Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary Information

An unusual *Burkholderia gladioli* double chain-initiating nonribosomal peptide synthetase assembles 'fungal' icosalide antibiotics

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1. Supplementary Methods

1.1 Strains and Culture Conditions

All *Burkholderia gladioli* isolates were drawn from previous studies[1] and had been accurately identified to the species level by either MLST[2] or *recA* sequence analysis[3]. Genome-sequenced BCC strains used were as follows: *B. gladioli* BCC0238, *B. gladioli* BCC0252, *B. gladioli* BCC1677, *B. gladioli* BCC1698, *B. gladioli* BCC1720 and *B. gladioli* BCC1713 (icosalide non-produer). The growth of *Burkholderia* (at 30 °C) was carried out on tryptic soya medium, whereas production of antibiotics was performed on a minimal salts medium with 4 g/L glycerol (BSM-G) as the carbon source, for 3 days at 30 °C.[4]

1.2 Extraction and HPLC Purification of Icosalide A1

B. gladioli growing on BSM-G agar was manually removed from the surface, and the agar was chopped into small pieces and extracted with EtOAc (4 mL per plate). The crude extract was fractionated by HPLC on a semi-preparative C_{18} Betasil column (21.2 mm × 150 mm), eluting as detailed in Table S3. Sixty fractions were collected at 1 min intervals and icosalide-containing fraction was identified by mass spectrometry.

1.3 Insertional Mutagenesis of Icosalide Biosynthetic Gene *icoA*

Insertional mutagenesis was performed using the pGpΩTp suicide plasmid. Primers amplifying a 1371 bp region of the NRPS gene between the third and fourth C domains, icoA_CC_For: 5'-CGCTCTAGACGCATGCCGATTGCCAGA-3' and 5'-icoA_CC_Rev: CGCGAATTCCTGGTGCGCCTGTCTATCTG-3' were designed. The PCR product was amplified using Phusion DNA polymerase, and cloned, following *Xba*I and *Eco*RI digestion (NEB), into pGpΩTp. The integrity of the mutagenesis construct was confirmed by DNA sequencing. The construct was introduced into *E. coli* SY327 by electroporation and subsequently mobilised into *B. gladioli* BCC0238 via triparental mating. Transconjugants were selected using trimethoprim (100 µg/mL) and polymyxin B (600 U/mL). A single *B. gladioli* BCC0238 *icoA* mutant was selected and integration of the plasmid at the intended locus was confirmed by PCR using the following primers: 5'-GCCACGAGCGTTACCAATGG-3' and 5'- GCCAGGGATGTAACGCACTG-3', which yielded a 1510 bp product.

1.4 Alkaline Hydrolysis of Icosalide A1

Purified Icosalide A1 (2.5mg) was suspended in 0.4 N KOH and left at room temperature for 4 h. LC-MS analysis showed that all of the starting material had been consumed and compounds giving rise to ions with *m/z* = 361.2314 and 389.2635, corresponding to $[M+H]^+$ for the expected hydrolysis products, were formed (Fig. S4). The hydrolysis products were purified using reverse phase HPLC on a C_{18} Betasil column (21.2 mm × 150 mm). A linear gradient from 30 to 100% acetonitrile was applied over 50 min, followed by isocratic elution with 100% acetonitrile for 10 min. The flow rate was 9 mL/min.

1.5 High Resolution Mass Spectrometry and NMR Spectroscopy

UHPLC-ESI-Q-TOF-MS/MS analyses were performed using a Dionex UltiMate 3000 UHPLC connected to a Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 µm) coupled to a Bruker MaXis IMPACT mass spectrometer. Mobile phases consisted of water (A) and acetonitrile (B), each supplemented with 0.1% formic acid. A gradient of 20% B to 100% B over 30 minutes was employed at a flow rate of 0.2 mL/min. The mass spectrometer was operated in positive ion mode with a scan range of 50-3000 m/z. Source conditions were: end plate offset at −500 V; capillary at −4500 V; nebulizer gas (N_2) at 1.6 bar; dry gas (N_2) at 8 L min−1; dry temperature at 180 °C. Ion transfer conditions were: ion funnel RF at 200 Vpp; multiple RF at 200 Vpp; quadrupole low mass at 55 m/z; collision energy at 5.0 eV; collision RF at 600 Vpp; ion cooler RF at 50–350 Vpp; transfer time at 121 µs; pre-pulse storage time at 1 µs. Calibration was performed with 1 mM sodium formate through a loop injection of 20µL at the start of each run. For NMR analysis, purified icosalide A1 was dissolved in d_6-acetone or d_6-DMSO, and ^1H, ^{13}C, COSY,

NOESY, TOCSY, HSQC and HMBC spectra were recorded on a Bruker Avance 700MHz spectrometer equipped with a TCl cryoprobe at 25 °C.

1.6 Absolute Stereochemistry Determination of Amino Acid Residues

Marfey's method was used to determine the stereochemistry of the constituent amino acids in each hydrolytic fragment of icosalide A1[5]. Purified hydrolytic fragments of icosalide A1 (~0.5 mg) were dissolved in 500 µL of 6N HCl and incubated at 110°C for 5 h. The resulting mixture was lyophilised and the residue was dissolved in 20 µL of H_2O and 40 µL of 1 M $NaHCO_3$ was added. Then 340 µL of a 1% Marfey's reagent solution in acetone was added and the mixture was heated to 37 °C for 1 h. The reaction was quenched with 40 µL of 1 M HCl. Marfey's derivatises of L-Leu, L-Ser, D-Leu and D-Ser were prepared using the same method. Samples were diluted 5-fold in a 1:1 MeCN:H_2O mixture containing 0.1% formic acid to a final volume of 500 µL and analysed by LC-MS on a C_{18} column using the elution conditions detailed in Table S4.

1.7 PCR-Screening of Fungal total DNA Extract

Total DNA was extracted from the *Aureobasidium* sp (MSX59166) fungal culture using a Zymo fungal-bacteria DNA miniprep kit, following the manufacturer's procedures. Extracted gDNA was subjected to PCR screening for the *Burkholderia recA* gene using primers; Burk_recA_For-(GATAGCAAGAAGGGCTCC) and Burk_recA_Rev-(CTCTTCTTCGTCCATCGCCTC). The 393 bp PCR product was sequenced and phylogenetically analysed as detailed in Section 1.8, to obtain species-level information. The extracted gDNA was also screened for the presence of the *icoA* gene using the following primers: IcoA_C_I (Module 3)_For-(GATGTCTCGCGTCTGGTCGA) and IcoA_C_I (Module 3)_Rev-(GGTAGCCTCGGGCGTGCGCTT), which specifically amplify the 1447 bp region of *icoA* encoding the module 3 C_I domain.

1.8 Phylogenetic Analysis of *Burkholderia recA* Sequences

The *recA* sequences from 25 representative *Burkholderia* species were downloaded from the MLST database[2]. The sequence of the icosalide-producing strain, determined by PCR product sequencing, was included in the analysis. The 26 *recA* sequences were aligned using MAFFT[6]. A phylogeny was reconstructed using FastTreeMP using the generalised time-reversible (GTR) model of nucleotide evolution. The resulting tree was visualised using FigTree.

1.9 Sequencing and Assembly of *Burkholderia* DNA from the Fungal Extract

Sequencing of the DNA extracted from *Aureobasidium* sp MSX59166 was conducted on an Illumina instrument. Reads were randomly subsampled to an estimated coverage of 50x based on the assumption of an 8.5 Mb genome and assembled with SPAdes v3.11.0[7] using the careful option and the coverage cut-off value set to 5. The resulting assembly was subjected to an automatic annotation using Prokka[8].

1.10 Overproduction and Purification of IcoA C_I-A-PCP (Module 3) and IcoA C_I-A-PCP(H164A) (Module 3)

The amplification of the region of *icoA* encoding the moduel 3 C_I-A-PCP tri-domain from *B. gladioli* BCC0238 gDNA was performed with Phusion DNA polymerase (NEB). The primers used were as follows: IcoA_C_I-A-PCP (Module 3)_For-(ATACATATGGATGTCTCGCGTCTGGT) and IcoA_C_I-A-PCP (Module 3)_Rev-(ATAAAGCTTTCACGCGACGCTTCGGCT). The PCR products was separated on a 1% agarose gel and the band was excised and purified with a Gene Jet gel extraction kit (Thermo Scientific). The insert and pET28a (NEB) were separately digested with the *Nde*I and *Hin*dIII restriction enzymesand ligated to each other using DNA ligase. The H164A mutant was constructed using the Q5 Site-Directed Mutagenesis Kit (NEB) with the following primers IcoA_C_I-A-PCP(H164A) (Module 3)_For-(GCGTTCGCCTGCTGTGCAGAACGAC) and IcoA_C_I-A-PCP(H164A) (Module 3)_Rev-(AACGCCACCAGCACGCTA). The resulting vectors were used to transform *E. coli* TOP10 cells (Invitrogen), which were plated on LB agar containing kanamycin (50 µg/mL). Colonies were picked

and grown overnight in LB medium. Plasmids were isolated from the culture using a miniprep kit (Thermo), and the inserts were sequenced to verify their integrity. A single colony of *E. coli* BL21(DE3) that had been transformed with the expression vector was picked and used to inoculate LB medium (10 mL) containing kanamycin (50 µg/mL). After incubation overnight at 37 °C and 180 rpm, the culture was inoculated into LB medium (1 L) containing kanamycin (50 µg/mL). The resulting culture was incubated at 37 °C and 180 rpm until the optical density at 595 nm reached 0.6, then IPTG (0.25mM) was added and growth was continued overnight at 15 °C and 180 rpm. The cells were harvested by centrifugation (4,000 x g, 15 min, 4 °C) and re-suspended in buffer (20 mM Tris-HCl, 300mM NaCl, 20 mM Imidazole, pH 8.0) at 10 mL/L of growth medium then lysed using a Constant Systems cell disruptor. The lysate was centrifuged (37,000 x g, 30 min, 4 °C) and the resulting supernatant was loaded onto a HiTrap Chelating Column (GE Healthcare), which had been pre-loaded with 100 mM $NiSO_4$ and equilibrated in re-suspension buffer (20 mM Tris-HCl, 300mM NaCl, 20 mM Imidazole, pH 8). Proteins were eluted in a stepwise manner using re-suspension buffer containing increasing concentrations of imidazole – 50 mM (5 mL), 100 mM (3 mL), 200 mM (3 mL) and 300 mM (3 mL). The presence of the protein of interest in fractions was confirmed by SDS-PAGE, and an additional gel filtration step (Superdex 75/200, GE Healthcare) was used to further purify the proteins where necessary. Fractions containing the protein of interest were pooled and concentrated to 80-100 µM and exchanged into storage buffer (20 mM Tris-HCl, 300 mM NaCl pH 7.4) using a 50 kDa MWCO Viva-Spin centrifugal concentrator. Glycerol was added to the concentrated protein samples to a final concentration of 10% (v/v) and they were snap-frozen in liquid N_2 and stored at -80 °C.

1.11 Overproduction and purification of Sfp

The phosphopantetheinyltransferase Sfp from *Bacillus subtilis* was overproduced and purified as an N-terminal hexahistidine fusion using the procedure described in section 1.10 and the pET28a(+)-Sfp vector (kindly provided by Dr M. Tosin, Department of Chemistry, University of Warwick). Fractions containing the purified protein were concentrated to ~500 µM and exchanged into storage buffer (20 mM Tris-HCl, 300 mM NaCl pH 7.4) using a 10 kDa MWCO Viva-Spin centrifugal concentrator. The molecular weight of the purified protein was confirmed by UHPLC-ESI-Q-TOF-MS analysis.

1.12 IcoA C_I-A-PCP Condensation Domain Assays

All condensation reactions were conducted in storage buffer (20 mM Tris-HCl, 300 mM NaCl pH 7.4). The *apo*- forms of IcoA C_I-A-PCP tridomain (~100 µM) and IcoA C_I-A-PCP(H164A) were pre-treated with 3U of thrombin (Sigma-Aldrich) for 16 h at 4 °C to remove the N-terminal His-Tag. The cleaved tri-domains were then converted to their *holo* forms by incubation in 20 mM Tris, 300 mM NaCl, 10% glycerol, 2 µM Sfp, 500 µM CoA and 10 mM $MgCl_2$ in a total volume of 50 µL at room temperature for 1 h. Addition of L-Ser (500 µM), ATP (500 µM) and one of (3*R*)-3-hydroxyoctanoyl pantetheine (**4**), (*3S*)-3-hydroxyoctanoyl pantetheine (**4a**), (3*R*)-3-hydroxydecanoyl pantetheine (**5**) and (3*S*)-3-hydroxydecanoyl pantetheine (**5a**) thioesters (1 mM) initiated the loading reaction, which was allowed to proceed for 6 h at room temperature. The competition assay between the (3*R*)-3-hydroxyoctanoyl (**4**) and (3*R*)-3-hydroxydecanoyl (**5**) pantetheine thioesters was conducted using a final concentration of 0.5 mM for each substrate. The reaction was diluted and analysed by UHPLC-ESI-Q-TOF-MS.

1.13 UHPLC-ESI-Q-TOF-MS Analysis of Intact Proteins

All acyl-transfer assays were analyzed on a Bruker MaXis II ESI-Q-TOF-MS connected to a Dionex 3000 RS UHPLC fitted with an ACE C_4-300 RP column (100 x 2.1 mm, 5 µm, 30 °C). The column was eluted with a linear gradient of 5–100% MeCN containing 0.1% formic acid over 30 min. The mass spectrometer was operated in positive ion mode with a scan range of 200–3000 *m/z*. Source conditions were: end plate offset at −500 V; capillary at −4500 V; nebulizer gas (N_2) at 1.8 bar; dry gas (N_2) at 9.0 L min^{-1}; dry temperature at 200 °C. Ion transfer conditions were: ion funnel RF at 400 Vpp; multiple RF at 200 Vpp; quadrupole low mass at 300 *m/z*; collision energy at 8.0 eV; collision RF at 2000 Vpp; transfer time at 110.0 µs; pre-pulse storage time at 10.0 µs.

1.14 Minimum Inhibitory Concentration (MIC) Measurements

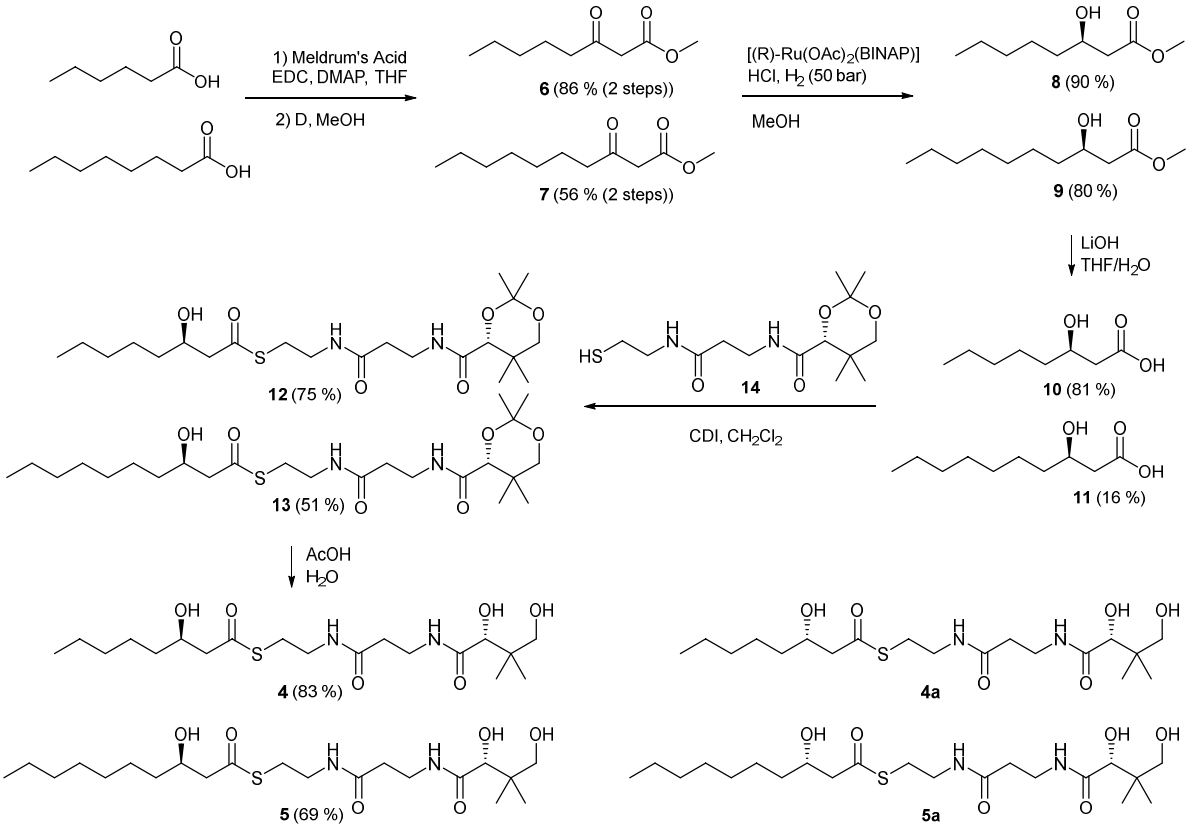
Minimal inhibitory concentration measurements were carried out using the broth microdilution method, following CLSI guidelines. Briefly, reference bacterial and yeast strains were grown overnight in Mueller-Hinton (MH) broth at 30/37 °C. In a 96-well microtiter plate, 50 µl of serial twofold dilutions of icosalide A1 in MH broth were mixed with 50 µl of bacterial suspension, diluted to a concentration of 106 CFU/ml in MH broth. The desired inoculum density was achieved using a 0.5 McFarland turbidity standard. Following incubation for 18 h at 30 or 37 °C, as appropriate, MICs were defined as the lowest concentrations that visibly inhibited bacterial growth. All MIC determinations were performed in triplicate.

2. Synthesis of Substrate Analogues

2.1 General Procedures

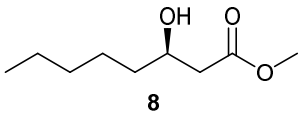
Room temperature refers to ambient temperature (20-22 °C), 5 °C refers to a cold water bath and 0 °C refers to an ice slush bath. Heated experiments were conducted using thermostatically controlled oil baths. All commercially available solvents and chemicals were used without any further purification. NMR spectra were recorded on Bruker Advance AV-300 and HD-500 MHz spectrometers at room temperature (298 K). Chemical shifts are reported in parts per million (ppm) referenced to $CHCl_3$ (δ_H: 7.26 ppm and δ_C: 77.0 ppm). Coupling constants (*J*) are quoted to the nearest 0.5 Hertz (Hz). Multiplicities are given as multiplet (m), singlet (s), doublet (d), triplet (t), quartet (q), quintet (quin.), sextet (sext.), septet (sept.), octet (oct.) and nonet (non.). ^1H and ^{13}C assignments were established on the basis of COSY, DEPT, HMQC and HMBC correlations. Infra-red spectra were recorded using either a Perkin Elmer Spectrum 100 FT-IR spectrometer or an Alpha Bruker Platunium ATR single reflection diamond ATR module. Optical rotations were measured using an Optical Activity Ltd AA-1000 millidegree auto-ranging polarimeter (589 nm). Specific rotations are given in units of 10^{-1} deg cm^2 g^{-1}. Melting points were recorded on a Stuart scientific melting point apparatus and are uncorrected. Silica column chromatography was performed on 40-60 Å silica gel. Thin layer chromatography (TLC) was carried out on aluminum sheets coated with 0.2 mm silica gel 60 F_{254}. Compounds were visualised using UV light (254 nm) or by staining with potassium permanganate solution followed by heating. Low resolution mass spectra (LRMS) were recorded using an Agilent 6130B single Quad (ESI). High resolution mass spectra (HRMS) were obtained by either Dr Lijiang Song or Mr Philip Aston using a Bruker MaXis ESI-Q-TOF mass spectrometer.

2.2 Synthesis of 3-hydroxyoctanoyl/3-hydroxydecanoyl pantetheine thioesters



Scheme 1: Synthesis of 3-hydroxyoctanoyl and 3-hydroxydecanoyl pantetheine substrates 4/4a and 5/5a. Compound **14** was synthesized according to literature procedures[14,15]. Substrates **4a/5a** were synthesized via the same route using [(*S*)-Ru(OAc)$_2$(BINAP)].The stereochemistry and *e.e.* of the (3*R*) and (3*S*)-hydroxyl esters of **8/9** was confirmed by Mosher's ester analysis.

2.2.1 Methyl (3*R*)-hydroxyoctanoate (**8**)



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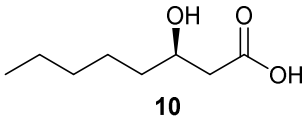
To a solution of [(*R*)-Ru(OAc)$_2$(BINAP)] (14.5 mg, 0.01 equiv., 0.02 mmol) and 1M HCl in MeOH (0.02 mL, 0.01 equiv., 0.02 mmol) in MeOH (2 mL) was added methyl 3-oxooctanoate **6** (300 mg, 1 equiv., 1.70 mmol) in a dry 10 mL high pressure reaction vessel containing a magnetic stirring bar. The reaction was heated to 60 ^{o}C at 50 bar hydrogen pressure for 16 hours. The mixture was filtered through celite and concentrated *in vacuo* to afford the product as a yellow oil (272 mg, 90.0 %). The absolute stereochemistry and *e.e.* (>95%) were confirmed by derivatisation with (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride. Spectroscopic data were consistent with those previously reported in the literature.[9]

δ_H (500 MHz; $CDCl_3$) 4.05-3.95 (1H, m, C*H*OH), 3.71 (3H, s, COOC*H*$_3$), 2.84 (1H, d, J 4, O*H*), 2.52 (1H, dd, J 16.5 and 3.5 C*H*$_2$CO$_2$Me), 2.41 (1H, dd, J 16.5 and 9, C*H*$_2$CO$_2$Me), 1.56-1.22 (8H, m, CH$_3$C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$), 0.89 (3H, d, J 7, CH$_2$C*H*$_3$); δ_C (125 MHz, $CDCl_3$) 173.7 (*C*OOCH$_3$), 68.2 (*C*HOH), 51.9 (COO*C*H$_3$), 42.2 (*C*H$_2$CO$_2$Me), 36.6 (CH*C*H$_2$), 31.8 (CHCH$_2$*C*H$_2$), 25.3 (CHCH$_2$CH$_2$*C*H$_2$), 22.7 (*C*H$_2$CH$_3$), 14.1 (CH$_2$*C*H$_3$); HRMS (ESI) cald. for $C_9H_{18}NaO_3$ (M + Na^+) requires 197.1148, found 197.1150.

2.2.2 (3*R*)-hydroxyoctanoic acid (**10**)



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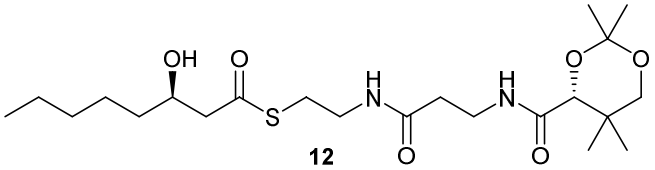
To a solution of methyl (3*R*)-hydroxyoctanoate **8** (250mg, 1.44 mmol, 1.0 equiv.) in a 2:1 mixture of THF (4 mL) and water (2 mL), was added LiOH (56 mg, 2 equiv., 2.88 mmol) and the resulting mixture was stirred for 16 hours. THF was removed in *vacuo*, the residual aqueous solution was acidified to pH 2.0 using 1 M HCl and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The combined organics were washed with brine (20 mL), dried ($MgSO_4$) and concentrated in *vacuo* to afford the product as a colourless oil (178 mg, 81 %). Spectroscopic data were consistent with those previously reported in the literature.[9]

δH (500 MHz; $CDCl_3$) 4.03 (1H, dddd, J 9, 7.5, 4.5 and 3, C*H*OH), 2.57 (1H, dd, J 16.5 and 3 C*H*$_2$CO$_2$H), 2.47 (1H, dd, J 16.5 and 9, C*H*$_2$CO$_2$H), 1.59-1.24 (8H, m, CH$_3$C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$), 0.89 (3H, t, J 7, CH$_2$C*H*$_3$); δ_C (125 MHz, CDCl3) 177.9 (*C*OOH), 68.2 (*C*HOH), 41.2 (*C*H$_2$CO$_2$H), 36.6 (CH*C*H$_2$), 31.8 (CHCH$_2$*C*H$_2$), 25.3 (CHCH$_2$CH$_2$*C*H$_2$), 22.7 (*C*H$_2$CH$_3$), 14.2 (CH$_2$*C*H$_3$); HRMS (ESI) cald. for $C_8H_{15}O_3$ (M - H+) requires 159.1027, found 159.1028.

2.2.3 *S*-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanamido)ethyl) (*R*)-3-hydroxyoctanethioate (**12**)



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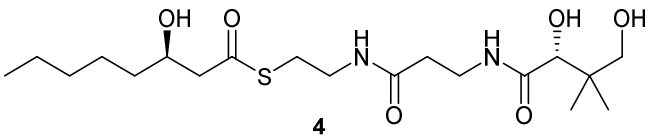
To a solution of (3*R*)-hydroxyoctanoic acid **10** (100 mg, 0.63 mmol, 1.1 equiv.) in CH_2Cl_2 (10 mL) was added CDI (102 mg, 0.63 mmol, 1.1 equiv.) and the resulting mixture was stirred at room temperature for 1 h. (*R*)-*N*-(3-((2-mercaptoethyl)amino)-3-oxopropyl)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamide **9** (180 mg, 0.57 mmol, 1.0 equiv.) was added and stirring was continued at room temperature for a further 16 hours. 1 M HCl (5 mL) was added and the mixture was extracted with CH_2Cl_2 (3 x 5 mL). The combined organics were washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL), dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil which was purified by silica chromatography (CH_2Cl_2 : MeOH 95 :5) to give the product as a colourless oil (196 mg, 75 %).

ν_{max}/cm^{-1} (neat) 3417 (OH), 2931 (NH), 1656, 1529 (C=O), 1160 (C-O); δ_H (500 MHz; $CDCl_3$) 6.99 (1H, br. t, J 6, N*H*), 6.38 (1H, br. s, N*H*CH_2CH_2S), 4.12-4.06 (1H, m, C*H*OH), 4.07 (1H, s, C*H*CONH), 3.67 (1H, d J 11.5, C*H*$_2$OC$(CH_3)_2$), 3.59-3.33 (5H, m, NHC*H*$_2$, C*H*$_2CH_2S$, O*H*), 3.27 (1H, d J 11.5, C*H*$_2$OC$(CH_3)_2$), 3.12 (1H, ddd, J 14, 7 and 5.5, C*H*$_2$S), 2.98 (1H, ddd, J 14, 6.5 and 5.5, C*H*$_2$S), 2.72 (1H, dd, J 15 and 4, C*H*$_2$COS), 2.68 (1H, dd, J 15 and 8.5, C*H*$_2$COS), 2.41 (2H, td J 6.5 and 1.5, C*H*$_2$CONH), 1.55-1.40 (CHOHC*H*$_2$C*H*$_2$), 1.46 (3H, s, OC(C*H*$_3)_2$), 1.42 (3H, s, OC(C*H*$_3)_2$), 1.37-1.23 (5H, m, C*H*$_2$C*H*$_2$C*H*$_2CH_3$), 1.03 (3H, s, CH_2C(C*H*$_3)_2$), 0.95 (3H, s, CH_2C(C*H*$_3)_2$), 0.88 (3H, t, J 7, CH_2C*H*$_3$); δ_C (125 MHz, $CDCl_3$) 199.4 (*C*OS), 171.3 (CH_2*C*ONH), 170.7 (CH*C*ONH), 99.3 (O*C*$(CH_3)_2$), 77.3 (*C*H), 71.6 (*C*H_2OC$(CH_3)_2$), 69.0 (*C*HOH), 51.4 (*C*H_2COS), 39.3 (*C*H_2CH_2S), 37.2 (C*H*$_2$CHOH), 36.4 (*C*H_2CONH), 35.4 (*C*H_2NH), 33.1 (CH_2*C*$(CH_3)_2$), 31.9 (CHOHCH$_2$*C*H$_2$), 29.6 ((OC(*C*$H_3)_2$), 28.8 (*C*H_2S), 25.3 (CH_3CH_2*C*H$_2$), 22.7 (CH_3*C*H$_2$), 22.3 (CH_2C(*C*$H_3)_2$), 19.0 (CH_2C(*C*$H_3)_2$), 18.9 (OC(*C*$H_3)_2$), 14.2 (CH_2*C*H$_3$); HRMS (ESI) cald. for $C_{22}H_{40}N_2NaO_6S$ (M + Na^+) requires 483.2499, found 483.2500; $[\alpha]_D^{26}$ (c 0.2, $CHCl_3$): +17.5.

2.2.4 *S*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (*R*)-3-hydroxyoctanethioate (**4**)

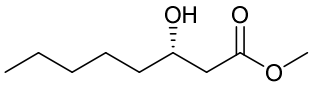


4

S-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanamido)ethyl) (*R*)-3-hydroxyoctanethioate **12** (100 mg, 0.22 mmol, 1.0 equiv.) was stirred in AcOH : H_2O (2 : 1, 3 mL), for 16 h at room temperature. The mixture was concentrated *in vacuo* and purified using silica chromatography (CH_2Cl_2 : MeOH, 85 : 15) to give the product as a colourless oil (76 mg, 83 %).

ν_{max}/cm^{-1} (neat) 3405 (OH), 2871 (NH), 1642, 1529 (C=O), 1076 (C-O); δ_H (500 MHz; CD_3OD) 4.05-3.99 (1H, m, C*H*OH), 3.89 (1H, s, C*H*), 3.53-3.32 (6H, m, NHC*H*$_2$, C*H*$_2CH_2$S, C*H*$_2$CHOH), 3.02 (2H, t, J 6.5, C*H*$_2$S), 2.71 (1H, dd, J 15 and 5, C*H*$_2$COS), 2.68 (1H, dd, J 15 and 7.5, C*H*$_2$COS), 2.41 (2H, t, J 6.5, C*H*$_2$CONH), 1.50-1.25 (8H, m, CH_3C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$), 0.92 (6H, s, CH_2C(C*H*$_3)_2$), 0.91 (3H, t, J 7.5, CH_2C*H*$_3$); δ_C (125 MHz, CD_3OD) 198.9 (*C*OS), 176.0 (CH*C*ONH), 173.9 (CH_2*C*ONH), 77.3 (*C*H), 70.3 (*C*H_2OH), 69.6 (*C*HOH), 52.6 (*C*H_2COS), 40.3 (CH_2*C*$(CH_3)_2$), 40.0 (*C*H_2CH_2S), 38.1 (*C*H_2CONH), 36.4 (*C*H_2OH), 36.3 (*C*H_2NH), 32.8 ($CH_3CH_2CH_2$C*H*$_2$), 29.3 (*C*H_2S), 26.3 (CH_3CH_2*C*H$_2$), 23.6 (CH_3*C*H$_2$), 21.3 (CH_2C(*C*$H_3)_2$), 20.9 (CH_2C(*C*$H_3)_2$), 14.4 (*C*H_3CH_2); HRMS (ESI) cald. for $C_{19}H_{36}N_2NaO_6S$ (M + Na^+) requires 443.2186, found 443.2189; $[\alpha]_D^{26}$ (c 0.1, $CHCl_3$): +16.5.

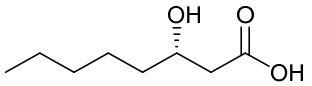
2.2.5 Methyl (*S*)-3-hydroxyoctanoate (**8a**)



8a

Methyl (*S*)-3-hydroxyoctanoate **8a** was synthesized using the same procedure as for the synthesis of Methyl (*R*)-3-hydroxyoctanoate **8** using methyl 3-oxooctanoate **6** (300 mg, 1.70 mmol) and [(*S*)-$Ru(OAc)_2$(BINAP)] (14.5 mg, 0.01 equiv., 0.02 mmol) to afford the product as a yellow oil (268 mg, 88 %). The absolute stereochemistry and *e.e.* (>95%) were confirmed by derivatisation with (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride. Spectroscopic data were consistent with those previously reported in the literature.[10]

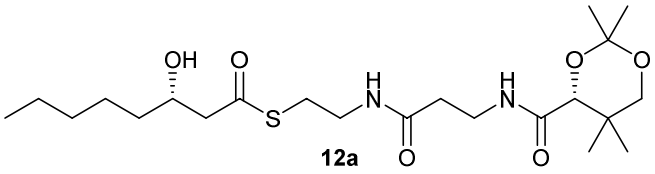
2.2.6 (*S*)-3-hydroxyoctanoic acid (**10a**)



10a

(*S*)-3-hydroxyoctanoic acid **8a** was synthesized using the same procedure as for the synthesis of (*R*)-3-hydroxyoctanoic acid **10** using methyl (*S*)-3-hydroxyoctanoate **8a** (220mg, 1.2.6 mmol, 1.0 equiv.) to give the product as a colourless oil (139 mg, 69 %). Spectroscopic data were consistent with those previously reported in the literature.[11]

2.2.7 *S*-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanamido)ethyl) (*S*)-3-hydroxyoctanethioate (**12a**)



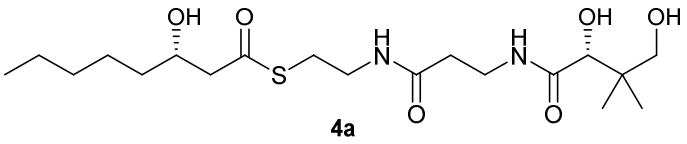
12a

S-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanamido)ethyl) (*S*)-3-hydroxyoctanethioate **12a** was synthesized using the same procedure as for the synthesis of *S*-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido) propanamido)ethyl) (*R*)-3-hydroxyoctanethioate **12** using (*S*)-3-hydroxyoctanoic acid **10a** (100 mg, 0.63 mmol, 1.1 equiv.) to give the product as a colourless oil (202 mg, 77 %).

ν_{max}/cm^{-1} (neat) 3324 (OH), 2930 (NH), 1666, 1542 (C=O), 1159 (C-O); δ_H (500 MHz; $CDCl_3$) 6.99 (1H, br. t, J 6.5, N*H*), 6.38 (1H, br. t, J 5.5, N*H*CH_2CH_2S), 4.11-4.05 (1H, m, C*H*OH), 4.06 (1H, s, C*H*CONH), 3.67 (1H, d J 11.5, $CH_2OC(CH_3)_2$), 3.59-3.39 (5H, m, $NHCH_2$, CH_2CH_2S, O*H*), 3.27 (1H, d J 11.5, $CH_2OC(CH_3)_2$), 3.09 (1H, dt, J 14 and 6, CH_2S), 3.00 (1H, dt, J 14 and 6, CH_2S), 2.72 (1H, dd, J 15 and 3.5, CH_2COS), 2.67 (1H, dd, J 15 and 9, CH_2COS), 2.40 (2H, td J 7 and 2.5, CH_2CONH), 1.55-1.39 ($CHOHCH_2CH_2$), 1.45 (3H, s, $OC(CH_3)_2$), 1.41 (3H, s, $OC(CH_3)_2$), 1.37-1.21 (5H, m, $CH_2CH_2CH_2CH_3$), 1.02 (3H, s, $CH_2C(CH_3)_2$), 0.96 (3H, s, $CH_2C(CH_3)_2$), 0.88 (3H, t, J 7, CH_2CH_3); δ_C (125 MHz, $CDCl_3$)

199.4 (*C*OS), 171.3 (CH_2*C*ONH), 170.7 (CH*C*ONH), 99.3 (O*C*(CH_3)$_2$), 77.3 (*C*H), 71.5 (*C*H_2OC(CH_3)$_2$), 69.2 (*C*HOH), 51.4 (*C*H_2COS), 39.4 (*C*$H_2$$CH_2$S), 37.3 (C*H*$_2$CHOH), 36.5 (*C*$H_2$CONH), 35.3 (*C*$H_2$NH), 33.1 ($CH_2$*C*($CH_3$)$_2$), 31.9 (CHOHCH$_2$*C*$H_2$), 29.6 ((OC(*C*$H_3$)$_2$), 28.8 (*C*$H_2$S), 25.3 ($CH_3$$CH_2$*C*$H_2$), 22.7 ($CH_3$*C*$H_2$), 22.3 ($CH_2$C(*C*$H_3$)$_2$), 19.0 ($CH_2$C(*C*$H_3$)$_2$), 18.8 (OC(*C*$H_3$)$_2$), 14.2 ($CH_2$*C*$H_3$); HRMS (ESI) cald. for $C_{22}H_{40}N_2NaO_6S$ (M + Na^+) requires 483.2499, found 483.2498; $[\alpha]_D^{26}$ (c 0.075, $CHCl_3$): +34.

2.2.8 *S*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (*S*)-3-hydroxyoctanethioate (**4a**)

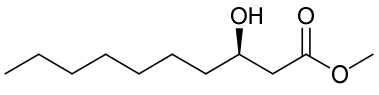


4a

S-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (*S*)-3-hydroxyoctanethioate **4a** was synthesized using the same procedure as for the synthesis of *S*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (*R*)-3-hydroxyoctanethioate **4** using *S*-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanamido)ethyl) (*S*)-3-hydroxyoctanethioate **12a** (100 mg, 0.22 mmol, 1 equiv.) to give the product as a colourless oil (78 mg, 85 %).

ν_{max}/cm^{-1} (neat) 3293 (OH), 2870 (NH), 1642, 1529 (C=O), 1076 (C-O); δ_H (500 MHz; CD_3OD) 4.05-3.99 (1H, m, C*H*OH), 3.89 (1H, s, C*H*), 3.53-3.32 (6H, m, NHC*H*$_2$, C*H*$_2$$CH_2$S, C*H*$_2$CHOH), 3.02 (2H, t, J 6.5, C*H*$_2$S), 2.71 (1H, dd, J 15 and 5, C*H*$_2$COS), 2.68 (1H, dd, J 15 and 7.5, C*H*$_2$COS), 2.41 (2H, t, J 6.5, C*H*$_2$CONH), 1.49-1.26 (8H, m, CH_3C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$), 0.92 (6H, s, CH_2C(C*H*$_3$)$_2$), 0.91 (3H, t, J 7.5, CH_2C*H*$_3$); δ_C (125 MHz, CD_3OD) 198.9 (*C*OS), 176.0 (CH*C*ONH), 173.9 (CH_2*C*ONH), 77.3 (*C*H), 70.4 (*C*H_2OH), 69.6 (*C*HOH), 52.6 (*C*H_2COS), 40.4 (CH_2*C*(CH_3)$_2$), 40.0 (*C*$H_2$$CH_2$S), 38.1 (*C*$H_2$CONH), 36.4 (*C*$H_2$OH), 36.3 (*C*$H_2$NH), 32.9 ($CH_3$$CH_2$$CH_2$C*H*$_2$), 29.3 (*C*$H_2$S), 26.3 ($CH_3$$CH_2$*C*$H_2$), 23.7 ($CH_3$*C*$H_2$), 21.3 ($CH_2$C(*C*$H_3$)$_2$), 20.9 ($CH_2$C(*C*$H_3$)$_2$), 14.4 (*C*$H_3$$CH_2$); HRMS (ESI) cald. for $C_{22}H_{40}N_2NaO_6S$ (M + Na^+) requires 483.2499, found 483.2500; $[\alpha]_D^{26}$ (c 0.15, $CHCl_3$): +10.3.

2.2.9 Methyl (3*R*)-hydroxydecanoate (**9**)

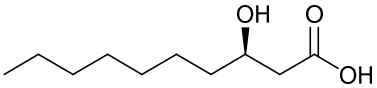


9

To a solution of [(*R*)-Ru(OAc)$_2$(BINAP)] (19.3 mg, 0.03 mmol) and HCl in MeOH (1 M 0.03 mL, 0.03 mmol) in MeOH (3 mL) was added methyl 3-oxooctanoate **7** (400 mg, 2.0 mmol) in a dry 10 mL high pressure reaction vessel containing a magnetic stirring bar. The resulting mixture was heated to 60 °C under 50 bar hydrogen pressure for 16 hours. The mixture was filtered through celite and concentrated *in vacuo* to afford the desired product as a yellow oil (320.0 mg, 1.6 mmol, 80 %). The absolute stereochemistry and *e.e.* (>95%) were confirmed by derivatisation with (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride. Spectroscopic data were consistent with those previously reported in the literature.[12]

δ_H (400 MHz; $CDCl_3$): 3.97 (1H, br s), 3.69 (3H, s), 2.84 (1H, br s), 2.53-2.41 (1H, m), 2.40 (1H, dd *J* = 16.0 and 9.0 Hz), 1.63-1.56 (1H, m), 1.54-1.47 (1H, m), 1.44-1.38 (2H, m), 1.31-1.21 (8H, m), 0.86 (3H, t *J* = 6.5 Hz). δ_C (100 MHz, $CDCl_3$): 173.6, 68.3, 52.3, 41.1, 36.6, 32.0, 29.6, 29.3, 25.6, 22.8, 14.2. MS (ES^+): *m/z* (%): 427 (100, $[M_2+Na]^+$), 225 (12, $[M+Na]^+$).

2.2.10 (3*R*)-hydroxydecanoic acid (**11**)

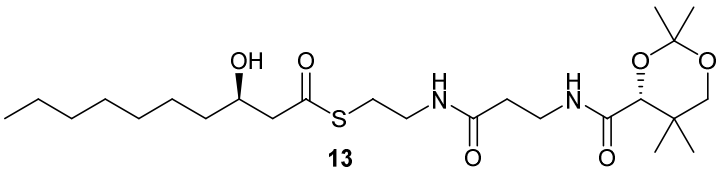


11

To a solution of methyl (3*R*)-hydroxydecanoate (**9**) (290.0 mg, 1.4 mmol) in a mixture of THF (4.0 mL) and water (2.0 mL) was added LiOH (55.8 mg, 2.9 mmol). The resulting mixture was stirred at room temperature for 16 hours. The THF was removed *in vacuo*, the residual aqueous solution was acidified with HCl (1 M) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organics were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to afford the desired product as a waxy solid (42 mg, 0.2 mmol, 16 %). Spectroscopic data were consistent with those previously reported in the literature.[12]

δ_H (400 MHz; $CDCl_3$): 4.01-3.98 (1H, m), 2.56 (1H, dd *J* = 16.5 2.5 Hz), 2.46 (1H, dd *J* 16.5 9.0 Hz), 1.52-1.40 (3H, m), 1.35- 1.24 (9H, m), 0.88 (3H, t *J* 6.5 Hz). δ_C (100 MHz, $CDCl_3$): 177.2, 68.1, 41.2, 36.6, 31.9, 29.5, 29.3, 25.5, 28.8, 14.2. MS (ES^+): *m/z* (%): 187 (100, $[M-H]^-$)

2.2.11 *S*-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanamido)ethyl) (*R*)-3-hydroxydecanethioate (**13**)

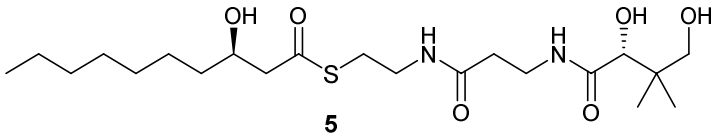


13

To a solution of (3*R*)-hydroxydecanoic acid (**11**) (32.0 mg, 0.2 mmol) in CH_2Cl_2 (3 mL) was added CDI (31.0 mg, 0.2 mmol) and the resulting mixture was stirred at room temperature for 1 hour. (*R*)-*N*-(3-((2-Mercaptoethyl)amino)-3-oxopropyl)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamide (54.0 mg, 0.2 mmol) was then added and the resulting solution was stirred at room temperature for 16 hours. HCl (1M, 5 mL) was added and the mixture was extract with CH_2Cl_2 (3 × 5 mL). The combined organics were washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL), dried ($MgSO_4$) and concentrated *in vacuo*, to give a yellow oil which was purified by silica chromatography (ethyl acetate) to give the desired product as a colourless oil (42 mg, 0.1 mmol, 51 %). Spectroscopic data were consistent with those previously reported in the literature.[13]

δ_H (400 MHz; $CDCl_3$): 6.98 (1H, br s), 6.26 (1H, br s), 4.08 (1H, s), 3.68 (1H, d *J* = 12.0 Hz), 3.61-3.46 (2H, m), 3.43-3.31 (2H, m), 3.28 (1H, m), 3.18-3.07 (1H, m), 3.03-2.94 (1H, m), 2.42 (2H, t *J* = 7.0 Hz), 1.66-1.50 (2H, m), 1.47 (3H, s), 1.42 (3H, s) 1.33-1.22 (12H, m), 1.04 (3H, s), 0.97 (3H, s), 0.88 (3H, t *J* = 7.0 Hz). δ_C (100 MHz, $CDCl_3$): 199.2, 171.2, 170.5, 99.1, 77.1, 71.4 68.8, 51.3, 39.1, 37.1, 36.2, 35.2, 33.0, 31.8, 29.5, 29.2 29.2, 28.7, 25.5, 22.6, 22.1, 18.9, 18.7, 14.1. MS (ES^+): *m/z* (%): 511 (100, $[M+Na]^+$)

2.2.12 *S*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (*R*)-3-hydroxydecanethioate (**5**)

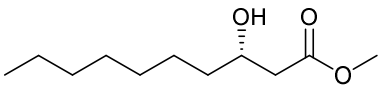


5

S-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanamido)ethyl) (3*R*)-hydroxydecanethioate (**13**) (38 mg, 0.1 mmol) was storred in AcOH:H_2O (2:1, 2 mL) for 16 hours at room temperature. The reaction mixture was concentrated *in vacuo* and purified using silica chromatography (ethyl acetate) to give the desired product as a colourless oil (24 mg, 0.1 mmol, 69 %). %). Spectroscopic data were consistent with those previously reported in the literature.[13]

δ_H (400 MHz; $CDCl_3$): 7.45 (1H, br t *J* = 6.0 Hz), 6.64 (1H, br t *J* = 5.5 Hz) 4.08 (1H, br s), 4.00 (1H, s), 3.66-3.53 (2H, m), 3.53-3.43 (4H, m), 3.35-3.26 (1H, m), 3.21-3.11 (1H, m), 2.71-2.66 (2H, m), 2.46-2.38 (2H, m), 1.49-1.40 (2H, m), 1.31-1.23 (10H, m), 1.01 (3H, s), 0.93 (3H, s), 0.88 (3H, t *J* = 6.0 Hz). δ_C (100 MHz, $CDCl_3$): 199.4, 174.9, 171.2, 77.7, 70.8, 69.0, 51.4, 39.4, 38.9, 37.2, 35.8, 35.4, 31.9, 29.5, 29.2, 26.8, 25.6, 22.7, 21.7, 20.5, 14.2. MS (ES^+): *m/z* (%): 471 (100, $[M+Na]^+$)

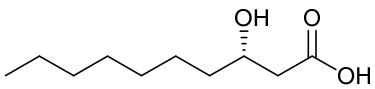
2.2.13 Methyl (*S*)-3-hydroxydecanoate (**9a**)



9a

Methyl (3*S*)-hydroxydecanoate was synthesized using the same procedure as for the synthesis of methyl (3*R*)-hydroxydecanoate (**9a**) using methyl 3-oxodecanoate (**7**) (400 mg, 2.0 mmol) and [(*R*)-$Ru(OAc)_2$(BINAP)] (19.3 mg, 0.03 mmol) to afford the desired product as a yellow oil, with identical spectroscopic data as methyl (3*R*)-hydroxydecanoate (348 mg, 1.7 mmol, 87 %). The absolute stereochemistry and *e.e.* (>95%) was determined by derivatisation with (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride. Spectroscopic data were consistent with those previously reported in the literature.[12]

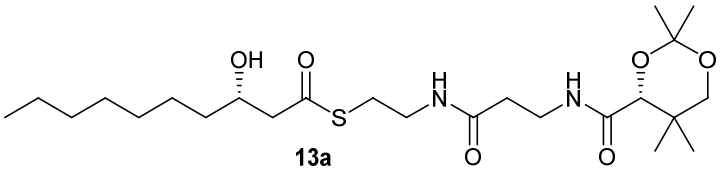
2.2.14 (*S*)-3-hydroxydecanoic acid (**11a**)



11a

(3*S*)-Hydroxydecanoic acid (**11a**) was synthesized using the same procedure as for the synthesis of (3*S*)-hydroxydecanoic acid (**11**) using (3*S*)-hydroxydecanoate (**9a**) (348.0 mg, 1.7 mmol) to give the desired product as a colourless oil (56 mg, 0.3 mmol, 18 %). %). Spectroscopic data were consistent with those previously reported in the literature.[12]

2.2.15 *S*-(2-(3-((*R*)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanamido)ethyl) (*S*)-3-hydroxydecanethioate (13a)

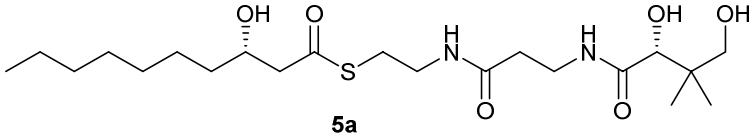


13a

S-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (3*S*)-hydroxyodecanethioate (**13a**) was synthesised using the same procedure as for the synthesis of *S*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (3*R*)-hydroxyodecanethioate (**13**) using (3*S*)-hydroxydecanoic acid (**11a**) (35 mg, 0.2 mmol) to give the desired product as a colourless oil (41 mg, 0.1 mmol, 44 %). Spectroscopic data were consistent with those previously reported in the literature.[13]

δ_H (400 MHz; $CDCl_3$): 6.99 (1H, br t *J* = 6.0 Hz), 6.30 (1H, br t *J* = 5.5 Hz), 4.08 (1H, s), 3.68 (1H, d *J* = 12.0 Hz), 3.61-3.51 (1H, m), 3.51-3.44 (3H, m), 3.28 (1H, d *J* = 12.0 Hz), 3.15-3.06 (1H, m), 3.06-2.98 (1H, m), 2.76-2.67 (2H, m), 2.41 (2H, t *J* = 6.5 Hz), 1.70-1.58 (2H, m), 1.47 (3H, s), 1.42 (3H, s), 1.32-1.24 (12H. m), 1.04 (3H, s), 0.96 (3H, s), 0.88 (3H, t *J* = 7.0 Hz). δ_C (100 MHz, $CDCl_3$):.199.2, 171.3, 170.6, 99.1, 77.1, 71.4, 69.9, 51.3, 39.1, 37.1, 36.2, 35.3, 33.0, 31.8, 29.4, 29.4, 29.2, 28.7, 25.5, 22.6, 22.2, 18.9, 18.7, 14.1. MS (ES^+): *m/z* (%): 511 (100, $[M+Na]^+$)

2.2.16 *S*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (*S*)-3-hydroxyoctanethioate (5a)

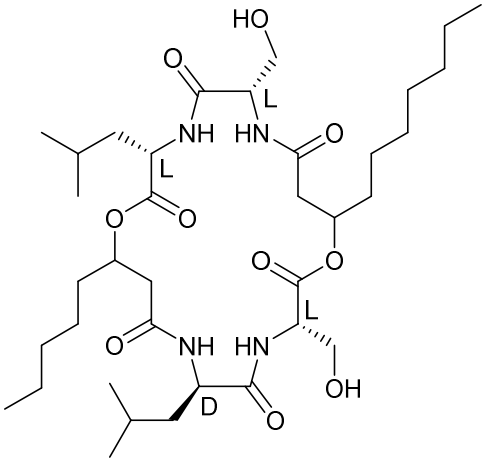


5a

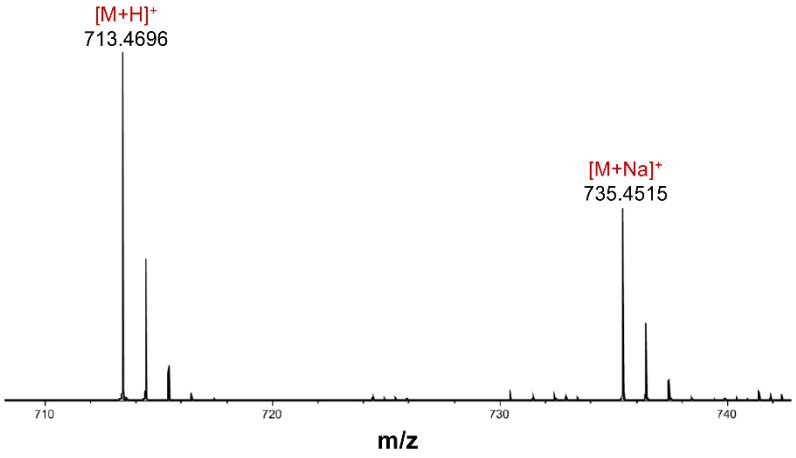
S-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (3*S*)-hydroxyodecanethioate (**5a**) was synthesised using the same procedure as for the synthesis of *S*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (3*R*)-hydroxyodecanethioate (**5**) using *S*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl) (3*S*)-hydroxyodecanethioate (**13a**) (38 mg, 0.1 mmol) to give the desired product as a colourless oil (21 mg, 0.1 mmol, 60 %). Spectroscopic data were consistent with those previously reported in the literature.[13]

δ_H (400 MHz; $CDCl_3$): 7.47 (1H, br t *J* = 6.0 Hz), 6.78 (1H, br t *J* = 6.0 Hz), 4.12-4.04 (1H, m), 3.97 (1H, s), 3.59-3.49 (2H, m), 3.48-3.41 (4H, m), 3.13-3.04 (1H, m), 3.04-2.95 (1H, m), 2.74-2.64 (2H, m), 2.41 (2H, t *J* = 6.0 Hz), 1.49-1.39 (2H, m), 1.33-1.21 (10H, m), 0.98 (3H, s), 0.91 (3H, s), 0.87 (3H, t *J* = 6.5 Hz). δ_C (100 MHz, $CDCl_3$): 199.4, 174.5, 172.4, 77.6, 70.8, 69.2, 53.4, 39.4, 39., 37.3, 35.8, 35.5, 31.9, 29.6, 29.3, 28.9, 25.6, 22.7, 21.6, 20.7, 14.1. MS (ES^+): *m/z* (%): 471 (100, $[M+Na]^+$)

3. Supplementary Figures

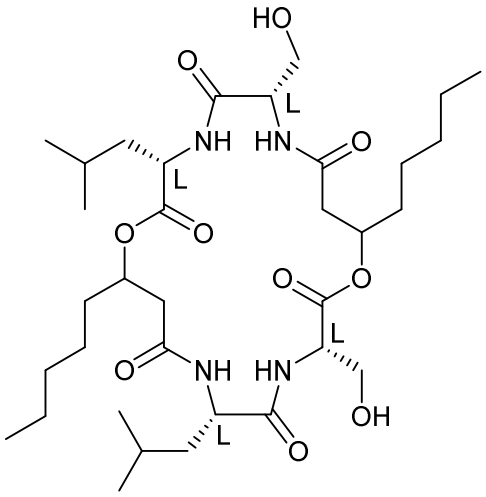


icosalide A1 (**1**)

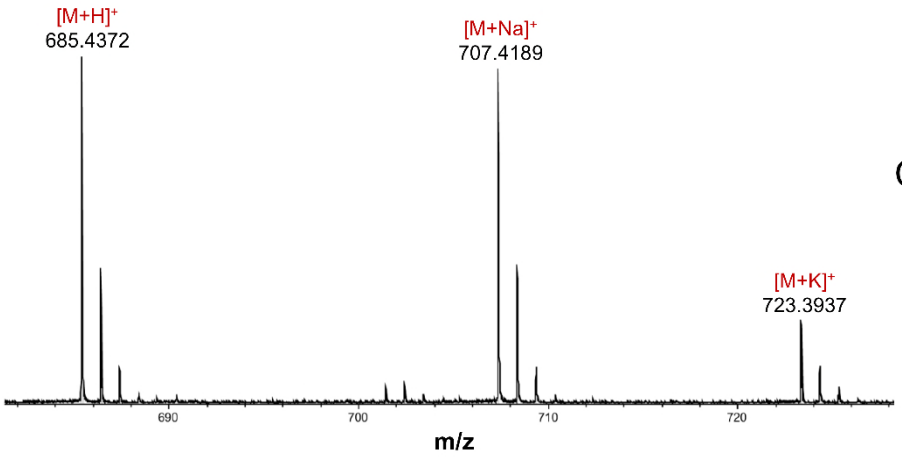


$C_{36}H_{64}N_4O_{10}$

Figure S1. High-resolution mass spectrometry analysis of icosalide A1. Calculated *m/z* values for icosalide A1 are: $[M+H]^+$ = 713.4701, and $[M+Na]^+$ = 735.4520. The molecular formula deduced from the *m/z* values of the detected ions is displayed, and is consistent with the molecular formula of icosalide A1.



icosalide B (**3**)



$C_{34}H_{60}N_4O_{10}$

Figure S2. High-resolution mass spectrometry analysis of the metabolite with a molecular formula corresponding to icosalide B. Calculated *m/z* values for icosalide B are: $[M+H]^+$ = 685.4388, $[M+Na]^+$ = 707.4207 and $[M+K]^+$ = 423.3947. The molecular formula deduced from the *m/z* values of the detected ions is displayed, and is consistent with the molecular formula of icosalide B.

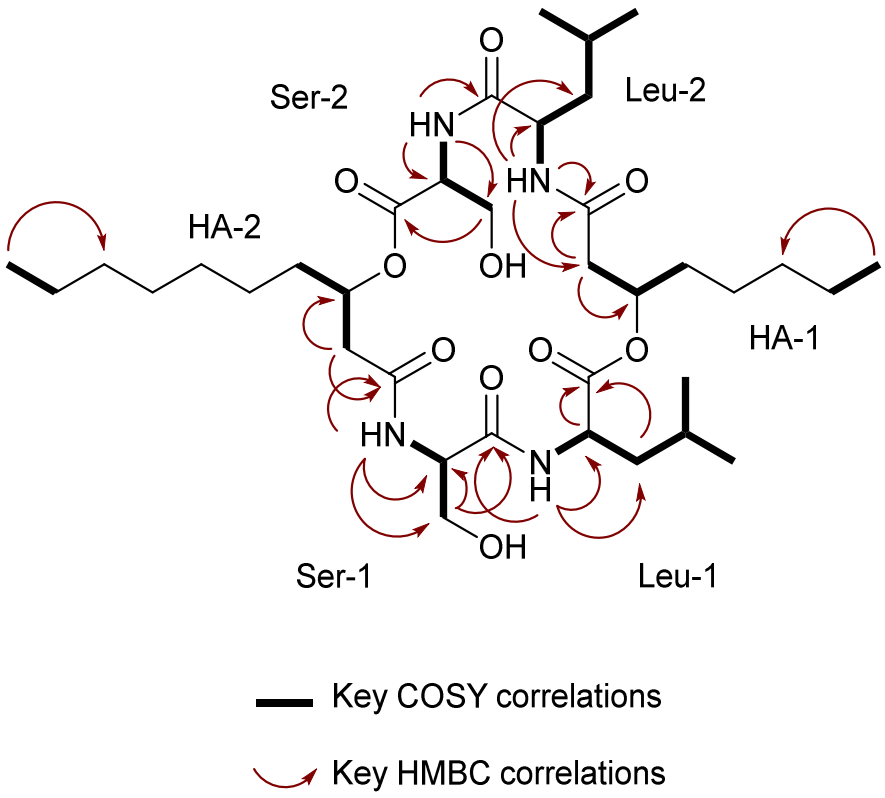


Figure S3. Structural analysis of icosalide A1 by NMR spectroscopy (d_6-acetone, ^{1}H 700 MHz, ^{13}C 175 MHz). Bold lines and arrows, respectively, indicate key COSY and HMBC correlations.

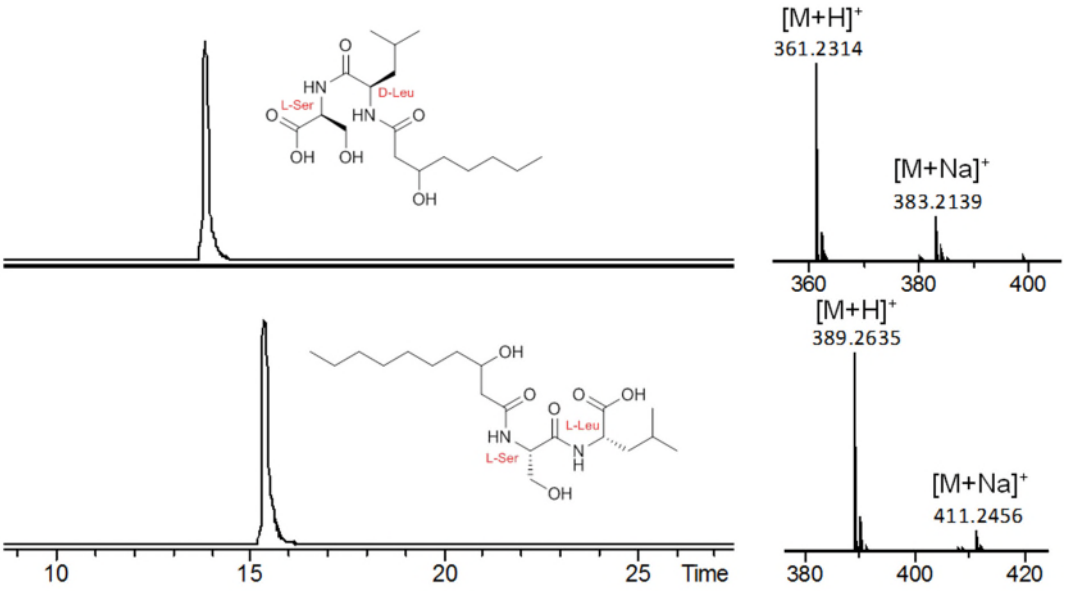


Figure S4. Purification of 3-hydroxyacyl-dipeptides following alkaline hydrolysis of icosalide A1. Reverse-phase C18 HPLC chromatograms showing separation of the two acyl-deipeptides resulting from alkaline hydrolysis of icosalide A1. The mass spectrum for each species is shown to the right of the chromatograms.

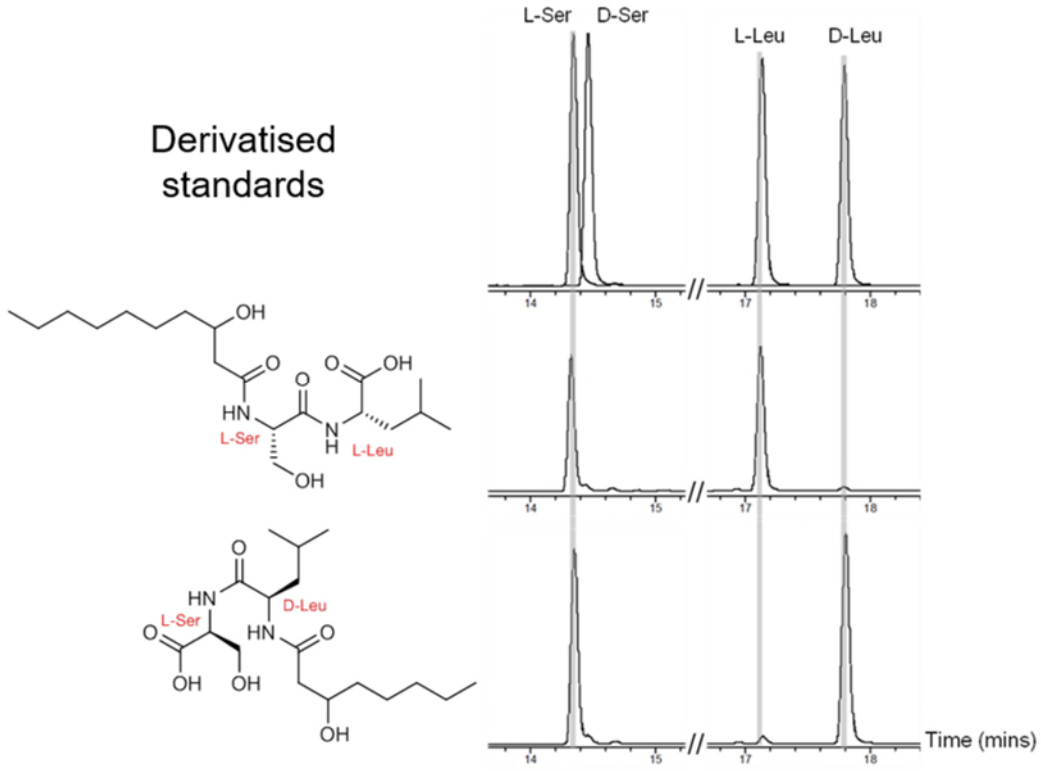


Figure S5. Stereochemical determination of amino acids in icosalide A1. Extracted ion chromatograms at *m/z* = 384.15 ± 0.02 (corresponding to $[M+H]^+$ for the Marfey's derivative of leucine - right), and *m/z* = 358.01 ± 0.02 (corresponding to $[M+H]^+$ for the Marfey's derivative of serine - left). The top chromatogram is for the derivatised standards of L/D-Leu and L/D-Ser. The bottom two chromatograms are for the derivatised hydrolysis products of the 3-hydroxyacyl-dipeptides shown to the left of the chromatograms.

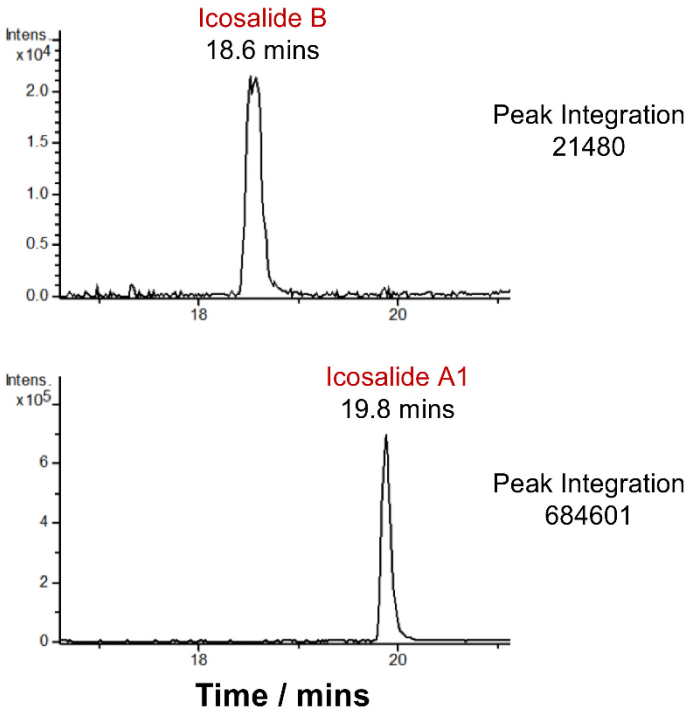


Figure S6. Relative production level of icosalide A1 and the species with molecular formula corresponding to icosalide B in *B. gladioli* BCC0238. Extracted ion chromatograms at *m/z* = 685.44 ± 0.02 (corresponding to the $[M+H]^+$ for icosalide B), and *m/z* = 713.47 ± 0.02 (corresponding to the $[M+H]^+$ for icoasalide A1). Peak integration values (shown on right) were used to calculate that 3% of the total icosalides produced have a molecular formula corresponding to icosalide B.

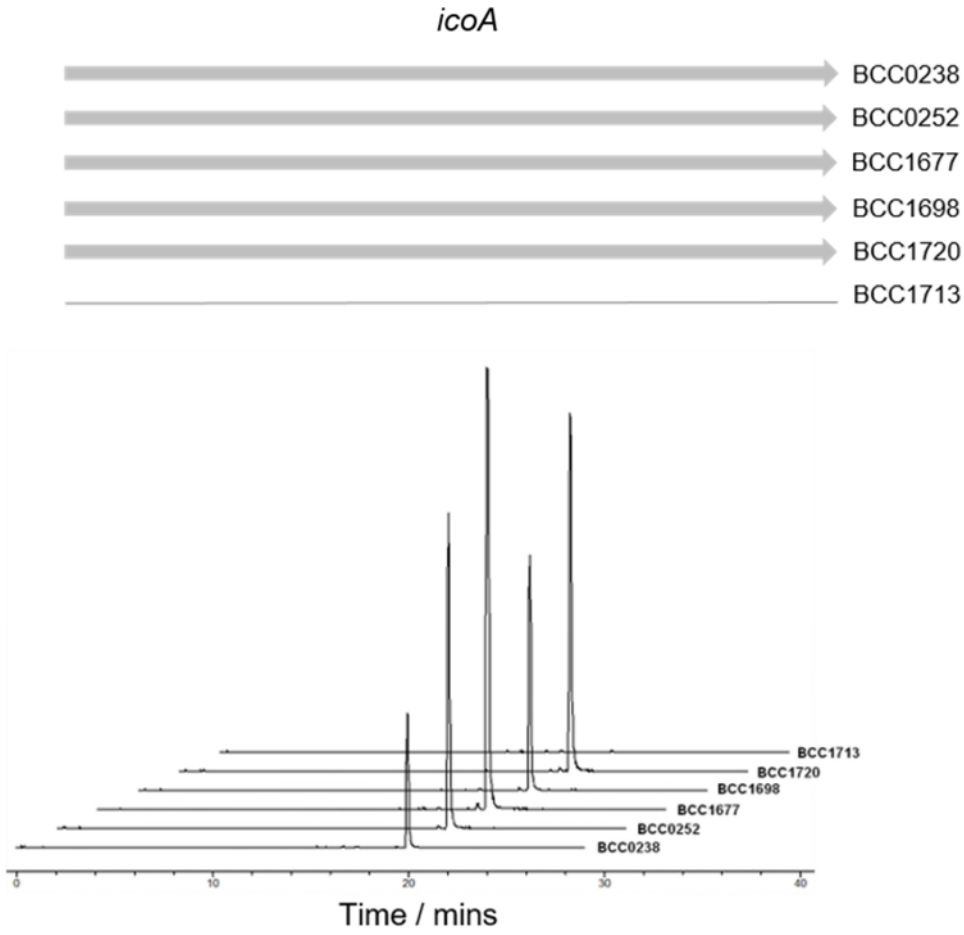


Figure S7. Production of icosalide A1 by genome sequenced *B. gladioli* strains. Extracted ion chromatograms at *m/z* = 713.47 ± 0.02 (corresponding to the $[M+H]^+$ for icoasalide A1) for extracts of *B. gladioli* strains BCC0238, BCC0252, BCC1677, BCC1698, BCC1720 and BCC1713. No icosalide A1 production was observed for BCC1713, because it does not possess the *icoA* gene.

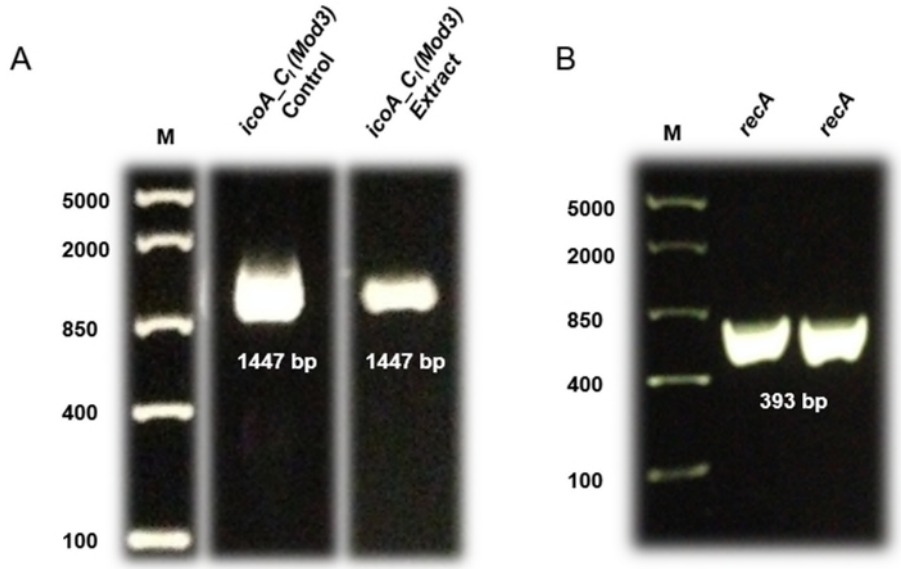


Figure S8. Results of PCR screens for the presence of *icoA* and *recA* genes in total gDNA extracts from *Aureobasidium* sp. MSX 59166. 1% agarose gel electrophoresis of PCR reactions with primers (**A**) designed to detect the *icoA* gene by amplification of a 1447 bp region encoding the fourth C-domain (IcoA C_I (Module 3)); and (**B**) designed to specifically amplify a 393 bp region of the *recA* gene from *Burkholderia* species. The *recA* PCR product was sequenced to obtained species-level information.

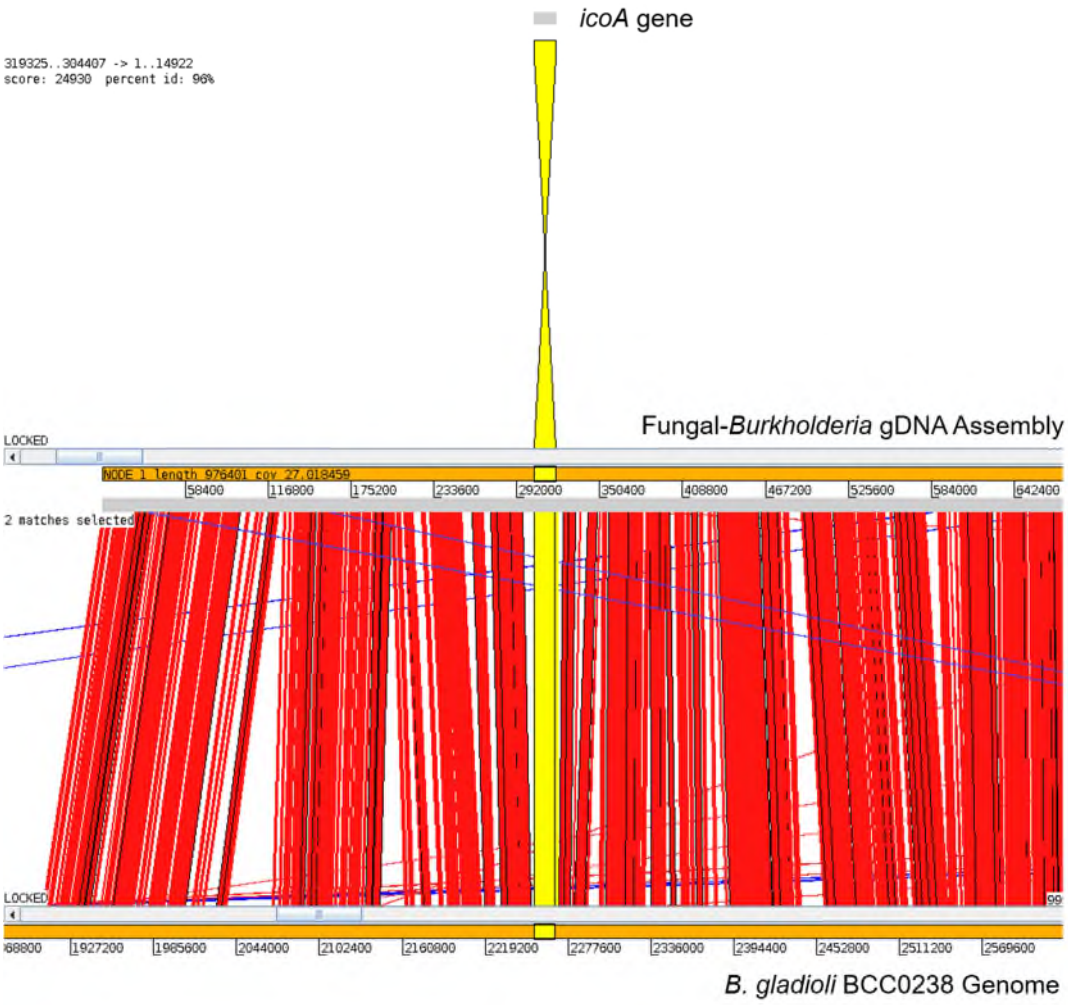


Figure S9. Comparison of *icoA* and surrounding genes from *B. galdioli* BCC0238 and fungal-associated *B. gladioli*. Gene comparison diagram between: *icoA* (top), ~1Mb sequencing assembly from fungal-associated *Burkholderia* gDNA (middle) and the *B. gladioli* BCC0238 genome (bottom). The *icoA* gene is highlighted in yellow, and the location of homologous genes either side of the *icoA* locus is highlighted by the red lines.

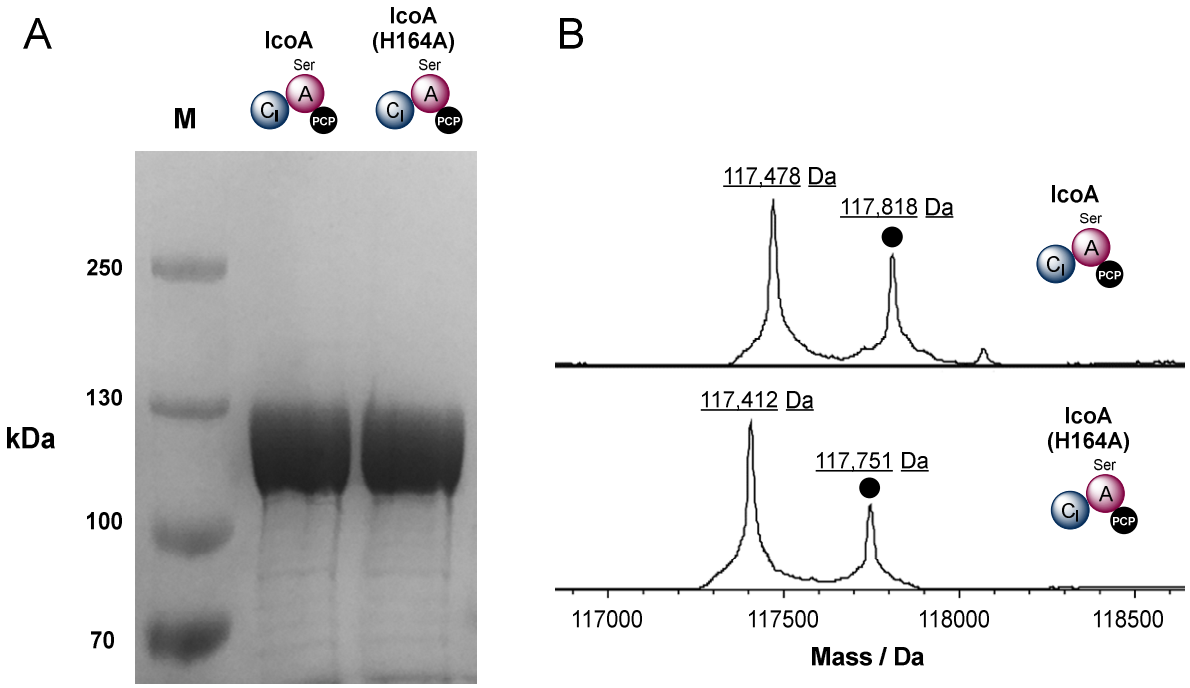


Figure S10. SDS-PAGE and mass spectrometry analysis of the purified recombinant C_I-A-PCP tri-domain construct from module 3 of IcoA. (**A**) 6% SDS-PAGE gel containing (from left to right): wild type IcoA C_I-A-PCP (Module 3) (119 kDa) and IcoA C_I-A-PCP(H164A) (Module 3) (119 kDa). (**B**) Deconvoluted mass spectra of wild type IcoA C_I-A-PCP (Module 3) (left) and IcoA C_I-A-PCP(H164A) (Module 3) (right) following His-Tag cleavage using thrombin. Measured masses are given for each protein. The signal marked ● is the *holo*-protein resulting from partial phosphopantetheinylation of the PCP domain by an *E. coli* phosphopantetheinyl transferase.

Table S1. Comparison of ^{1}H and ^{13}C NMR spectroscopic data observed for icosalide A1 isolated from *B. gladioli* BCC0238 (500 MHz, acetone-d_6) and *Aureobasidium* sp. (400 MHz, acetone-d_6)

	Icosalide A1 (*B. gladioli* BCC0238)		**Icosalide A1 (*Aureobasidium* sp. MSX 59166)**	
Position	**^{13}C (ppm)**	**^{1}H (ppm)**	**^{13}C (ppm)**	**^{1}H (ppm)**
Ser-1				
C=O	173.6		173.4	
α	55.3	4.72 (1H, m)	55.5	4.68 (1H, m)
β	63.6	3.74 (1H, dd, 11.1, 5.1) 3.65 (1H, dd, 11.1, 7.3)	63.4	3.73 (1H, dd, 11.0, 7.0) 3.66 (1H, dd, 11.0, 7.0)
NH		7.38 (1H, d, 8.7)		7.37 (1H, d, 8.6)
Leu-1				
C=O	173.3		173.0	
α	53.9	4.20 (1H, m)	53.8	4.21 (1H, m)
β	40.0	1.72 (2H, m)	40.0	1.72 (2H, m)
γ	25.5	1.73 (1H, m)	25.6	1.73 (1H, m)
γ-CH3	22.9	0.96 (3H, d, 6.0)	23.0	0.96 (3H, d, 6.5)
γ-CH3	22.2	1.02 (3H, d, 6.0)	22.3	1.02 (3H, d, 6.0)
NH		8.42 (1H, d, 4.7)		8.38 (1H, d, 4.4)
HA-1				
C=O	169.1		168.7	
2	41.4	2.77 (1H, dd, 14.0, 3.9) 2.30 (1H, dd, 5.0, 3.4)	41.4	2.77 (1H, dd, 14.0, 3.8) 2.29 (1H, dd, 14.0, 3.4)
3	73.2	4.99 (1H, m)	73.2	4.99 (1H, m)
4	32.6	1.64 (2H, m)	32.7	1.63 (2H, m)
5	26.0	1.39 (2H, m)	26.0	1.40 (2H, m)
6	32.4	1.28 (2H, m)	32.5	1.28 (2H, m)
7	23.3	1.27 (2H, m)	23.4	1.27 (2H, m)
8	14.4	0.88 (3H, t, 7.0)	14.5	0.87 (3H, t, 6.8)
Leu-2				
C=O	174.1		173.8	
α	52.9	4.60 (1H, ddd, 12.6, 9.3, 3.5)	52.8	4.60 (1H, ddd, 12.1, 9.3, 3.4)
β	42.0	1.79 (1H, ddd, 13.3, 13.0, 3.4) 1.55 (1H, m)	42.0	1.79 (1H, ddd, 13.3, 13.0, 3.4) 1.54 (1H, m)
γ	25.2	1.99 (1H, m)	25.3	2.00 (1H, m)
γ-CH3	24.2	0.98 (3H, d, 6.6)	24.3	0.98 (3H, d, 6.0)
γ-CH3	20.9	0.94 (3H, d, 6.5)	21.1	0.94 (3H, d, 6.5)
NH		7.99 (1H, d, 9.3)		7.97 (1H, d, 9.3)
Ser-2				
C=O	171.3		171.1	
α	55.1	4.50 (1H, ddd, 8.9, 2.6, 2.5)	55.1	4.49 (1H, ddd, 9.0, 2.7, 2.4)
β	64.3	4.16 (1H, dd, 10.4, 2.4) 3.85 (1H, dd, 10.4, 2.7)	64.1	4.14 (1H, dd, 10.3, 2.4) 3.84 (1H, dd, 10.4, 2.7)
NH		7.59 (1H, d, 8.9)		7.59 (1H, d, 9.0)
HA-2				
C=O	169.6		169.1	
2	41.5	2.64 (1H, dd, 14.0, 3.9) 2.27 (1H, dd, 4.9, 3.5)	41.5	2.63 (1H, dd, 13.7, 3.9) 2.25 (1H, dd, 13.7, 3.5)
3	72.6	5.07 (1H, m)	72.6	5.06 (1H, m)
4	32.9	1.55 (2H, m)	33.0	1.54 (2H, m)
5	26.6	1.26 (2H, m)	26.7	1.26 (2H, m)
6	30.2	1.28 (2H, m)	30.3	1.28 (2H, m)
7	30.0	1.28 (2H, m)	30.1	1.28 (2H, m)
8	32.1	1.28 (2H, m)	32.2	1.28 (2H, m)
9	23.1	1.32 (2H, m)	23.2	1.32 (2H, m)
10	14.2	0.89 (3H, t, 6.9)	14.3	0.89 (3H, t, 6.4)

Table S2. MIC values determined for icosalide A1 (**1**) against *C. albicans* and a range of Gram-positive and Gram-negative bacteria.

Strain	MIC (μg/mL)
Gram-negative Bacteria	
Klebsiella pneumonia DSM 26371	>32
Acinetobacter baumannii DSM25645	>32
Pseudomonas aeruginosa DSM29239	>32
Enterobacter cloacae DSM 16690	>32
Burholderia multivorans	>32
Escherichia coli SY327	>32
Gram-positive Bacteria	
Enterococcus faecium DSM25390	16
Staphylococcus aureus DSM21979	>32
Bacillus subtilis DSM10	>32
Fungi	
Candida albicans SC 5313	>32

Table S3. Predicted substrate specificities of adenylation domains of IcoA

A-domain	Residues in Binding Pocket	Prediction
A1	D-A-W-F-L-G-N-V	Leu
A2	D-V-W-H-I-S-L-I	Ser
A3	D-V-W-H-I-S-L-I	Ser
A4	D-A-W-F-L-G-N-V	Leu

Table S4. Elution conditions for HPLC purification of icosalide A1.

Time (mins)	Water / 0.1% FA (%)	MeCN / 0.1% FA (%)	Flow Rate (mL/min)
0.0	95	5	9.0
5.0	5	5	9.0
50.0	0	100	9.0
60.0	0	100	9.0
70.0	95	5	9.0

Table S4. Elution conditions for LC-MS analysis of amino acids derivatised with Marfey's reagent.

Time (mins)	Water / 0.1% FA (%)	MeCN / 0.1% FA (%)	Flow Rate (mL/min)
0.0	95	5	0.2
5.3	5	5	0.2
18.3	0	100	0.2
22.3	0	100	0.2
25.3	95	5	0.2
34.0	95	5	0.2

4. Protein Sequences

The sequence of the N-terminal fusion containing the His-Tag is coloured red. Calculated molecular weights for the proteins minus the N-terminal Met residue, which is removed during overproduction in *E. coli*, are given below the sequence.

4.1 IcoA C_I-A-PCP (Module 3) and IcoA C_I-A-PCP(H164A – mutated residue highlighted in yellow) (Module 3).

MGSSHHHHHHSSGLVPRGSHMDVSRLVDRPGSPAETWYALSPAQQAVWLQEQAARPGTSFFSVAAMRCAPEIDRM
RLVAASRALIAQNQAFWIQVSDAGLQCEASTPTTRFQHFVEPTTMTGEAMRRAVIEWHEKLNEDPRDKSGAVAVF
DSPGSVLVALRSPHVQNDGWSALRYFERIGRNYAALESDPARAFDMDRIFLDTLSLDERYLCSPTYERDAAFWQS
ACARIDGQPLVTLVADHAHPVDARGVVRSLRKVFPQTLQERVLNAARKLSLSPAECLTALTALYLMRVTGERSTV
LGVSFLNRTREALDIPGQFAKVIPLPVSIGQGDIPLSSTLNGIRDAFKDVMQHGRFPFGEMVRRYGFDPRHIEIS
VNTLFLRHPVEVGGQPAHVQWLSGPEHGLSFLFTQFGRSAPIDIELRYNGNAFDSESVERHARRLLDFIERACED
DSVSARGIELVSSEERALLIDALNATDAPYDRNQYLHGLFEAQAKRTPEATALIAGDERLSYAELDARANRFARY
LVDLGVGPDALVAVCLERSTAMVVSLIGILKAGGAYVPIDPAYPGPRIAHIVSDSAPAVVLVDATGREALVDALG
DEKLAEYGLIDVSAASTPWNKLSSDSLSSNALGLNPRHLAYVIYTSGSTGMPKGVQNEHDALVNRLTWMQEAYRL
GGQDVVLQKTPFSFDVSVWEFFWTLANGATLVIAEPGAHRDADYLTEIIAKHGVTTLHFVPSMLAGFLEAQDLTR
CKTLSRIICSGEALPAPIARRCLERLPHAQLHNLYGPTESAIDVTAFTCPPDFDAQAVPIGKPIANTRIYLLDER
QAPVPLGAIGELYIGGVGVARGYLNRADLTAQRFLADPFARAAGHPEARMYRTGDLARYLPDGNIVFLGRNDDQV
KIRGFRIELGEIEVQLAKHEAVRDAIVIARQDSTGNARLLAYVTPQESASREELARSLREHLTARLPEYMVPAAF
VVLETLPLTPNGKLDRRALPEPADDAFVQSRYEAPQGETEQAVAALWAELLGVERIGRHDNFFALGGHSLLAVRM
LNRLRAMQAGDLSLSSLFDHPTVSAVAQAIDSGSRSVA

MW (Wild Type): (**-His-Tag**): 117481 Da

MW (H164A): (**-His-Tag**): 117415 Da

4.2 Sfp (*Bacillus subtilis*) sequence

MGSSHHHHHHSSGLVPRGSHMKIYGIYMDRPLSQEENERFMSFISPEKQEKCRRFYHKEDAHRTLLGDVLVRSVI
SRQYQLDKADIRFSAQEYGKPCIPDLPDAHFNISHSGRWVICAFDSHPIGIDIEKMKPISLEIAKRFFSKTEYSD
LLAKNKDEQTDYFYHLWSMKESFIKQEGKGLSLPLDSFSVRLHQDGQVSIELPDSHTPCYIKTYEVDPGYKMAVC
AAHPDFPEDITMLSYEAL

MW: 28134 Da

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