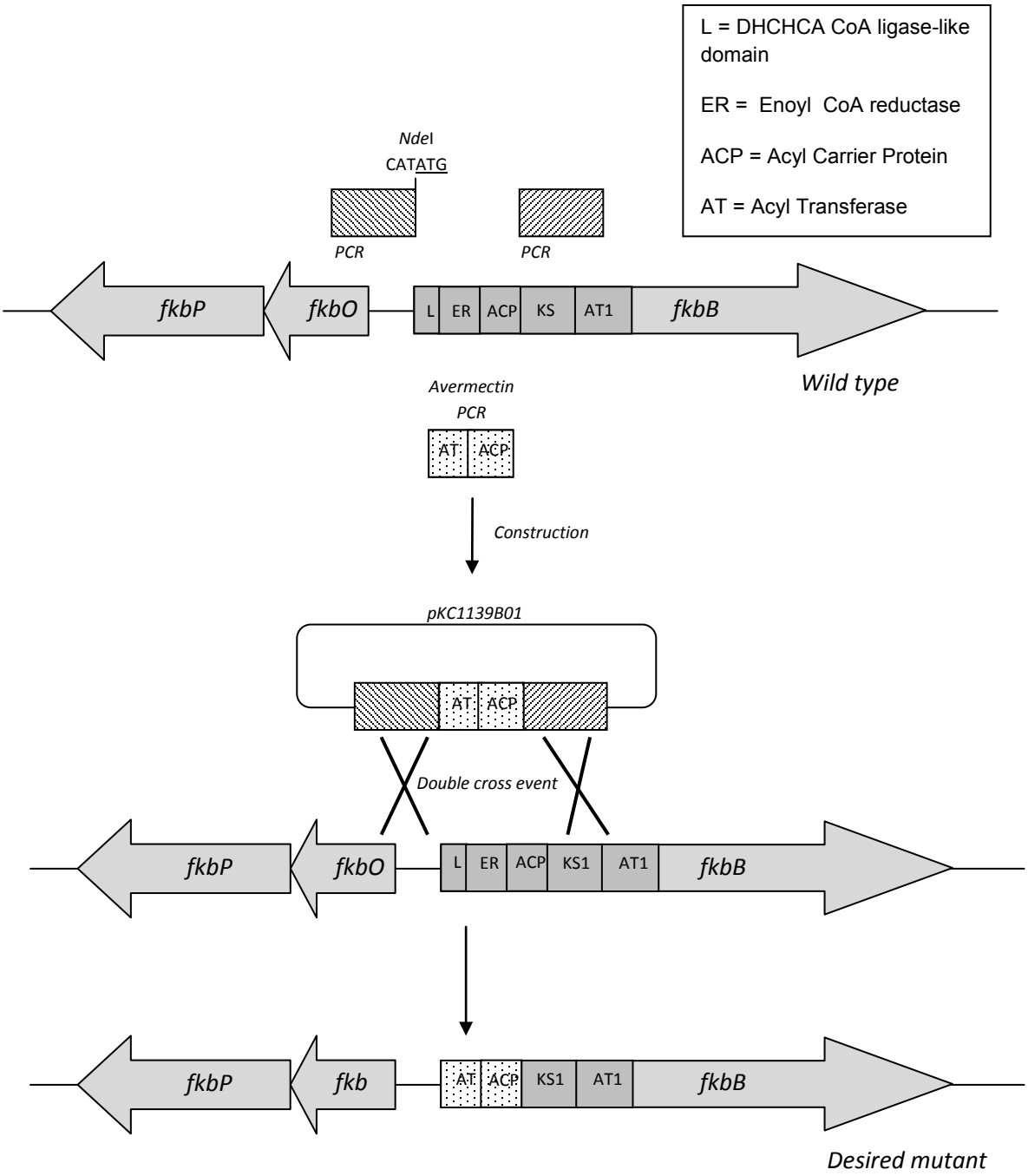


Figure S2: Strategy to replace FK loading domain with the avermectin loading domain.

Plasmid pUS14 was transformed into *Streptomyces tsukubaensis* by spore conjugation as described above. Exconjugants were screened as before. A total of 26 out of 51 isolates had undergone the desired double recombination event. One strain was selected and designated as BIOT-4276$_{506}$ and was then used exclusively for analytical and preparative biotransformations.

Construction of BIOT-4225$_{520}$

Oligos PCR11F (5'-CGACTAGTGCAGCGCGAGCGTGTTGACGAACATGCCGATCAGG-3') and PCR11R (5'-GGCATATGAACACCTTTCTCTCGACCAACCGCACAACAGCACG-3') were used to amplify a 2.19 kb region of DNA from *Streptomyces hygroscopicus* subsp. *hygroscopicus* (DSM40822) using genomic DNA template (Kieser *et al*., 2000) and the KOD Polymerase kit from Novagen. PCR samples were supplemented with 10% DMSO. Annealing temperatures between 65 and 70 °C and extension for 1 min at 70 °C were used. A 5' extension was designed for each oligo to introduce restriction sites (*Spe*I, *Nde*I) to aid cloning of the amplified fragment. The *Nde*I site of PCR11R comprises the loading module start codon (ATG), and the 3 non-coding bases upstream are mutated to CAT (replacing TCC). The 2.19 kb PCR fragment was ligated with *Sma*I digested and SAP-treated pUC19, resulting in plasmid pUC19 PCR11. The insert sequence was verified by sequencing.

Oligos PCR13F2 (5'-CTCGGCTCCGGCGACCCGCTAGCGATCGTCGGCATGGCGT-3) and PCR13R (5'-GCTCTAGACACCGGCTCGGTCACCCAGGCGCTGTCCAC-3') were used to amplify a 2.20 kb region of DNA from *Streptomyces hygroscopicus* subsp. *hygroscopicus* (DSM40822) using genomic DNA template (Kieser et al., 2000) as above. A 5' extension was designed for each oligo to introduce restriction sites (*Nhe*I, *Xba*I) to aid cloning of the amplified fragment. The 2.20 kb PCR fragment was ligated with *Sma*I digested and SAP-treated pUC19. A construct was selected with the insert orientated such that the *Nhe*I site of the PCR fragment would be close to the *Nde*I site of pUC19. This was relevant for the subsequent cloning procedure and the resulting plasmid was named pUC19 PCR13F. The insert sequence was verified by sequencing. PCR13F2 comprises the 5' end of KS1 and the choice of *Nhe*I introduces a mutation resulting in the KS1 N-terminal sequence of 'DPLA' instead of the original FK520 KS1 sequence 'DPVA'.

Oligos PCR12F (5'- TTCATATGCAGAGGATGGACGGCGGGGAAGAACCCCGCCCTGCGG-3') and PCR12R (5'- TTGCTAGCGGGTCGTCCGCCGCTGCCGTGCCTCCGTGGCCGCT-3') were used to amplify a 1.46 kb region of DNA from *Streptomyces avermitilis* (DSM41443) using genomic DNA template (Kieser et al., 2000) as above. The 1.46 kb PCR fragment was ligated with *Sma*I digested and SAP-treated pUC19, resulting in plasmid pUC19 PCR12 and the insert verified by sequencing. PCR12F comprises the 5' end of *ave* loading module and the choice of *Nde*I introduces a mutation resulting in the N-terminal sequence of the loading module being 'MQR' instead of 'VQR' in the avermectin PKS. PCR12R comprises the 5' end of KS1 and as mentioned for PCR13F2 the choice of *Nhe*I introduces a mutation resulting in the KS1 N-terminal sequence of 'DPLA' instead of 'DPVA' in the FK520 cluster and 'DPIA' in the avermectin cluster.

The *Spe*I/*Nde*I fragment of pUC19PCR11 was ligated with *Spe*I/*Nde*I cut pKC1139B01 resulting in pUS1. The *Nde*I/*Nhe*I fragment of pUC19PCR12 was ligated with *Nde*I/*Nhe*I cut pUC19PCR13F resulting in pUS2. The *Nde*I/*Xba*I fragment of pUS2 was then cloned into pUS1 digested with *Nde*I/*Xba*I, the final plasmid being pUS4. (See Figure S2.)

Escherichia coli ET12567 (pUZ8002) was transformed with pUS4. This strain was used to transform *Streptomyces hygroscopicus* subsp. *hygroscopicus* by spore conjugation (as previously). Exconjugants were plated on R6 agar, incubated at 37 °C and overlaid with nalidixic acid (25 mg/L) and apramycin (50 mg/L) the next day. pUS4 is not able to self-replicate in *Streptomyces hygroscopicus* subsp. *hygroscopicus* at 37 °C and is forced to integrate into the genome by recombination. Six days later, transformants were subcultured on MAM plates containing apramycin (50 mg/L) at 37 °C to ensure the pUS4 plasmid with resistance marker was present. Subculturing for two subsequent rounds at 37 °C on MAM plates without apramycin was carried out to allow secondary recombination. This event would either cause the loss of the plasmid via the second region of homology, not the one by which it had originally integrated resulting in the desired gene replacement; or the loss of the plasmid via the same region of homology as the original integration resulting in a wildtype revertant. This required single spore isolation of subcultured patches. The transformants were subcultured on MAM plates without apramycin at 28 °C and subsequently incubated at 28 °C on ISP3 plates for spore harvest. Serial dilutions were made from the collected spores and plated on MAM plates to achieve single colonies.

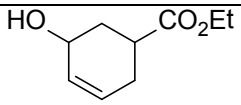
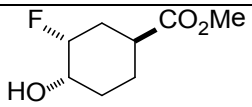
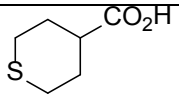
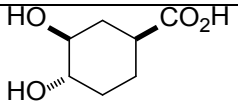
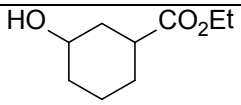
Single colonies were patched in duplicate onto MAM supplemented with 50 mg/L apramycin and MAM containing no antibiotics, and grown at 28 °C for 3-4 days. Patches that grew on the no antibiotic plate but did not grow on the apramycin plate were screened for production to test if the desired double recombination event had occurred. A 6 mm circular plug from each patch that had lost the marker was used to inoculate individual 50 mL falcon tubes containing 7 mL FK seed medium (see Media Recipes) without antibiotics and grown for 2 days at 28 °C, 300 rpm, 2.5 cm throw. These were then used to inoculate (0.5 mL into 7 mL - 7% inoculum) FK production medium (see Media Recipes) in a 50 mL falcon tube and incubated at 28 °C, 300 rpm, 2.5 cm throw. The cultures were harvested after 6 days growth and analysed by LC-MS, using the methods described below. 61 out of 93 apramycin sensitive strains tested had undergone the desired recombination event.

Six of these strains were selected and feeding experiments were undertaken. After inoculation of FK production medium (see Media Recipes), tubes were incubated at 28 °C, 300 rpm, 2.5 cm throw. After 24 h, each falcon tube was fed with 50 μL 0.32 M cyclobutylcarboxylic acid (**85**) to give a final concentration of 2.12 mM and shaking incubation was continued for five days. Culture extracts were analysed by LC-MS, using the methods described above. All six isolates used **85** as starter. One strain was selected and designated BIOT-4225$_{520}$ and was then used exclusively for analytical and preparative biotransformations.

Chemistry

Starting materials

The mutasynthons were sourced from commercial suppliers except for the following:

Name	structure	Source
Ethyl 5-hydroxycyclohex-3-enecarboxylic acid		Synthesised as per Lowden 1997
(1*S**,3*R**,4*S**)-methyl 3-fluoro-4-hydroxycyclohexanecarboxylate		Synthesised as per WO2004007709
tetrahydro-2H-thiopyran-4-carboxylic acid		Synthesis as per Strässler *et al.* 1997
(1S*,3S*,4S*)-3,4-dihydroxycyclohexanecarboxylic acid		Synthesised as per Lowden 1997
3-hydroxycyclohexanecarboxylic acid		Synthesised as per Goss *et al*. 2010

Mutasynthesis analysis methods

Qualitative analysis

Culture broth (0.9 mL) was added to an Eppendorf tube (2.0 mL) and ethyl acetate (0.9 mL) added. The broth was mixed with the solvent for 30 minutes on a shaking platform (vibrax) at 400 rpm. The phases were then separated by centrifugation (10 minutes, 13,200 rpm). An aliquot of the organic layer (0.1 mL) was then transferred to either a clean glass LC-vial or a vial containing 0.005 mg of pimecrolimus (as an internal standard for quantification). The solvent was removed *in vacuo* and then residue re-dissolved in methanol (1 mL) by gentle agitation on a shaking platform (5 minutes).

The HPLC system comprised an Agilent HP1100 equipped with a Hyperclone ODS2, C18, 3 micron 4.6 x 150 mm column (Phenomonex). Injection volume 10 µL, oven 50°C, A: 0.1% formic acid, B: 0.1% formic acid in MeCN. 1mL/min; 0-1 min 65% B; 6.5 min 100% B; 10 min 100% B; 10.05 min 65% B, 12 min 65% B. The HPLC system described above was coupled to a Bruker Daltonics Esquire3000 electrospray mass spectrometer. Positive-negative switching was used over a scan range of 500 to 1000 Dalton. Samples were initially analysed by LC-MS to identify potential FK-analogs, which were then quantified by the method described below.

Quantitative analysis

LC samples that had been spiked with 0.005 mg/mL pimecrolimus were analysed on the same instrument and with the same chromatographic conditions. However the MS was conducted in multiple reaction monitoring mode (MRM mode) in order to quantify the amount of FK analog in the sample. Details of the quantification are:

negative scan mode, m/z = 450-850

MRM setup:

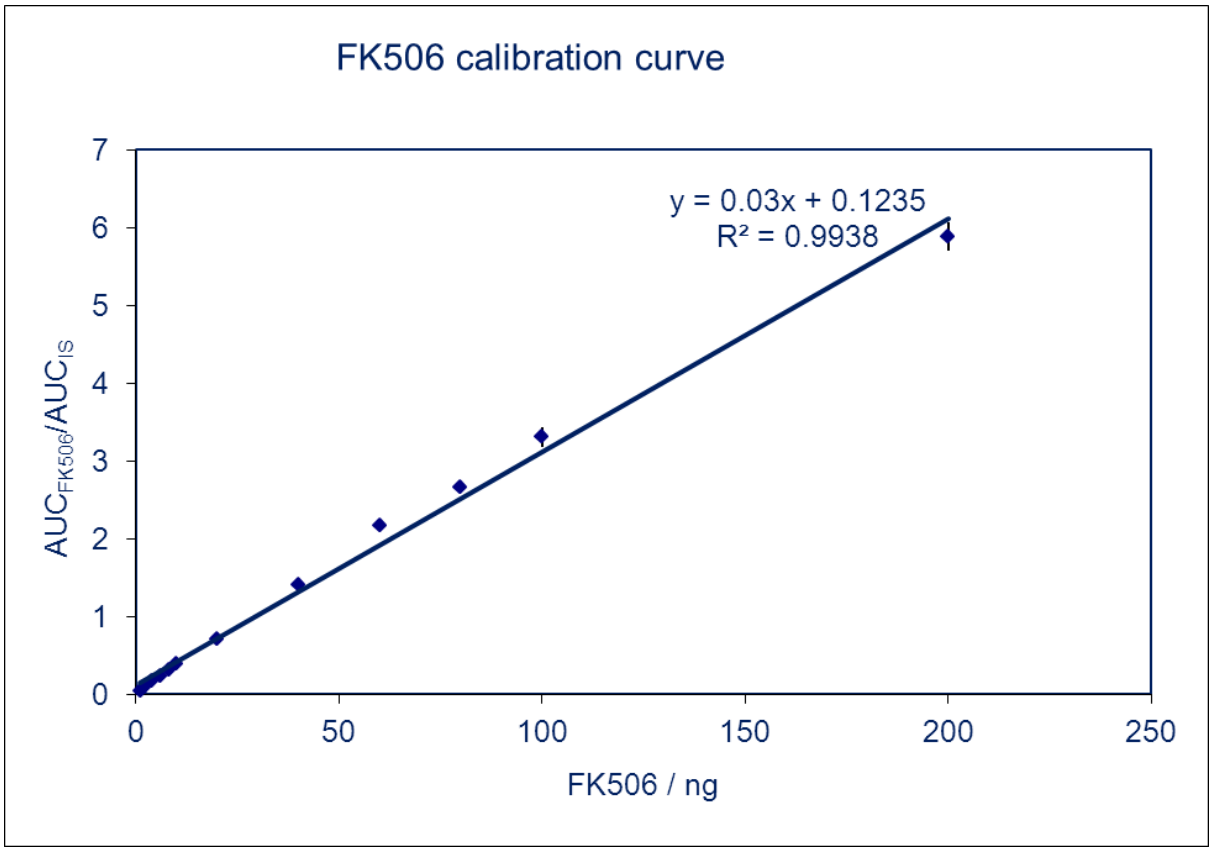
	transitions [Da]	fragmentation amplitude [V]
pimecrolimus (IS):	808.4 → 548.3	1.15
FK520 (**2**)	790.5 → 548.3	1.15
FK506 (**1**)	802.4 → 560.3	1.15

all parent ions are isolated with a width of 3 amu.

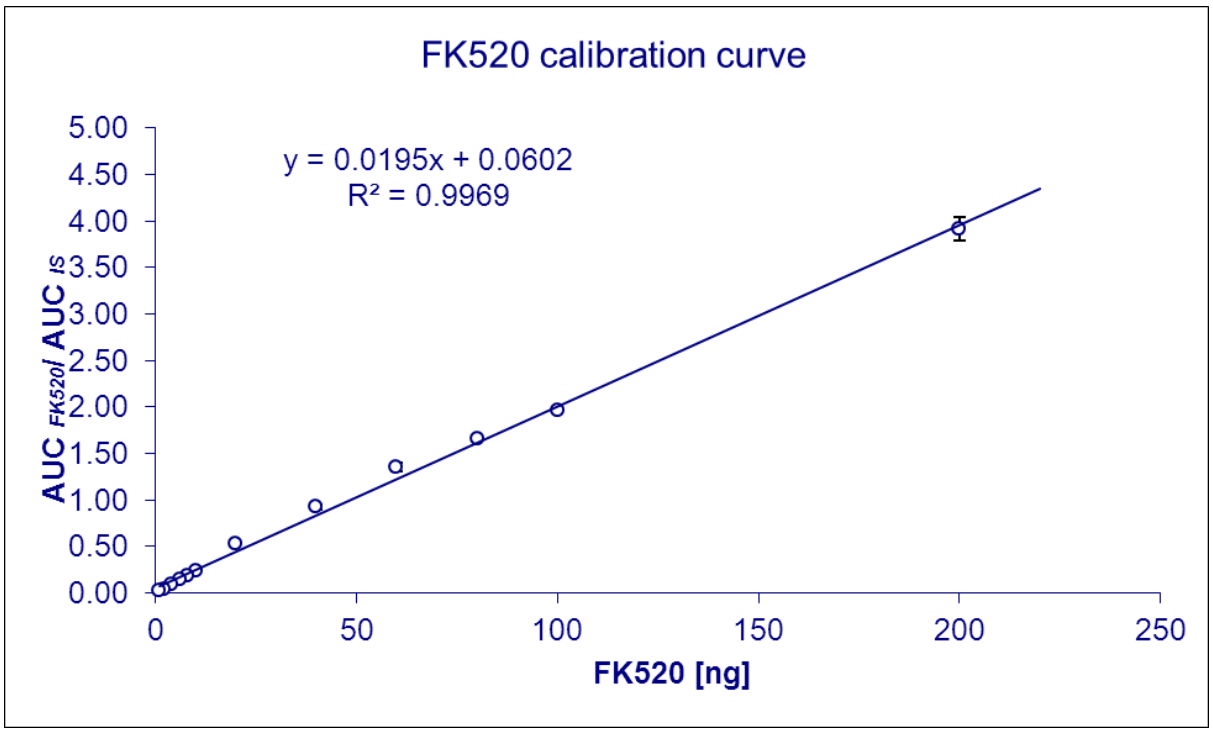
All FK520 and FK506 analogues can be quantified in this manner, with the parent ion isolated as [M-H]- and the transition to 548.2 (for FK520 analogues) or 560.2 (for FK506 analogues) used.

The amount of analyte present is then calculated by dividing the integral for the analyte transition (as detailed above) with that for the internal standard, pimecrolimus. This ratio is then compared with a standard calibration curve for FK520 or FK506 up to 100 ng on column with 50 ng on column pimecrolimus.

Calibration curve for FK506 (1) based on triplicate LC-MS/MS analyses



Calibration curve for FK520 (2) based on triplicate LC-MS/MS

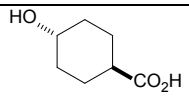
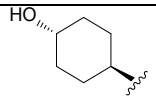
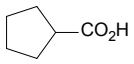
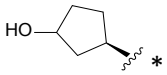
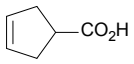
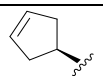
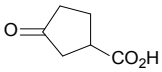
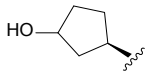
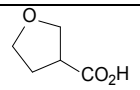
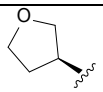
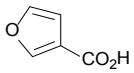
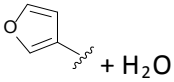
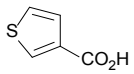
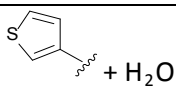
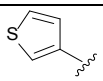
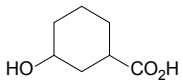
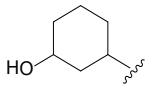
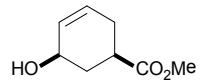
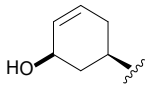
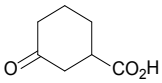
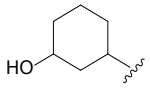
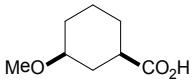
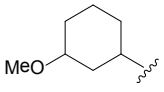
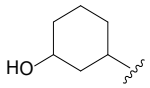
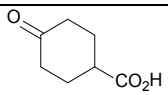
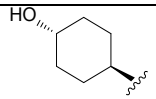


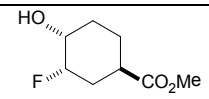
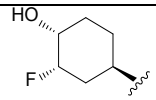
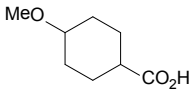
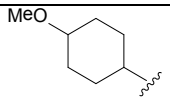
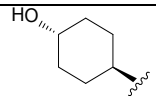
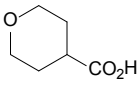
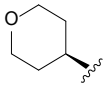
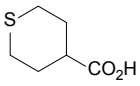
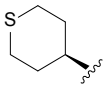
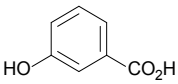
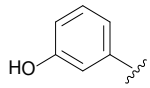
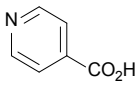
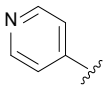
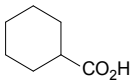
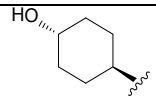
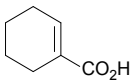
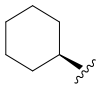
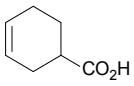
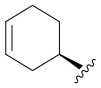
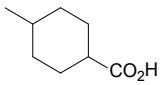
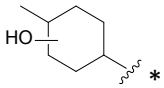
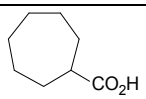
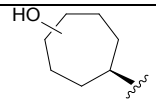
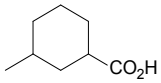
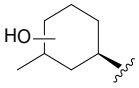
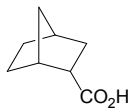
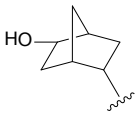
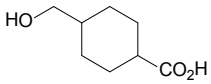
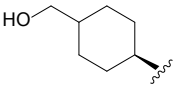
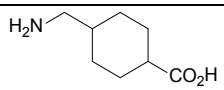
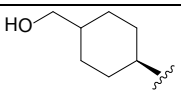
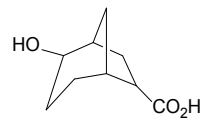
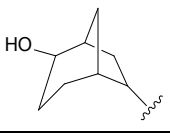
Mutasynthesis results

General Method

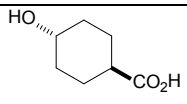
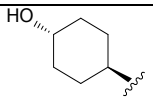
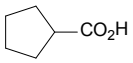
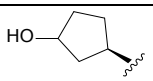
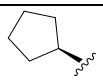
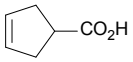
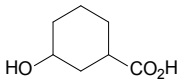
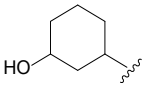
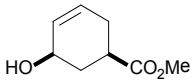
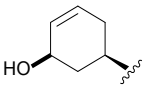
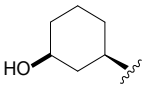
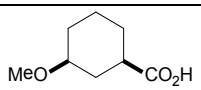
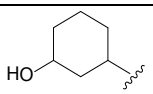
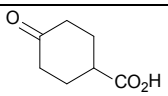
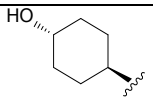
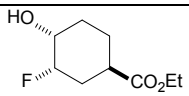
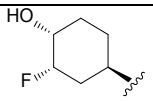
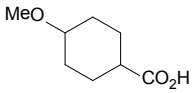
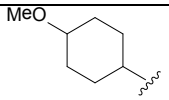
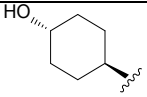
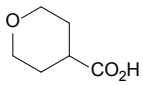
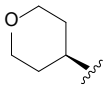
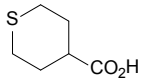
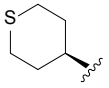
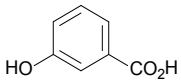
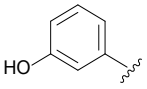
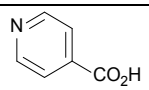
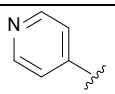
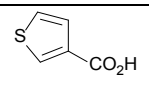
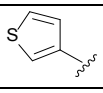
Spore stocks of BIOT-4254$_{506}$, BIOT-4131$_{520}$, BIOT-4276$_{506}$ and BIOT-4225$_{520}$ were cultured on MAM, ISP4, ISP3 or ISP2 plates, and preserved in 20% (w/v) glycerol and stored at -80 °C. Spores were recovered on plates of MAM, ISP4, ISP3 or ISP2 and incubated for 5-21 days at 28 °C. Vegetative cultures (seed culture) were prepared by removing one agar plug (6 mm in diameter) from the MAM, ISP4, ISP3 or ISP2 plate and transferring into 7 mL medium NGY in 50 mL polypropylene centrifuge tubes with foam plugs. The culture tubes were incubated at 28 °C, 300 rpm, 2.5 cm throw for 48 h. From the seed culture 0.5 mL were transferred into 7 mL production medium PYDG+MES in 50 mL centrifuge tubes with foam plugs. Cultivation was carried out for 6 days at 28 °C and 300 rpm (2.5 cm throw). When necessary a selected precursor was fed to the production medium 24 h post inoculation. The feed compound was dissolved in 0.05 - 0.1 mL methanol and added to the culture to give a final concentration of 2.12 mM of the feed compound. Results are presented as the mean average of three separate cultures $\pm$ the standard deviation unless stated otherwise.

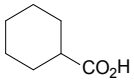
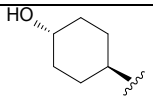
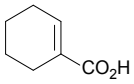
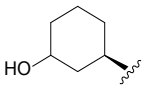
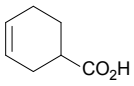
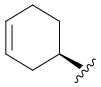
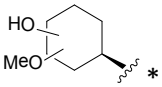
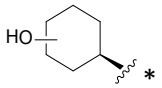
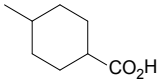
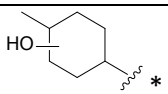
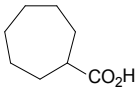
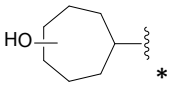
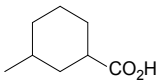
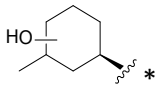
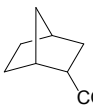
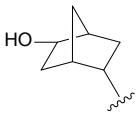
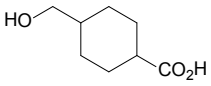
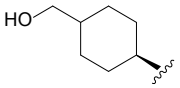
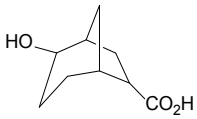
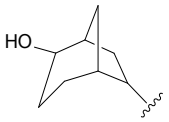
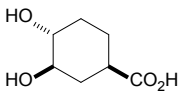
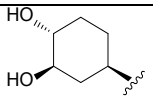
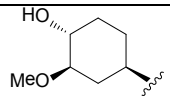
BIOT-4254$_{506}$

Feed	Compound Number	Retention time (minutes)	Molecular weight	Titre / mg/L ± SD	Putative product	Compound Number
	**12**	5.5	773.4	60 ± 14		**10**
	**49**	5.8	759.4	trace		**52**
		8.4	743.4	8 ± 5		**50**
	**46**	8.0	741.2	trace		**47**
	**54**	5.8	759.2	trace		**52**
	**70**	6.1	745.3	trace		
	**90**	4.2	759.3	< 1		**93**
		6.8	741.3	trace		**91**
	**73**	4.9	774.3	< 1		**100**
		7.3	756.3	trace		**74**
	**27**	6.1	773.5	14 ± 12		**16**
	**28**	5.9	771.5	15 ± 9		**29**
	**31**	6.0	773.5	6 ± 0.4		**16**
	**32**	7.6	787.5	trace		**33**
		6.1	772.5	trace		**16**
	**35**	5.4	773.5	41 (n = 1)		**10**

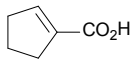
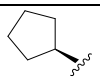
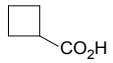
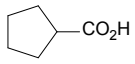
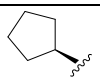
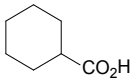
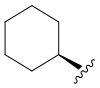
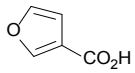
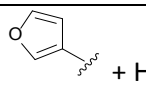
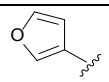
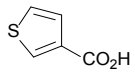
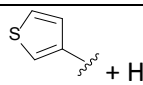
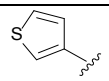
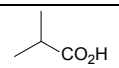
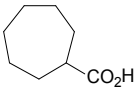
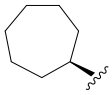
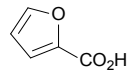
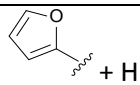
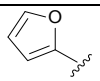
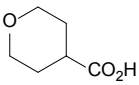
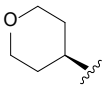
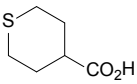
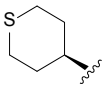
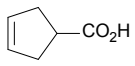
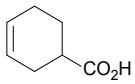
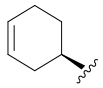
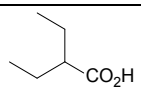
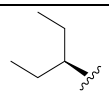
	**39**	5.3	791.5	50 ± 5		**40**
	**36**	7.1	787.5	44 ± 22		**37**
		5.6	773.2	trace		**10**
	**64**	6.6	759.5	53 ± 16		**65**
	**67**	7.7	775.5	30 ± 15		**68**
	**76**	5.9	767.5	3 ± 1		**77**
	**79**	3.8	752.5	3 ± 1		**80**
	**9**	5.6	773.5	trace		**10**
	**13**	9.0	757.5	15 ± 2		**14**
	**21**	8.5	755.5	20 ± 2		**22**
	**18**	6.0	787.5	trace	 *	**19**
	**55**	6.1	787.5	trace		**56**
	**24**	6.1	787.5	trace		**25**
	**58**	5.9	785.5	trace		**59**
	**42**	6.3	787.5	trace		**43**
	**45**	6.3	787.5	trace		**43**
	**61**	6.3	799.5	10 ± 3		**62**

BIOT-4131$_{520}$

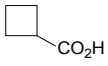
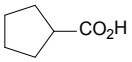
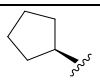
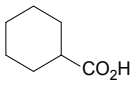
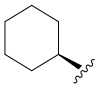
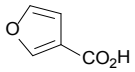
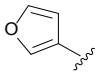
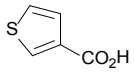
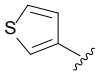
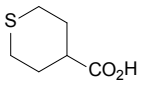
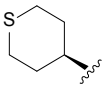
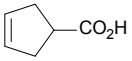
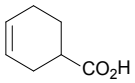
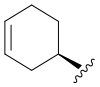
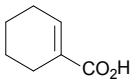
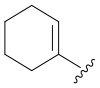
Feed	Compound Number	Retention time (minutes)	Molecular weight	Titre / mg/L ± SD	Putative product	Compound Number
	**12**	6.1	761.4	24 ± 10		**11**
	**49**	5.8	747.2	3 ± 1		**53**
		9.2	731.2	1 ± 0		**51**
	**46**	8.8	729.2	trace		**48**
	**27**	6.8	761.2	23 ± 2		**17**
	**28**	6.6	759.5	50 ± 5		**30**
		6.5	761.2	20 ± 3		**17**
	**32**	6.8	761.2	15 ± 4		**17**
	**35**	6.1	761.2	31 ± 6		**11**
	**39**	5.7	779.5	144 ± 17		**41**
	**36**	8.2	775.2	15 ± 2		**38**
		6.1	761.2	20 ± 3		**11**
	**64**	6.8	747.2	95 ± 16		**66**
	**67**	8.0	763.2	13 ± 1		**69**
	**76**	6.5	755.2	trace		**78**
	**79**	3.8	740.2	9 ± 12		**81**
	**73**	8.1	745.2	1 ± 0		**75**

	**9**	5.8	761.2	24 ± 4		**11**
	**13**	6.0	761.2	29 ± 6		**17**
	**21**	8.6	743.2	trace		**23**
		5.8	791.2	36 ± 24		**2**
		6.0	761.2	1 ± 2		**17**
	**18**	6.5	775.2	22 ± 3		**20**
	**55**	6.1	775.2	14 ± 3		**57**
	**24**	6.4	775.2	13 ± 1		**26**
	**58**	6.0	773.2	9 ± 2		**60**
	**42**	7.0	775.2	1 ± 1		**44**
	**61**	6.8	787.2	5 ± 3		**63**
	**7**	4.8	777.2	8 ± 4		
		5.7	791.2	75 ± 28		**2**

BIOT-4276$_{506}$

Feed	Compound Number	Retention time (minutes)	Molecular weight	Titre / mg/L ± SD	Putative product	Compound Number
		8.1	743.2	1 ± 1		**50**
	**85**	7.9	729.5	< 3		**86**
	**49**	8.3	742.5	< 3		**50**
	**9**	8.8	757.5	3 ± 1		**14**
	**90**	4.1	759.5	< 3		**93**
		6.4	741.5	< 3		**91**
	**73**	4.7	775.5	< 3		**100**
		7.1	757.5	< 3		**74**
	**82**	7.5	717.5	< 3		**83**
	**55**	9.0	771.5	< 3		**88**
	**95**	4.1	759.5	< 3		**98**
		8.5	745.5	< 3		**96**
	**64**	6.4	759.5	9 ± 3		**65**
	**67**	7.5	775.5	4 ± 1		**68**
	**46**	7.8	741.5	< 3		**47**
	**21**	8.2	755.5	27 ± 10		**22**
		8.5	745.5	< 3		

BIOT-4225$_{520}$

Feed	Compound Number	Retention time (minutes)	Molecular weight	Titre / mg/L ± SD	Putative product	Compound Number
	**85**	8.2	717.5	7 ± 4		**87**
	**49**	8.5	731.5	27 ± 4		**51**
	**9**	9.0	745.5	8 ± 1		**15**
	**90**	6.8	729.5	< 3		**97**
	**73**	7.3	745.5	< 3		**75**
	**67**	7.7	763.5	trace		**69**
	**46**	8.1	729.5	2 ± 1		**48**
	**21**	8.5	743.5	42 ± 4		**23**
	**13**	8.1	743.5	trace		

Isolation and Characterization of Analogs

Cultures of BIOT-4254$_{506}$ or BIOT-4131$_{520}$ were grown at an appropriate scale (typically 5 or 15 L) and the media supplemented with a carboxylic acid precursor at 24 h. Following a total of 6 d fermentation the cells were collected by centrifugation and then extracted with organic solvent. Typically the resultant crude extract was purified by normal-phase column chromatography followed by reversed-phase preparative HPLC. The compounds were assessed to be >95 % pure following QC procedures and their structures elucidated by standard 2D NMR experiments. **1** and **2** are known to exist in rotomeric forms in solution and typically only the major rotamer has been assigned (Baumann et al. 1995)

Isolation of 10

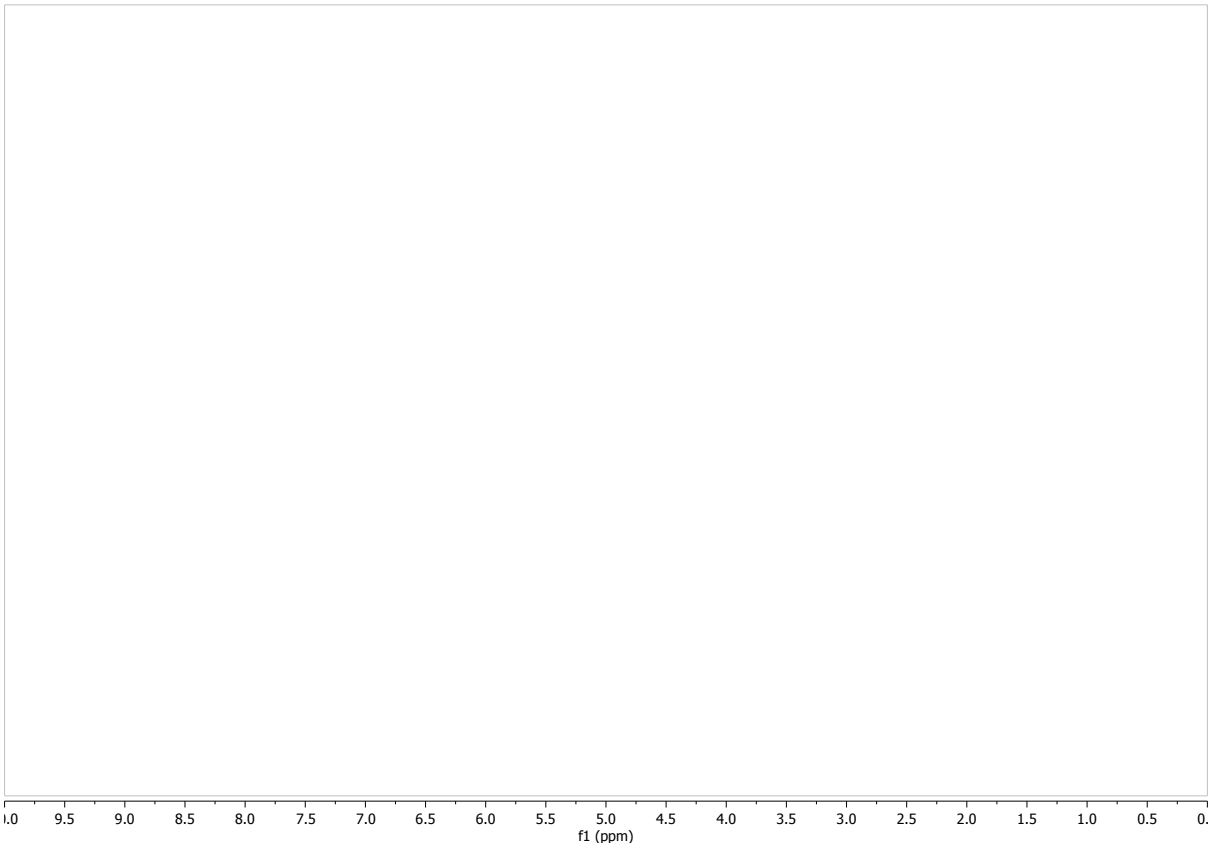
Spore stocks of BIOT-4254 were recovered onto plates of ISP4 medium and incubated for 2 weeks at 28°C. Vegetative cultures (seed culture) were prepared by adding 0.2 ml of BIOT-4254 working stock and inoculating into 400 ml NGY medium in 2 litre Erlenmeyer flasks with a foam bung. Cultivation was carried out for 48 hours at 28°C, 250 rpm (2.5 cm throw). The entire seed culture in one flask was transferred into 15 litres of medium PYDG pre-adjusted at pH 7.0 in 22 L Braun Biostat Fermenters. The fermentation was carried out at 28 ℃, with starting agitation at 200 rpm, aeration rate at 0.5 V/V/M (7.5 SL/min) and dissolved oxygen (DO) level controlled with the agitation cascade

at 30% air saturation. At 24 hours post-inoculation 5ml of 0.2 M trans-4-hydroxycyclohexanecarboxylic acid (TCI Europe) in methanol was added to the vessel. After 24 hours post inoculation the aeration rate was increased to 0.7 V/V/M (10.5 litres/min) and control pH 6.4 ± 0.8 with 1.0M NaOH and 0.5 M H_2SO_4. The culture was harvested after 93 hours post-inoculation.

The broths were combined and the cells harvested by centrifugation. The cells were extracted with EtOAc/MeOH (50:50) and the organics were dried *in vacuo* to yield a crude extract (19.5 g). The extract was subjected to normal phase column chromatography (on Silica Gel 60 (40 x 2.5 cm)) eluted with $CHCl_3$/MeOH (from 100:0 to 95:5 in incremental changes). Fractions containing the target compound were combined and reduced *in vacuo*. The enriched extract was then subjected to normal phase column chromatography (on Silica Gel 60 (25 x 2 cm)) eluted with 50:50 Hexane and EtOAc. Fractions containing the target compound were combined and taken to dryness, to yield **10** as an off-white solid (667 mg)

HRMS: $[C_{43}H_{67}NO_{11}Na]^+$ requires 796.460633, found 796.460123 (Δ0.64 ppm)

NMR: ^1H (500 MHz) and ^{13}C (125 MHz), referenced to residual solvent, $CDCl_3$



position	group	carbon [ppm]		proton [ppm]	
		major	minor	major	minor
1	C=O	169.0	-	-	-
2	CH	56.6	52.8	4.58	4.97
3	CH_2	27.7	-	2.05, 1.90	-
4	CH_2	21.0	20.8	1.72, 1.37	-
5	CH_2	24.5	-	1.70, 1.43	
6	CH_2	43.8	39.2	3.69, 4.40	3.25, 2.99
8	C=O	164.7	-	-	-
9	C=O	196.1	-	-	-
10	C-O	97.0	98.6	-	-
10-OH	OH	-	-	4.23	
11	CH	34.6	-	2.11	-
11-Me	CH_3	16.2	-	0.97	-
12	CH_2	32.6	-	1.46, 2.13	
13	CH	73.6	-	3.36	-
13-OMe	OCH_3	56.3	-	3.37	
14	CH	72.8	72.2	3.65	-
15	CH	75.2	76.6	3.55	-
15-OMe	OCH_3	57.0	57.5	3.27	-
16	CH_2	33.0	35.2	1.04, 1.49	
17	CH	26.2	26.0	2.29	
17-Me	CH_3	20.4	19.4	0.90	-
18	CH_2	48.6	48.3	2.14, 1.78	-
19	C	138.9	-	-	-
19-Me	CH_3	15.9	-	1.60	
20	=CH	122.5	-	5.00	-
21	CH	52.8	54.9	3.37	-
22	C=O	212.6	-	-	-
23	CH_2	43.8	-	2.08, 2.74	-
24	CH	72.2	69.9	3.88	-
25	CH	39.8	40.4	1.90	
25-Me	CH_3	9.5	9.8	0.85	-
26	CH	77.6	77.9	5.29	5.16
27	C	131.8	-		
27-Me	CH_3	13.9	14.2	1.63	
28	=CH	131.0	-	5.05	-
29	CH	35.6	-	2.14	
30	CH_2	31.2	-	1.67, 1.08	
31	CH_2	35.2	-	1.95, 1.27	
32	CH	70.4	-	3.53	
33	CH_2	35.2	-	1.95, 1.27	
34	CH_2	31.2	-	1.67, 1.08	
35	CH_2	35.2	-	2.44, 2.14	
36	CH	135.5	-	5.68	
37	CH_2	116.7	116.3	4.99	

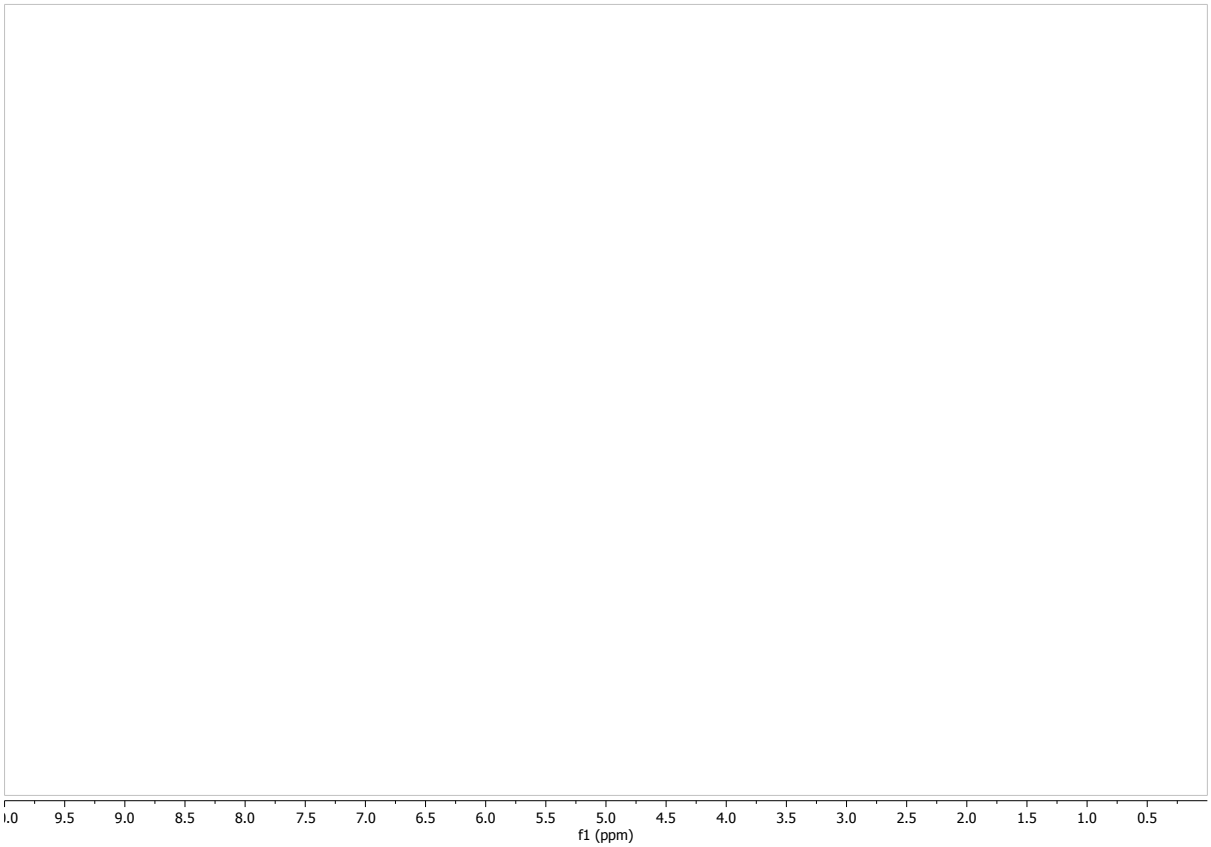
Isolation of 11

Spore stocks of BIOT-4131 were recovered onto plates of MAM medium and incubated for 5 days at 28°C. Vegetative cultures (seed culture) were prepared by removing 4 agar plugs (6 mm in diameter) from the MAM plate and inoculating into 250 ml FK seed medium in 2 litre Erlenmeyer flasks with foam bung. Cultivation was carried out for 50 hours at 28°C, 250 rpm (2.5 cm throw). The entire seed culture in one flask was transferred into 5 litres of FK production medium (pre-sterilisation pH adjusted at pH 6.4) in 7 L Applikon Fermenter. The fermentation was carried out at 28 °C, with starting agitation at 350 rpm, aeration rate at 0.5 V/V/M (2.5 SL/min) and dissolved oxygen (DO) level controlled with the agitation cascade at 30% air saturation. At 24 hours post-inoculation 5 ml from a 2M stock solution of *trans*-4-hydroxycyclohexane carboxylic acid (TCI Europe) in methanol was added to the vessel. The culture was harvested after 116 hours post-inoculation.

A second fermentation batch was run as above except: NGY seed medium, PYDG production medium (pre-sterilisation pH adjusted at pH 7.0) and harvested after 117 hours. The combined broths were extracted with ethyl acetate (2 x 1 volume equivalents) and the resultant organics reduced *in vacuo* to a crude extract (16 g). This extract was dissolved in acetonitrile (1000 ml) and silica added (30 g). The solvent was removed *in vacuo* and the adsorbed silica added to the top of a packed silica column (170 mm x 55 mm diameter). The column was eluted with ethyl actate / hexanes (1:2, 0.9 L; 1:1, 1 L; 3:2, 1 L; 2:1, 1.8 L) and fractions containing the target compound combined and taken to dryness. This enriched extract was then purified by preparative HPLC. A C18 Phenomenex C18 Luna column (5 micron, 25 cm x 22.5 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The column was run isocratically at 60 % B for 5 minutes following the injection followed by a gradient to 100 % B at 25 minutes. Pure fractions were identified by HPLC-MS and combined. These fractions were taken to dryness under reduced pressure to yield **11** as a white solid (56 mg).

HRMS: $[C_{42}H_{67}NO_{11}Na]^+$ requires 784.460633, found 784.460247 (Δ0.49 ppm)

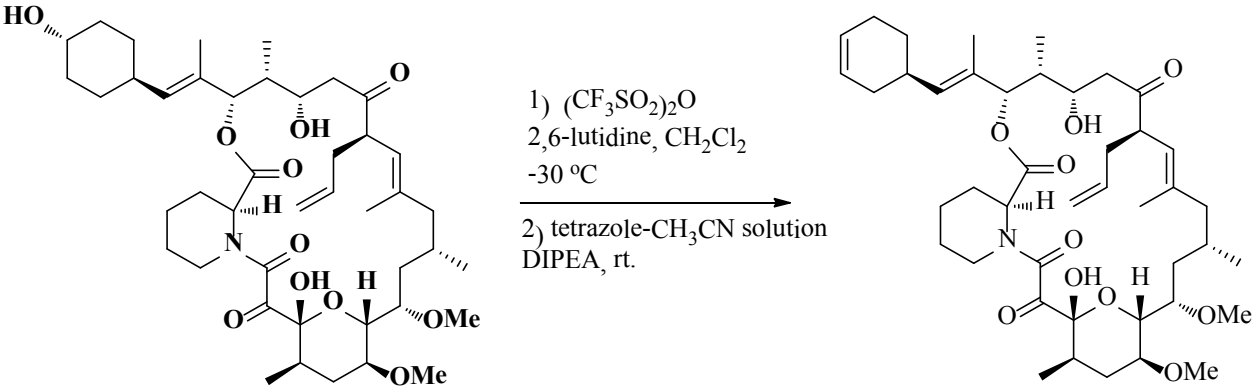
NMR: ^{1}H (500 MHz) and ^{13}C (125 MHz), referenced to residual solvent, $CDCl_3$



position	group	carbon [ppm]		proton [ppm]	
		major	minor	major	minor
1	C=O	169.0	168.7	-	-
2	CH	56.6	52.6	4.60	5.01
3	CH_2	27.6	26.2	2.09, 1.93	2.33, 1.75
4	CH_2	21.0	20.8	1.77, 1.62	1.47, 1.82
5	CH_2	24.2	24.5	1.78, 1.41	
6	CH_2	39.2	43.9	3.05, 4.44	3.29, 3.73
8	C=O	164.7	165.8	-	-
9	C=O	196.1	192.6	-	-
10	C-O	97.0	98.6	-	-
10-OH	OH	-	-	4.26	.
11	CH	34.6	33.5	2.19	2.30
11-Me	CH_3	16.2	16.0	1.01	0.97
12	CH_2	32.7	32.5	1.49, 2.16	
13	CH	73.7	73.7	3.39	3.49
13-OMe	OCH_3	56.3	56.1	3.40	
14	CH	72.8	72.2	3.69	3.89
15	CH	75.2	76.6	3.59	3.58
15-OMe	OCH_3	57.0	57.5	3.32	3.37
16	CH_2	33.0	35.5	1.09, 1.55	
17	CH	26.3	26.0	1.69	
17-Me	CH_3	20.4	19.5	0.95	0.87
18	CH_2	48.7	48.4	1.83, 2.18	1.92, 2.18
19	C	138.7	139.6	-	-
19-Me	CH_3	15.8	15.7	1.62	

20	=CH	123.1	123.3	5.03	5.01
21	CH	54.7	54.9	3.24	3.17
22	C=O	213.4	213.5	-	-
23	CH_2	43.4	43.6	2.10, 2.76	2.34, 2.70
24	CH	70.1	69.1	3.93	3.95
25	CH	39.7	40.4	1.90	
25-Me	CH_3	9.5	9.8	0.89	0.94
26	CH	77.7	78.0	5.34	5.20
27	C	131.8	131.4		
27-Me	CH_3	13.9	14.2	1.63	
28	=CH	131.0	130.6	5.09	5.05
29	CH	35.7	35.6	2.17	
30	CH_2	31.2	31.1	1.69, 1.08	
31	CH_2	35.2	35.2	1.99, 1.31	
32	CH	70.4	70.4	3.57	
s33	CH_2	35.2	35.2	1.99, 1.31	
34	CH_2	31.2	31.1	1.69, 1.08	
35	CH_2	24.5	24.6	1.75, 1.49	
36	CH_3	11.7	11.7	0.87	

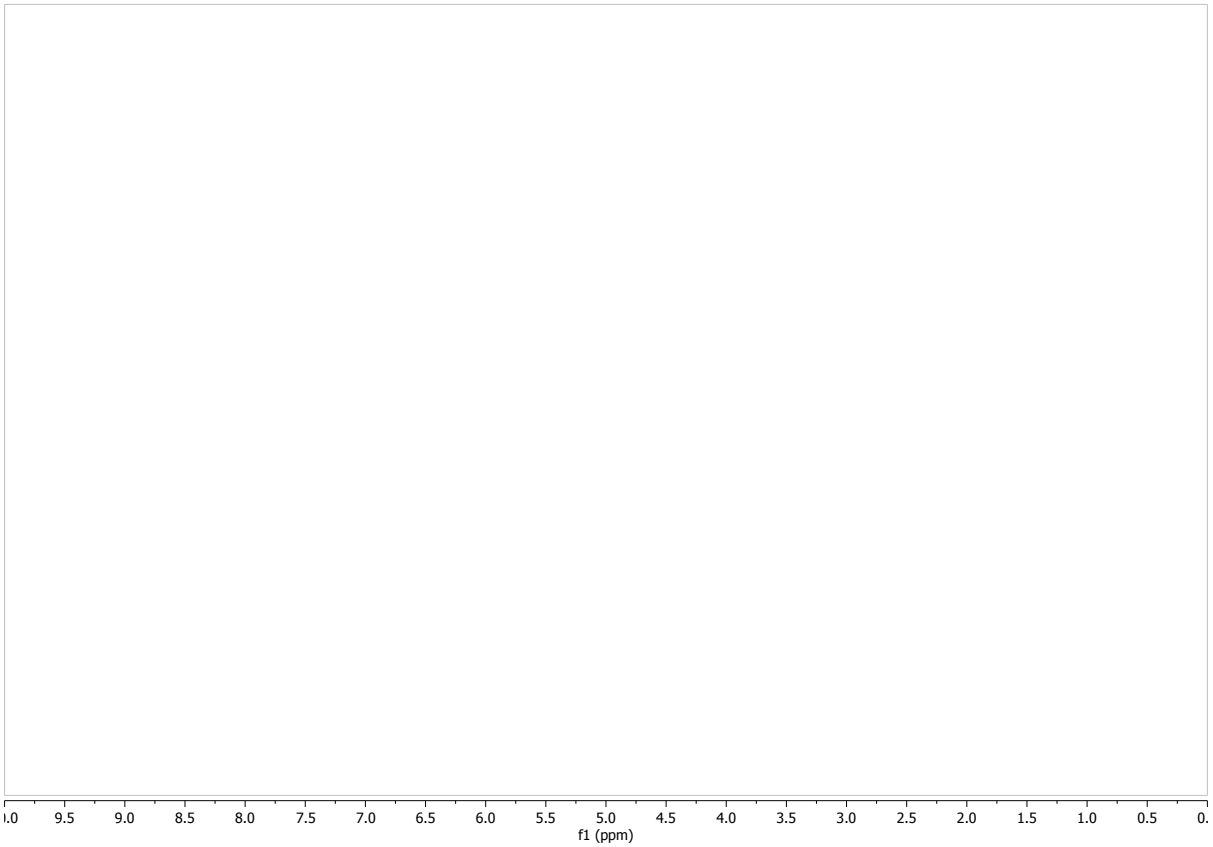
Isolation of 22



Compound **22** was synthesised whilst attempting to prepare a different target. The reaction that it was prepared from was as follows. To a solution of **10** (40 mg, 0.052 mmol) in CH_2Cl_2 (3 ml) was added 2,6-lutidine (24 µl, 0.208 mmol) and Tf_2O (17 µl, 0.104 mmol) at -30 °C. The resulting mixture was stirred at -30 °C for 1 hour. Then a solution of tetrazole-CH_3CN (0.45 M, 0.23 ml, 0.104 mmol) and DIPEA (18 µl, 0.104 mmol) were added at -30 °C. The resulting mixture was stirred at room temperature for 48 hours. The solvent was removed *in vacuo* and the residue was purified by preparative TLC (petroleum ether / acetone = 4:1) to give an oil **22** (25 mg).

HRMS: $[C_{43}H_{65}NO_{10}Na]^+$ requires 778.450068, found 778.451089 (Δ1.31 ppm)

NMR:



position	group	carbon [ppm]		proton [ppm]	
		major	minor	major	minor
1	C=O	169.4	169.1	-	
2	CH	56.7	53.2	4.63	
3	CH_2	26.4		2.09, 1.94	
4	CH_2	21.5		1.77, 1.40	
5	CH_2	24.8		1.68, 1.46	
6	CH_2	39.6		4.45, 3.71	
8	C=O	165.2	166.2	-	
9	C=O	196.6	193.0	-	
10	C-O	97.4	99.0	-	
11	CH	35.0	-	2.10	
11-Me	CH_3	16.6	16.4	0.96	
12	CH_2	33.1	-	2.13, 1.53	
13	CH	73.2	-	3.40	
13-OMe	OCH_3	57.1	-	3.42	
14	CH	74.1		3.61	
15	CH	75.6		3.60	
15-OMe	OCH_3	57.4		3.33	
16	CH_2	33.9		1.52, 1.09	
17	CH	26.7		2.35	
17-Me	CH_3	20.8	19.8	0.91	
18	CH_2	49.0	48.8	2.12, 1.77	
19	C	139.3	140.2	-	
19-Me	CH_3	16.3	-	1.62	
20	=CH	122.9	123.1	5.04	

21	CH	51.3	53.3	3.42	
22	C=O	213.2	213.0	-	
23	CH_2	44.3	-	2.80, 2.15	
24	CH	70.5	69.6	3.95	
25	CH	40.2		1.94	
25-Me	CH_3	10.2	10.0	0.84	
26	CH	78.3		5.23	
27	C	132.0	131.7		
27-Me	CH_3	14.4	14.7	1.63	
28	=CH	131.3	131.4	5.05	
29	CH	35.9	-	2.19	
30	CH_2	43.7	-	1.76, 1.09	
31	CH	127.4	127.4	5.74	
32	CH	126.4	-	5.69	
s33	CH_2	43.8	-	2.20, 1.55	
34	CH_2	31.9	-	1.67, 1.08	
35	CH_2	35.6	-	2.52, 2.35	
36	CH	135.9	135.8	5.37	
37	CH_2	117.1	116.3	5.23	

Isolation of 26

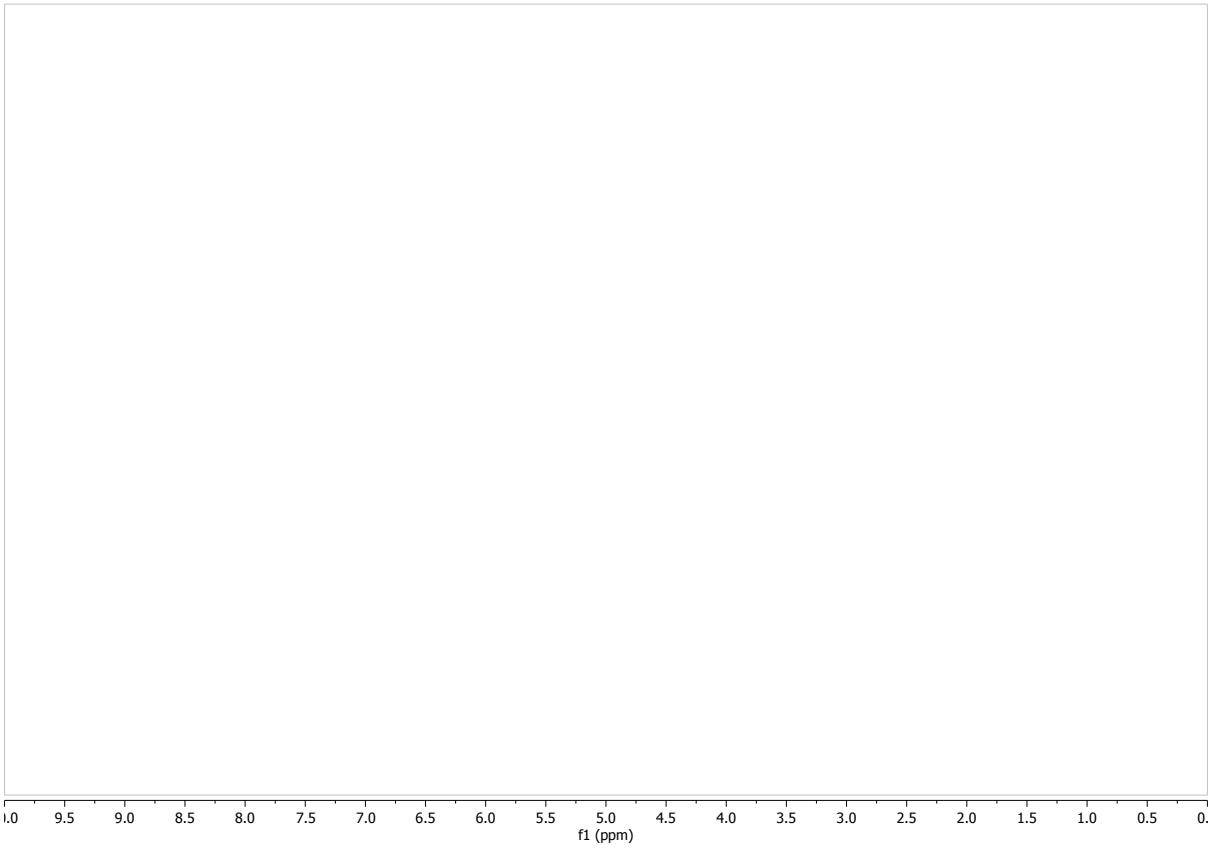
Spore stocks of BIOT-4131 were recovered onto plates of MAM medium and incubated for 8 days at 28 °C. Vegetative cultures (seed culture) were prepared by 3 x 8 plug of BIOT-4131 from MAM medium plate and inoculating into 3 x 350 ml NGY medium in 3 x 2 litre Erlenmeyer flasks with foam bung. Cultivation was carried out for 48 hours at 28 °C, 250 rpm (2.5 cm throw). The entire seed culture in one flask was transferred into 5 litres of medium PYDG in 3 x 7 L Applikon Fermenter. The fermentation was carried out at 28 °C, with starting agitation at 350 rpm, aeration rate at 0.5 V/V/M (2.5 SL/min) and dissolved oxygen (DO) level controlled with the agitation cascade at 30% air saturation. At 24 hours post-inoculation 3-methylcyclohexanecarboxylic acid (Sigma) in methanol was added to the vessel to a final concentration of 2 mM. After 24 hours post inoculation the aeration rate was increased to 0.8 V/V/M (4.0 litres/min). The cultures were harvested after 5 days hours post-inoculation.

The broths were combined and the cells removed by centrifugation. The supernatant was discarded and the cells were extracted into acetonitrile (2 x 15 liters, overhead stirrer, 1 hour). The solvent was reduced *in vacuo* to leave an aqueous slurry. The slurry was diluted with water and extracted with ethyl acetate (3 x 400 ml). The combined organics were taken to dryness *in vacuo* to yield a brown oil (70 g). The extract was dissolved in methanol (200 ml) and water (800 ml) added. This was washed with hexanes (2 x 1 litre) and the methanol removed from the aqueous layer and the resultant slurry extracted into methylene chloride (2 x 800 ml).. The first hexanes wash was reduced *in vacuo* and resuspended in methanol (100 ml) and water (400 ml) added. This was washed with hexanes and the methanol removed from the aqueous layer and the resultant slurry extracted into methylene chloride (2 x 500 ml). All of the organics were combined and reduced in vacuo to yield a brown oil (22 g). The extract was dissolved in methylene chloride and silica (173 g) added. The solvent was removed *in vacuo* and this adsorbed silica was added to the top of a silica column (20 cm x 5 cm diameter) and the column eluted with hexanes/ ethyl acetate (2:1, 1 litre; 1:1, 1 litres; 4:6,

1 litre). Active fractions were combined and taken to dryness to yield a dark yellow oil (0.8 g). The material;l was further purified by column chromatography over normal phase silica (40 g silica, eluted with 2:7 acetone/hexanes). Active fractions were combined and reduced *in vacuo* to yield a yellow solid (146 mg). This was then purified by successive rounds of preparative HPLC. Column Luna C18 (Phenomenex), 22.5 mm diameter x 25 cm. Solvent A = water and solvent B = acetonitrile. 21 ml/min, t = 0, B = 65%; t = 20 min, B = 75%; t = 35 min, B = 75%. Active fractions were combined and taken to dryness, 29.6 mg as a white solid.

HRMS: $[C_{43}H_{69}NO_{11}Na]^+$ requires 798.476283, found 798.475052 (Δ1.54 ppm)

NMR: ^{1}H (500 MHz) and ^{13}C (125 MHz), referenced to residual solvent, $CDCl_3$



position	group	carbon [ppm]		proton [ppm]	
		major	minor	major	
1	C=O	169.4	169.2	-	
2	CH	57.0	53.1	4.60	
3	CH_2	28.0	26.6	2.09, 1.94	
4	CH_2	21.5	21.3	1.76, 1.41	
5	CH_2	24.9	26.6	1.77, 1.46	
6	CH_2	39.7	44.0	4.44, 3.06	
8	C=O	165.2	166.2	-	
9	C=O	196.6	193.0	-	
10	C-O	97.5	99.0	-	
11	CH	35.0	34.0	2.19	
11-Me	CH_3	16.6	16.4	1.01	
12	CH_2	33.1	32.9	2.17, 1.53	

13	CH	74.1	74.0	3.39	
13-OMe	OCH_3	57.4	57.9	3.32	
14	CH	73.3	72.6	3.69	
15	CH	75.6		3.59	
15-OMe	OCH_3	56.8	56.5	3.41	
16	CH_2	33.4	34.0	2.16, 1.55	
17	CH	26.7	26.6	1.67	
17-Me	CH_3	20.9	19.9	0.95	
18	CH_2	49.1	48.9	2.19, 1.84	
19	C	139.1	140.0	-	
19-Me	CH_3	16.2	16.1	1.63	
20	=CH	123.5	123.8	5.02	
21	CH	55.1	55.3	3.21	
22	C=O	213.8	214.0	-	
23	CH_2	43.8	44.2	2.80, 2.11	
24	CH	70.5	69.6	3.39	
25	CH	40.5	40.8	1.93	
25-Me	CH_3	10.0	10.2	0.89	
26	CH	78.0	78.4	5.34	
27	C	131.3	131.0		
27-Me	CH_3	14.4	14.7	1.63	
28	=CH	132.1	131.7	5.08	
29	CH	34.0		2.22	
30	CH_2	32.1		1.68, 1.07	
31	CH_2	35.4	35.0	1.98, 1.33	
32	CH	76.5		3.14	
33	CH	36.5	35.9	2.26	
33-Me	CH_3	18.9		1.03	
34	CH_2	40.1		1.61, 0.85	
35	CH_2	24.6		1.78, 1.47	
36	CH_3	12.1		0.9	

Isolation of 41 and 102

Vegetative cultures (seed culture) were prepared by removing 8 agar plugs (6 mm in diameter) from a 9 day old MAM plate and inoculating into 250 mL medium NGY in 2000 mL Erlenmeyer flasks with foam plugs. The seed flasks were incubated at 28 °C, 250 rpm (2.5 cm throw) for 48 h. From the seed culture 5% (v/v) was transferred into 77 x 50 mL production medium PYDG in 250 mL Erlenmeyer flasks respectively with foam plugs. Cultivation was carried out for 6 days at 28 °C and 200 rpm with a 5 cm throw or 250 rpm with a 2.5 cm throw. The cultures were all fed with methyl 3-*cis*-fluoro-4-*trans*-hydroxycyclohexanecarboxylate in methanol to final concentrations as follows. 71 of the cultures were fed with 2 mM at 24 hours, 3 cultures were fed with 4 mM at 24 hours and 3 cultures were fed with 2 mM at 24 hours and a further 2 mM at 48 hours post inoculation.

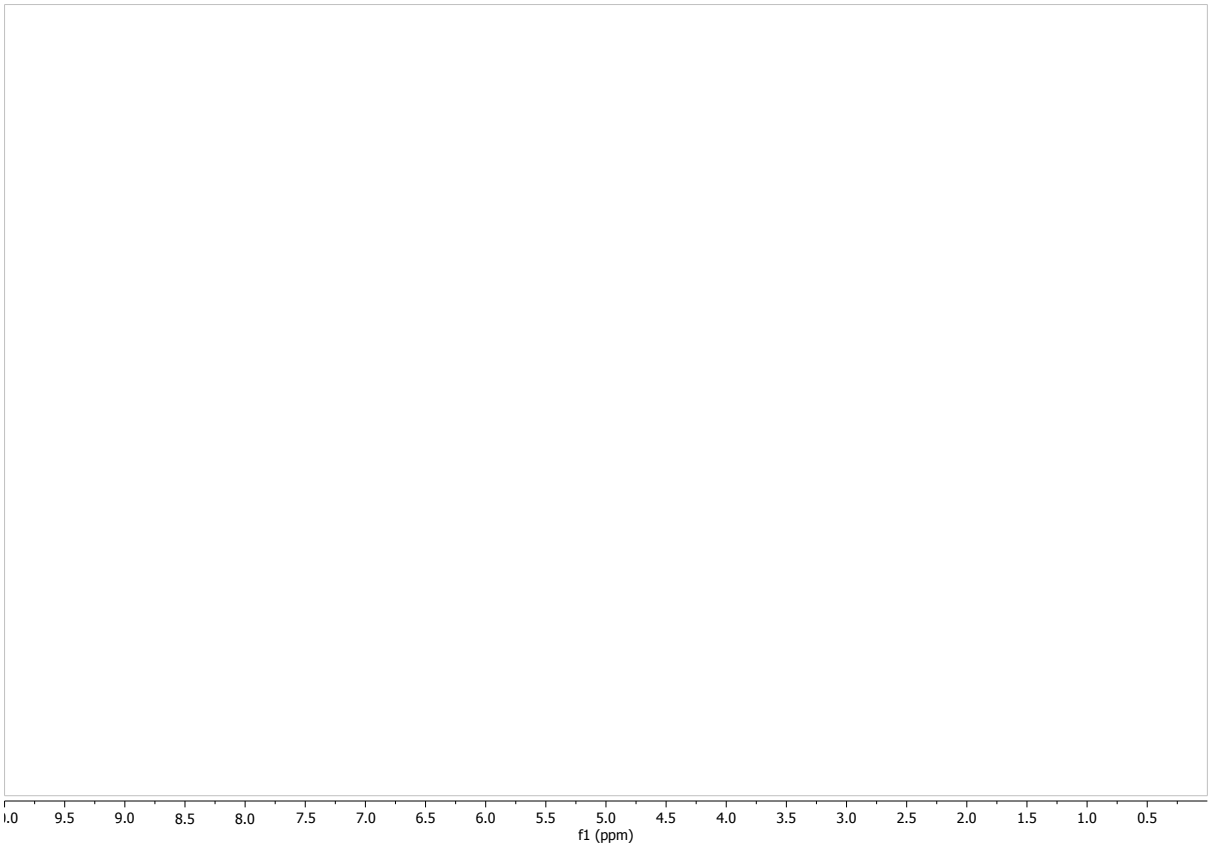
All of the fermentation broths were combined (approx 4 litres) and extracted twice with 1 volume equivalent of ethyl acetate. The organics were combined and the solvent removed *in vacuo* to yield a brown oil (13.5 g). The extract was dissolved in methylene chloride (500 ml) and adsorbed onto silica (25 g). The adsorbed silica was added to the top of a silica column (18 cm x 5 cm diameter) and

eluted with hexanes / ethyl acetate (2:1, 750 ml; 1:1, 1 litre; 4:6, 1 litre). Active fractions were combined and taken to dryness (0.5 g brown oil). This enriched extract was purified by preparative HPLC. Column Luna C18 (Phenomenex), 22.5 mm diameter x 25 cm. Solvent A = water and solvent B = acetonitrile. 21 ml/min, t = 0, B = 60%; t = 5 min, B = 60%; t = 25 min, B = 100%. **41** Eluted at 19 minutes and **102** eluted at 16 minutes. Active fractions were combined and taken to dryness, to yield **38** as a white solid (67 mg) and **102** as a white solid (16 mg).

41

HRMS: $[C_{42}H_{66}FNO_{11}Na]^+$ requires 802.451211, found 802.448568 (Δ3.29 ppm)

NMR: ^{1}H (500 MHz) and ^{13}C (125 MHz), referenced to residual solvent, $CDCl_3$



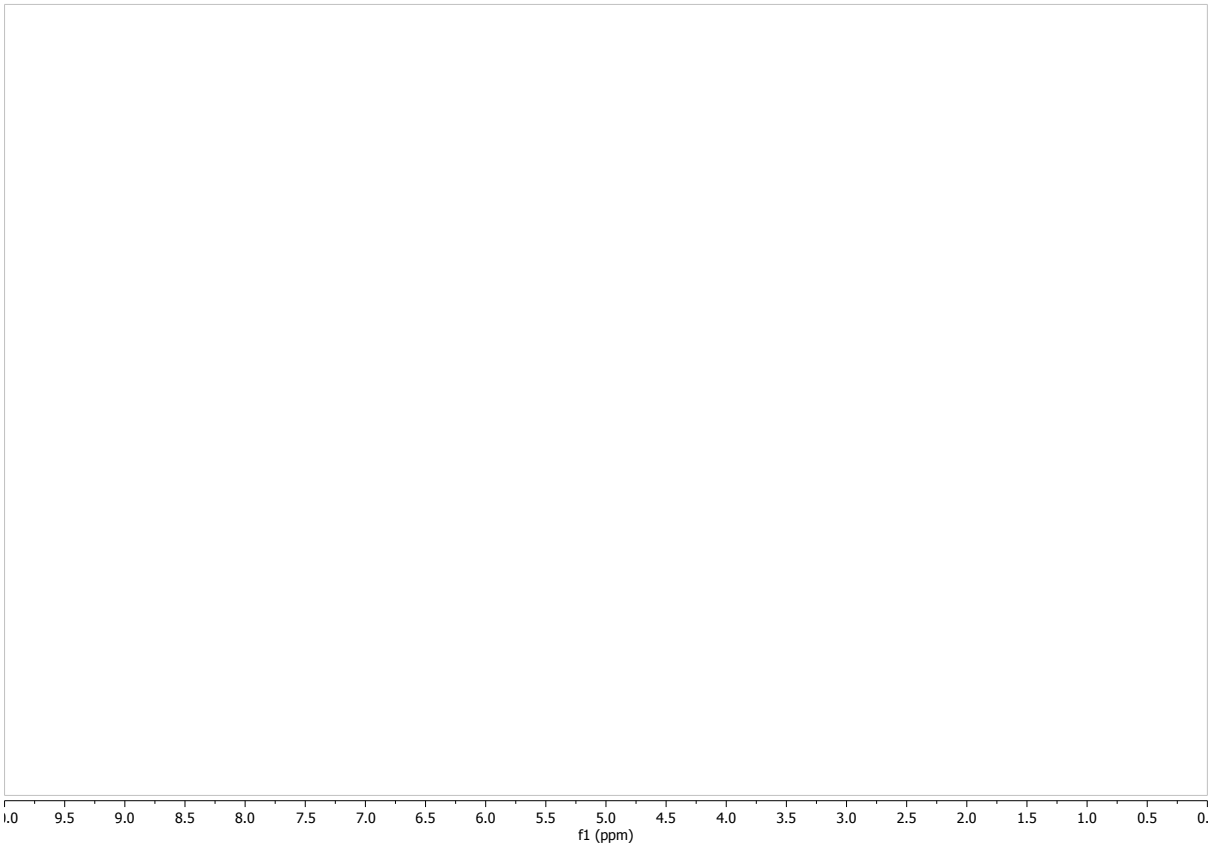
position	group	carbon [ppm]		proton [ppm]	
		major	minor	major	minor
1	C=O	169.0	168.7	-	
2	CH	56.6		4.62	
3	CH_2	26.0		2.34, 1.84	
4	CH_2	21.1		1.77, 1.40	
5	CH_2	24.2		1.77, 1.46	
6	CH_2	40.4	43.5	4.46, 3.05	
8	C=O	164.7	165.7	-	
9	C=O	196.1	192.7	-	
10	C-O	97.1	98.7	-	
11	CH	34.6		2.20	
11-Me	CH_3	16.0	16.2	1.01	

12	CH_2	32.7		2.19, 1.49	
13	CH	72.8	72.2	3.71	
13-OMe	OCH_3	56.3		3.41	
14	CH	73.6		3.39	
15	CH	75.2		3.60	
15-OMe	OCH_3	57.0		3.33	
16	CH_2	33.1		1.53, 1.09	
17	CH	26.3		1.69	
17-Me	CH_3	20.4		0.96	
18	CH_2	48.7	48.5	2.19, 1.84	
19	C	138.7	139.6	-	
19-Me	CH_3	15.7	15.8	1.62	
20	=CH	123.1	123.4	5.02	
21	CH	54.8	54.9	3.24	
22	C=O	213.3		-	
23	CH_2	43.6	43.9	2.80, 2.14	
24	CH	70.0	69.0	3.97	
25	CH	39.2		1.90	
25-Me	CH_3	9.6	9.8	0.91	
26	CH	77.8	78.5	5.35	
27	C	133.0	132.6	-	
27-Me	CH_3	13.8	14.1	1.65	
28	=CH	129.7	129.2	5.09	
29	CH	29.6		2.56	
30	CH_2	36.0 (d, 19 Hz)		2.04, 1.30	
31	CHF	91.7 (d, 170 Hz)		4.83 (d, 50 Hz)	
32	CH	70.6 (d, 20 Hz)		3.55	
33	CH_2	28.9 (d, 5Hz)		1.86, 1.70	
34	CH_2	30.2		1.70, 1.07	
35	CH_2	24.5		1.70	
36	CH_3	11.7		0.89	

102

HRMS: $[C_{41}H_{64}FNO_{11}Na]^+$ requires 788.435561, found 788.434209 (Δ1.72 ppm)

NMR:



position	group	carbon [ppm]		proton [ppm]	
		major	minor	major	minor
1	C=O	168.9			
2	CH	56.6		4.62	
3	CH_2	27.8		2.07, 1.90	
4	CH_2	21.2		1.70, 1.33	
5	CH_2	24.6		1.71, 1.43	
6	CH_2	39.2		4.41, 3.00	
8	C=O	164.6			
9	C=O	196.3			
10	C-O	96.9			
11	CH	34.6		2.13	
11-Me	CH_3	16.3		0.99	
12	CH_2	32.7		2.14, 1.44	
13	CH	73.6		3.35	
13-OMe	OCH_3	56.3		3.37	
14	CH	72.8		3.66	
15	CH	75.1		3.56	
15-OMe	OCH_3	56.9		3.28	
16	CH_2	32.6		1.58, 1.00	
17	CH	26.3		1.64	
17-Me	CH_3	20.6		0.93	
18	CH_2	48.3		2.14, 1.74	
19	C	137.9			
19-Me	CH_3	15.7		1.59	
20	=CH	124.1		5.07	

21	CH	47.0		3.33	
22	C=O	214.3			
23	CH_2	41.8		2.81, 1.97	
24	CH	70.4		3.87	
25	CH	39.9		1.91	
25-Me	CH_3	9.7		0.85	
26	CH	76.5		5.35	
27	C	133.5			
27-Me	CH_3	14.1		1.63	
28	=CH	129.1		5.03	
29	CH	29.6		2.54	
30	CH_2	36.1 (d, 20 Hz)		2.07, 1.71	
31	CHF	91.7 (d, 168 Hz)		4.78	
32	CH	70.6 (d, 20 Hz)		3.53	
33	CH_2	29.0 (d, 5Hz)		1.84, 1.66	
34	CH_2	30.3		2.07, 1.71	
35	CH_3	15.4		1.10	

Isolation of 57

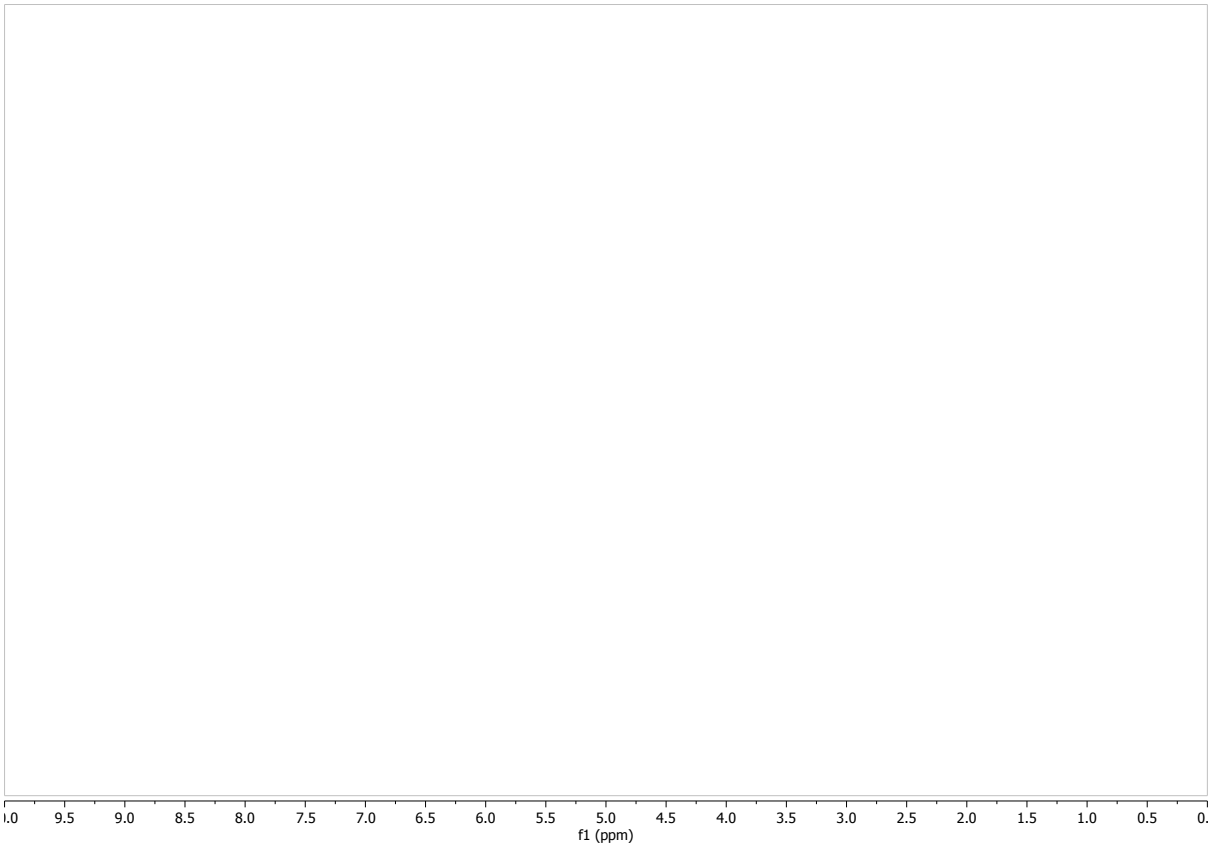
Spore stocks of BIOT-4131 were recovered onto plates of MAM medium and incubated for 8 days at 28 °C. Vegetative cultures (seed culture) were prepared by 5 x 8 plug of BIOT-4131 from MAM medium plate and inoculating into 5 x 350 ml NGY medium in 5 x 2 litre Erlenmeyer flasks with foam bung. Cultivation was carried out for 48 hours at 28 °C, 250 rpm (2.5 cm throw). The entire seed culture in one flask was transferred into 5 litres of medium PYDG pre-adjusted between pH 6.5 and 7.0 in 5 x 7 L Applikon Fermenter. The fermentation was carried out at 28 °C, with starting agitation at 350 rpm, aeration rate at 0.5 V/V/M (2.5 SL/min) and dissolved oxygen (DO) level controlled with the agitation cascade at 30% air saturation. At 24 hours post-inoculation cycloheptanecarboxylic acid (Sigma) in methanol was added to the vessel to a final concentration of 2 mM. After 24 hours post inoculation the aeration rate was increased to 0.8 V/V/M (4.0 litres/min). The cultures were harvested after 5 days hours post-inoculation.

The broths were combined and the cells removed by centrifugation. The supernatant was discarded and the cells were extracted into acetonitrile (2 x 10 liters, overhead stirrer, 1 hour). The solvent was reduced *in vacuo* to leave an aqueous slurry. The slurry was diluted with water and extracted with ethyl acetate twice. The combined organics were taken to dryness *in vacuo* to yield a brown waxy solid (72 g). The extract was dissolved in a mixture of methylene chloride, methanol, hexane and ethyl acetate (total 1 litre) and silica (173 g) added. The solvent was removed *in vacuo* and this adsorbed silica was added to the top of a silica column (350 g silica, 6.5 cm diameter) and the column eluted with hexanes/ ethyl acetate (2:1, 1.8 litres; 1:1, 1.8 litres; 1:2, 0.9 litres; 100% ethyl acetate). Active fractions were combined and taken to dryness to yield a yellow foam (34 g). The foam was suspended in acetone (100 ml) and mixed with hexanes (100 ml). A white solid did not dissolve and was collected by filtration and the filtrate kept. The white solid was washed with a further 1:1 acetone / hexanes (100 ml). The filtrates were combined and taken to dryness (27 g, yellow foam) before being dissolved in methanol (10 ml) and chloroform (250 ml) and silica (70 g)

added. The solvent was removed *in vacuo* and this adsorbed silica was put onto the top of a silica column (220 g, dry packed) and the column eluted with acetone/hexanes (1:3, 2.5 litres; 1:2, 2 litres). Active fractions were combined and reduced *in vacuo* to yield a yellow foam (1.4 g). This enriched extract was purified by column chromatography over silica (100 g), eluted with acetone/methylene chloride (1:19, 250 ml; 1:9, 1 litre; 1:4, 1 litre). Active fractions were combined and dried *in vacuo* to yield an off-white solid (155 mg). This was then purified by preparative HPLC. Column Luna C18 (Phenomenex), 22.5 mm diameter x 25 cm. Solvent A = water and solvent B = acetonitrile. 21 ml/min, t = 0, B = 65%; t = 20 min, B = 75%; t = 35 min, B = 75%. The target compound eluted at 23 minutes. Active fractions were combined and taken to dryness, 54 mg as a white solid.

HRMS: $[C_{43}H_{69}NO_{11}Na]^+$ requires 798.476283, found 798.472893 (Δ4.24 ppm)

NMR: ^{1}H (500 MHz) and ^{13}C (125 MHz), referenced to residual solvent, $CDCl_3$



position	group	carbon [ppm]		proton [ppm]	
		major	minor	major	
1	C=O	169.5	169.2	-	
2	CH	56.7	53.1	4.58	
3	CH_2	26.7	26.4	2.36, 1.67	
4	CH_2	21.4	21.2	1.76, 1.52	
5	CH_2	24.9	25.0	1.78, 1.42	
6	CH_2	39.9	44.3	3.05, 4.43	
8	C=O	165.2	166.2	-	
9	C=O	196.7	193.1	-	

10	C-O	97.5	98.9	-	
11	CH	35.0	34.9	2.23	
11-Me	CH_3	16.6	16.4	1.00	
12	CH_2	33.1	32.9	2.16, 1.50	
13	CH	73.3	no	3.68	
13-OMe	OCH_3	57.0	56.5	3.40	
14	CH	72.7	no	3.88	
15	CH	75.6	no	3.58	
15-OMe	OCH_3	57.4	57.9	3.32	
16	CH_2	33.5	35.9	2.13, 1.50	
17	CH	26.7	26.6	1.68	
17-Me	CH_3	20.8	19.9	0.94	
18	CH2	49.1	48.8	2.18, 1.83	
19	C	139.2	140.0	-	
19-Me	CH_3	16.2	16.1	1.63	
20	=CH	123.8	123.6	5.02	
21	CH	55.2	55.3	3.24	
22	C=O	213.7	no	-	
23	CH_2	44.0	44.1	2.79, 2.12	
24	CH	70.5	69.6	3.91	
25	CH	40.2	40.8	1.91	
25-Me	CH_3	10.0	10.3	0.91	
26	CH	78.4	78.5	5.32	
27	C	130.4	130.1		
27-Me	CH_3	14.2	14.5	1.60	
28	=CH	132.9	132.5	5.17	
29	CH	38.4	35.6	2.44	
30	CH_2	30.1	31.1	1.63, 1.22	
31	CH_2	24.9	35.2	1.74, 1.46	
32	CH_2	36.3	36.4	2.00, 1.56	
33	CH	74.1	70.4	3.84	
34	CH_2	38.0	35.2	1.91, 1.61	
35	CH_2	35.0	31.1	2.17, 1.73	
36	CH_2	24.7	26.4	1.75, 1.49	
37	CH_3	12.1	12.0	0.87	

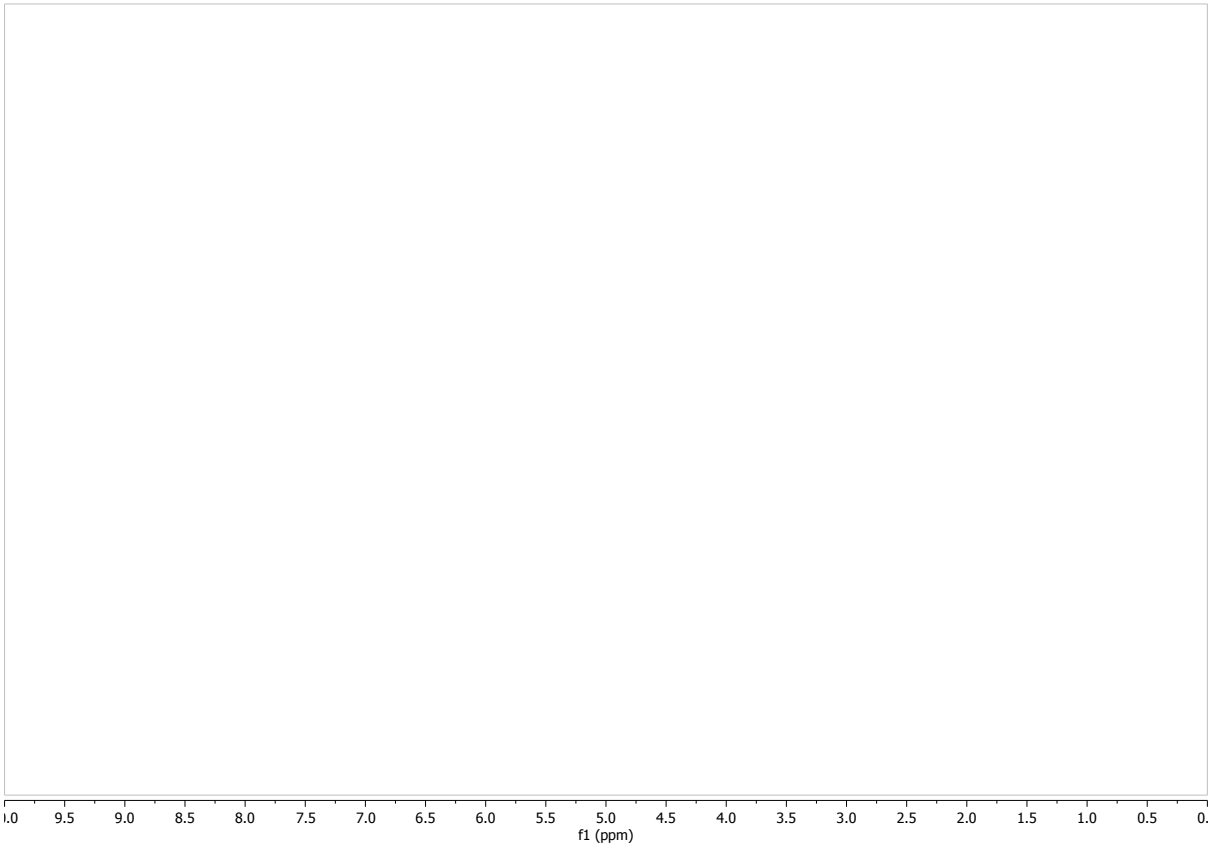
Isolation of 66

Spore stocks of BIOT-4131 were recovered onto plates of MAM medium and incubated for 10 days at 28°C. Vegetative cultures (seed culture) were prepared by removing 4-5 agar plugs (6 mm in diameter) from the MAM plate and inoculating into 50 mL medium NGY in 250 mL Erlenmeyer flasks with foam plug. Cultivation was carried out for 48 hours at 28°C and 300 rpm (2.5 cm throw). From the seed culture 2.5 mL was transferred into 50 mL production medium PYDG in 250 mL Erlenmeyer flasks with foam plug. After 24 hours cultivation at 28°C and 200 rpm (2.5 cm throw) (also at 250 rpm with similar results), 0.33 mL from a 0.32 M methanolic solution of tetrahydropyran-4-carboxylic acid was added to each production flask to give a final 2.12 mM concentration of the feed. Cultivation was continued for further five days at 28°C and 200 rpm (2.5 cm throw). The culture broths were pooled (to 3.75 L) and extracted with ethyl acetate three times. The combined organics were taken to dryness under reduced pressure to yield a brown oil (8 g). The oil was dissolved in

methanol and silica (10 g) added. The solvent was removed under reduced pressure to impregnate the silica with the crude extract. The impregnated silica was dry packed above silica (110 g) in a column with a 5 cm diameter. The column was conditioned with and eluted with ethyl acetate / hexane (1:1, 1 litre), ethyl acetate (1 litre). After a 250 mL pre-fraction, 50 mL fractions were collected and the target compound identified in fractions 2 – 10. The fractions were combined and taken to dryness (500 mg). This material was impregnated onto silica (4 g) and this material was dry-packed onto silica (20 cm x 2.5 cm diameter) and conditioned with ethyl acetate / hexane (1:2, 300 mL) and eluted with ethyl acetate / hexane (1:1, 300 mL), ethyl acetate / hexane (2:1, 300 mL) and finally ethyl actate. After a prefraction (150 mL) 15 mL fractions were collected and the target compound was identified in fractions 13 to 20. These were combined and taken to dryness under reduced pressure (133 mg). This material was dissolved in 0.9 mL methanol and purified by preparative HPLC in 2 injections. A Phenomenex C18 Luna column (5 micron, 25 cm x 22.5 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The column was run isocratically at 60 % B for 5 minutes following the injection followed by a gradient to 100 % B at 25 minutes. The compound started to elute at 21 minutes. Pure fractions were identified by HPLC-MS and combined. These fractions were taken to dryness under reduced pressure to yield **66** as a white solid (20 mg).

HRMS: $[C_{41}H_{65}NO_{11}Na]^+$ requires 770.444983, found 770.442851 (Δ2.77 ppm).

NMR



Compound QC

All compounds were shown to be >95% pure by the following QC criteria:

A stock solution of the compound is prepared in acetonitrile (0.1 mg/ml) and this solution analysed by HPLC-MS over 2 different stationary phases (one C18 reversed-phase silica, the other phenylhexyl reserved-phase silica) and two different concentrations (1 μl and 5 μl injections). Data is collected at three wavelengths (210, 254 and 280 nm), as well as by MS (in positive and negative switching mode). The chromatograms are then assessed for purity with a percentage purity given by the peak area of the compound divided by the total peak areas. The compounds were also assessed by ^{1}H NMR spectroscopy.

Profiling

HLM and MLM stability assay

A 5 mM stock solution of the test compound was prepared in DMSO. Liver microsomes were diluted with 0.1 M potassium phosphate buffer (pH 7.4) containing EDTA (1 mM) to a final concentration of 2.5 mg/mL. NADPH stock solution (50 mM) is prepared by dissolving NADPH into 0.1 M potassium phosphate buffer (pH 7.4) containing EDTA (1 mM). Test compound was added to the liver microsomal incubation system to a concentration of 1 μM and the final volume set to 250 μl with 0.1 M potassium phosphate buffer. The mixture was incubated at 37 °C for 5 minutes and the reaction initiated by the addition of NADPH (50 μL) to a final concentration of 2 mM. Aliquots (50 μL) were removed at 0, 5, 15, 30, and 60 minutes and quenched with 3 volumes of acetonitrile containing internal standard (150 μL). Protein was removed by centrifugation and the supernatants were then analyzed by LC/MS/MS. Area ratios are determined for the analyte and internal standard (pimecrolimus). Each test compound was assessed in triplicate.

The results are expressed as % remaining of the test compound at each time point:

% Remaining = Peak area ratio t_x / Peak area ratio t_0, where

t_0 = 0 minute incubation

t_x= any given incubation time.

The *in vitro* half-life is reported in minutes and is calculated as follows:

$T_{1/2}$ = - (0.693/slope)

slope = ln(%remaining) vs incubation time when y intercept = 100% at x = 0 minute

Note: Some compounds in human and/or mouse liver microsomes showed biphasic metabolic kinetics, an initial fast disappearance followed by a slow disappearance. The data points marked in red represent the slow disappearance rates and were excluded from half-life calculation.

Test Compound	Species	Percentage remaining					$t_{1/2}$ (min)
		0 min	5 min	15 min	30 min	60 min	
1	Human	100	23.7	BQL	BQL	BQL	2.42
	Mouse	100	78.6	56.7	38.3	18.4	22.08
2	Human	100	23.8	BQL	BQL	BQL	2.43
	Mouse	100	81.4	51.2	30.3	13.1	17.40
10	Human	100	25.8	BQL	BQL	BQL	2.57
	Mouse	100	63.5	37.2	17.9	4.97	8.98
22	Human	100	53.0	17.0	3.38	0.340	5.68
	Mouse	100	31.4	4.96	2.49	1.54	3.04
26	Human	100	38.7	6.80	1.38	BQL	3.69
	Mouse	100	47.7	6.73	BQL	BQL	4.42
41	Human	100	33.0	6.79	BQL	BQL	3.15
	Mouse	100	73.9	40.7	16.5	3.91	11.25
57	Human	100	32.0	5.55	BQL	BQL	3.10
	Mouse	100	41.5	4.23	BQL	BQL	3.81
66	Human	100	14.6	BQL	BQL	BQL	1.81
	Mouse	100	66.3	22.6	BQL	BQL	7.63

BQL – below quantifiable limit

Solubility assay

Test compounds were dissolved in DMSO to a concentration of 10 mM. Phosphate buffer saline (396 µL) was added to a well of a flat-bottom polystyrene plate and test stock solution (4 µL) added such that the final concentration is 100 µM with a final DMSO concentration of 1%.

The plate was shaken for 4 hours at 25 °C before the plate was centrifuged at 4000 rpm for 10 minutes to sediment any precipitates. The supernatants were transferred to a new plate and diluted

with acetonitrile/PBS 1:1 containing the internal standard provided by the sponsor for LCMS/MS analysis. Each test compound was assessed in triplicate and the mean average reported.

NFAT gene reporter assay

To assess the effect of compounds on inhibition of the IL-2 pathway, the Invitrogen GENEblazer NFAT gene reporter assay was used (Hanson, 2006; Qureshi, 2007). The general process of carrying out the assay was as follows: NFAT-bla Jurkat cells were thawed and resuspended in assay media (OPTI-MEM, 0.5 % dialyzed FBS, 0.1 mM NEAA, 1 mM sodium pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 781,250 cells/mL.. 4 µL of a 10X serial dilution of the test compounds were added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension was added to the wells and pre-incubated at 37 °C/5 % CO_2 in a humidified incubator with test compounds for 30 minutes. Anti CD4:CD8 activator at the pre-determined EC_{80} concentration was added to wells containing the test compounds. The plate was incubated for 5 hours at 37 °C/5 % CO_2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution was added to each well and the plate was incubated for 2 hours at room temperature. The plate was read on a fluorescence plate reader.

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