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Supporting Information

# Total Synthesis and Structure Correction of the Cyclic Lipodepsipeptide Orfamide A

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## Table of Contents

## 1 List of Abbreviations

| | |
|---|---|
| A.A. | Amino acid |
| Alloc | Allyloxycarbonyl |
| BINAP | 2,2‘-Bis(diphenylphosphino)-1,1‘-binaphthalene |
| Bz | Benzoyl |
| DIC | *N, N‘*-Diisopropylcarbodiimide |
| DIEA | *N, N*-Diisopropylethylamine |
| DMAP | 4-Dimethylaminopyridine |
| DMF | *N, N*-Dimethylformamide |
| DMSO | Dimethyl sulfoxide |
| EDCI | *N*-Ethyl-*N‘*-(3-dimethylaminopropyl)-carbodiimide hydrochloride |
| eq. | Equivalent |
| F.A. | Fatty acid |
| Fmoc | 9-fluorenylmethyloxycarbonyl |
| HATU | 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5*b*]pyridinium3-oxide hexafluorophosphate |
| HBTU | *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate |
| HFIP | Hexafluoro-2-propanol |
| HOAt | 1-Hydroxy-7-azabenzotriazole |
| HOBt | 1-Hydroxybenzotriazole |
| SPPS | Solid phase peptide synthesis |
| TFA | Trifluoroacetic acid |
| THF | Tetrahydrofuran |

## 2 General Methods

### 2.1 Reagents and reaction conditions

All reagents were purchased from Acros Chemicals, Alfa Aesar, ABCR, Carbolution Chemicals, Carbosynth, Fischer Chemical, fluoroChem, GL Biochem (Shanghai), GRÜSSING, Manchester Organics, Merck, Novabiochem, Santa Cruz Biotechnology, Sigma-Aldrich, TCI Europe and VWR. All solvents, if not purchased in purity or dryness suitable, were distilled. THF was refluxed under $N_2$ atmosphere with Na and benzophenone. $CH_2Cl_2$ was refluxed under $N_2$ atmosphere with $CaH_2$. DMF and MeOH (HPLC grade) were stored with 3Å or 4Å molecular sieves at least for 24 h prior to use. All solvents for flash chromatography were distilled prior to use. If necessary, solvents were degassed by purging with $N_2$ or Ar for minimum 15 minutes. Deionized water was used for all experiments. All reactions were performed under protective atmosphere ($N_2$ or Ar) if not stated otherwise.

### 2.2 Thin Layer Chromatography (TLC)

Merck precoated silica gel plates (60 $F_{254}$) was used for TLC. Compounds were visualized using ultraviolet light irradiation at 254 nm and/or by using the following staining agent (dip, dry & heat development).

Phosphomolybdic acid (PMA): $12MoO_3 \cdot H_3PO_4$ (5 g) in EtOH (100 mL).

### 2.3 Silica Gel Flash Liquid Chromatography

Purifications were performed using silica gel from MACHEREY-NAGEL (particle size 40-60 µm) under approximately 0.2-0.4 bar pressure.

### 2.4 NMR spectroscopy

$^{1}H$- and $^{13}C$-NMR spectra were recorded using Bruker Advance I 250, Fourier 300, Advance III 400, Advance III HD 500 or Advance III 600 system. Spectra were calibrated to appropriate residual solvent peaks (chloroform-d, methanol-$d_4$, DMSO-$d_6$).[1]

### 2.5 High Performance Liquid Chromatography (HPLC)

#### 2.5.1 Analytical Reverse Phase HPLC (RP-HPLC)

Analyses were performed on a SHIMADZU system consisting of a system controller (SLC- 10A VP), a column oven (CTO-10AC VP), an auto-injector (SIL-10ADVP), a degasser (DGU- 14A), three pumps (LC-10AT VP), a diode array detector (SPD-M20A), and an analytical column (MACHEREY-NAGEL NUCLEODUR C18 Gravity, 5 µm, 125 × 4 mm). The column was equilibrated to starting condition of each method prior to sample injections.

Eluent System: A = acetonitrile, B = water, C = 2% TFA in water, Flow rate: 1 mL/min, Column oven: 25 °C, Detection: diode array 190-800 nm.

Condition A (RP-A)

Gradient: Eluent A: 10% (1 min), 10-95% (10 min), 95% (5 min), 95-10% (0.2 min), 10% (5.8 min). Eluent C: 5% (22 min).

Condition B (RP-B)

Gradient: Eluent A: 30% (1 min), 30-95% (10 min), 95% (5 min), 95-30% (0.2 min), 30% (5.8 min). Eluent C: 5% (22 min).

Condition C (RP-C)

Gradient: Eluent A: 50% (1 min), 50-95% (9 min), 95% (5 min), 95-50% (0.2 min), 50% (5.8 min). Eluent C: 5% (22 min).

Condition D (RP-D)

Gradient: Eluent A: 70% (1 min), 70-95% (10 min), 95% (5 min), 95-70% (0.2 min), 70% (5.8 min). Eluent C: 5% (22 min).

### 2.5.2 Analytical Normal Phase HPLC (NP-HPLC)

Analyses were performed on a SHIMADZU system consisting of a communications bus module (CBM-20A), a column oven (CTO-10AC VP), an auto sampler (SIL-20A HT), a degasser (DGU- 14A), a pump (LC-20AD), a diode array detector (SPD-M20A), an UV-VIS detector (SPD-10A VP). The column was equilibrated to starting condition of each method prior to sample injections.

Eluent System: A = 10% *i*-PrOH/*n*-hexane, B = *n*-hexane, C = *i*-PrOH.

Column oven: 25 °C.

Condition A (NP-A)

Column : Phenomenex Lux 5 µm Amylose-1, LC Column 250 × 4.6 mm.

Gradient: 30% A, 70% B, 0% C for 20 min. (isocratic 3% *i*-PrOH/*n*-hexane)

Flow rate: 0.7 mL/min.

Detection: Diode array 190-800 nm.

Condition B (NP-B)

Column : Daicel chemical industries CHIRALPAK AD, 10 µm, 250 × 4.6 mm.

Gradient: 0% A, 70% B, 30% C for 30 min. (isocratic 30% *i*-PrOH/*n*-hexane)

Flow rate: 1 mL/min.

Detection: UV-VIS 220 nm.

### 2.5.3 Reverse Phase Preparative HPLC (RP-Prep. HPLC)

Purifications of final compounds were performed on a SHIMADZU system consisting of a system controller (SLC- 10A VP), two pumps (LC-8A), a UV-VIS detector (SPD-10AVP) and a fraction collector (FRC-10A). Columns were equilibrated to starting conditions of each method prior to sample injections.

Eluent System: A = 0.1% TFA in acetonitrile, B= 0.1% TFA in water, Detection: UV-VIS 220 nm.

Condition A (Prep.-A)

Column : MACHEREY-NAGEL NUCLEODUR C18 Gravity, 5 µm, 250 × 16 mm.

Gradient: Eluent A: 80-85% (5 min), 85-90% (20 min), 90-95% (5 min), 95% (15min), 95-80% (10 min), 80% (12 min).

Flow rate: 10 mL/min.

Condition B (Prep.-B)

Column : MACHEREY-NAGEL NUCLEODUR C18 Gravity, 5 µm, 250 × 16 mm.

Gradient: Eluent A: 85-95% (20 min), 95% (17 min), 95-85% (10 min), 85% (10 min).

Flow rate: 10 mL/min.

Condition C (Prep.-C)

Column : MACHEREY-NAGEL NUCLEODUR C18 Gravity, 5 µm, 250 × 16 mm.

Gradient: Eluent A: 90-95% (35 min), 95% (10 min), 95-90% (2 min), 90% (20 min).

Flow rate: 10 mL/min.

Condition D (Prep.-D)

Column : KNAUER Eurospher 100-5 C8, 250 × 32 mm.

Gradient: Eluent A: 70-95% (35 min), 95% (10 min), 95-70% (2 min), 70% (10 min).

Flow rate: 25 mL/min.

### 2.6 Mass Spectrometry

#### 2.6.1 Liquid Chromatography Mass spectrometry (LC/MS)

Analyses were performed on a SHIMADZU system consisting of a system controller (SLC- 10A VP), a column oven (CTO-10AC VP), an auto-injector (SIL-10AD VP), a degasser (DGU- 14A), two pumps (LC-10AT VP), an analytical column (MACHEREY-NAGEL NUCLEODUR C18 Isis, 3 µm), a post-column flow splitter (Thermo scientific, ICP-04-20), a UV-VIS detector (SPD-10A VP) and a MS spectrometer (Finnigan LCQ spectrometer). The column was equilibrated to starting conditions of each method prior to the sample injections.

Eluent System: A = 0.1% HCOOH in acetonitrile, B= 0.1% HCOOH in water, Flow rate: 1 mL/min, Detection: UV/VIS 220 or 254 nm, Column oven: 25 °C.

Condition A (LC/MS-A)

Gradient: Eluent A: 50-100% (10 min), 100% (5 min), 100-50% (0.2 min), 50% (4.8 min).

LCQ Method: Positive mode, MS range 100-2000 (to MS source 2.5-14.8 min).

Condition B (LC/MS-B)

Gradient: Eluent A: 50-100% (10 min), 100% (10 min), 100-50% (0.2 min),

50% (4.8 min).

LCQ Method: Positive mode, MS range 100-2000 (to MS source 2.54-19.55 min).

Condition C (LC/MS-C)

Gradient: Eluent A: 70% (1 min), 70-100% (9 min), 100% (10 min), 100-70% (0.2 min), 70% (4.8 min).

LCQ Method: Positive mode, MS range 100-2000 (to MS source 2.5-19.8 min).

### 2.6.2 Liquid Chromatography High Resolution Mass spectrometry (LC/HRMS)

Analyses were performed on a Thermo scientific UltiMate 3000 UHPLC system consisting of a pump, an auto sampler, a column compartment, a diode array detector and a high resolution Q-TOF MS spectrometer (BRUKER DALTRONICS, maXis Impact). The column was equilibrated to starting conditions of each method prior to the sample injections.

Eluent System: A = 0.1% HCOOH in acetonitrile, B= 0.1% HCOOH in water, Flow rate: 0.5 mL/min, Detection: Diode array 200 - 400 nm.

Condition A (LC/HRMS.-A) (Figure 2)

Column : MACHEREY-NAGEL NUCLEODUR 300-5 C4 ec, 5 µm, 150 × 3 mm.

Gradient: Eluent A: 10% (1 min), 10-95% (7 min), 95% (5 min), 95-10% (0.5 min), 10% (4.5 min).

Mass Method: Positive mode, MS range 100-3000 (to MS source from 3 min).

Condition B (LC/HRMS.-B) (Figure 4)

Column : Phenomenex Kinetex 2.6u C18 100A, 100 × 2.1 mm.

Gradient: Eluent A: 65% (7 min), 65-95% (3 min), 95% (5 min), 95-65% (0.5 min), 65% (4.5 min).

Mass Method: Positive mode, MS range 100-3000 (to MS source from 3 min).

### 2.7 Specific optical rotation

Optical rotations were recorded in a Jasco P-2000 polarimeter at 589 nm. Path length of cuvettes was d = 10 mm. Concentrations (c) are given in g/100 mL.

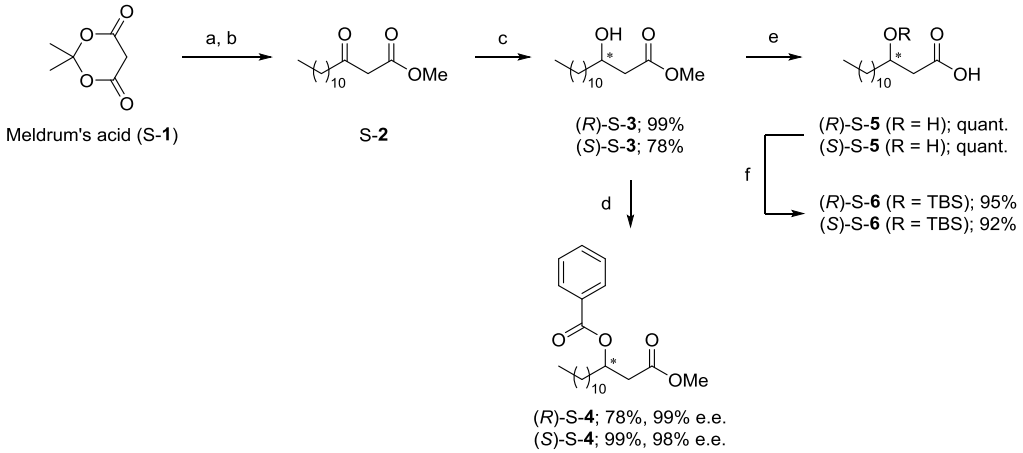
### 2.8 Fourier Transform Infrared Spectroscopy (FT-IR)

IR spectra were measured on SHIMADZU IRAffinity-1 filled with an ATR unit. Spectra were analyzed on ACD/Spectrus Processor 2018.2.3. Wavelengths are given in $cm^{-1}$. The following symbols show the abbreviations of relative intensities of absorptions: s (strong), m (medium), w (weak).

### 2.9 Melting points

Melting points were measured with Büchi B-545 melting point apparatus and one-side open capillaries were used.

## 3 Synthetic Procedures and Compound Characterization Data



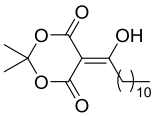

Reagents and conditions: a) lauroyl chloride, pyridine, $CH_2Cl_2$, 0 °C to rt; b) MeOH, reflux, 78% (over 2 steps from Meldrum's acid); c) $(COD)Ru(2\text{-methylallyl})_2$, (*R*) or (*S*)-BINAP, 48% HBr in MeOH, acetone, rt, 30 min; S-**2** in MeOH, $H_2$, 55 °C; d) BzCl, pyridine, $CH_2Cl_2$, 0 °C to rt; e) NaOH aq., THF, 0 °C to rt, quant.; f) i) TBSCl, imidazole, DMF, 0 °C to rt; ii) $K_2CO_3$, $H_2O$, MeOH, THF, 0 °C.

**Scheme S1.** Enantioselective synthesis of *β*-hydroxy tetradecanoic acid.

### 3.1 Synthesis of Building Blocks

Both enantiomers of 3-hydroxytetradecanoic acid (S-**6**) were prepared in the same procedures as published previously.[2, 3]

#### 3.1.1 5-(1-hydroxydodecylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (S-7)



S-**7**

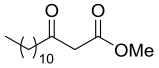
If necessary, Meldrum's acid (S-**1**) was recrystallized from acetone/$H_2O$ (1:2).[4] To Meldrum's acid (S-**1**) (0.971 g, 6.74 mmol, 1.0 eq.) in $CH_2Cl_2$ (3.2 mL) was added pyridine (1.3 mL, 16.1 mmol, 2.4 eq.) at 0 °C dropwise. The mixture was stirred for 20 min and then lauroyl chloride (1.77 g, 8.09 mmol, 1.2 eq.) in $CH_2Cl_2$ (2.4 mL) was added dropwise. The mixture was warmed up to room temperature gradually and stirred for 18 h. The reaction mixture was poured onto 2 M HCl (20 mL) and the organic layers were separated. The aqueous phase was extracted with $CH_2Cl_2$ (2 × 60 mL). The combined organic layers were washed with 2 M HCl (2 × 20 mL), $H_2O$ (1 × 20 mL) and brine (1 × 20 mL), dried over $MgSO_4$ and evaporated to dryness after filtration to give an orange oil. The obtained compound was used for the next reaction without any further purification.

**TLC**: $R_f$ = 0.23 (petroleum ether/EtOAc = 1:1).

**$^1$H-NMR** ($CDCl_3$, 250 MHz, 297 K): $\delta$ = 0.88 (t, $J$ = 6.5 Hz, 3 H), 1.26 - 1.49 (m, 20 H), 1.70 (m, 2 H), 1.73 (s, 6 H), 3.06 (t, $J$ = 7.6 Hz, 2 H), 15.29 (s, 1 H) ppm.

**$^{13}$C-NMR** ($CDCl_3$, 126 MHz, 297 K): $\delta$ = 14.3, 22.8, 26.3, 27.0, 29.4, 29.5, 29.5, 29.6, 29.7, 32.0, 35.9, 91.4, 104.9, 160.4, 170.7, 198.5 ppm.

**Methyl 3-oxo-tetradecanoate (S-2)**



S-**2**

5-(1-hydroxydodecylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (S-**7**) (2.46 g) in dry MeOH (8.1 mL) was warmed up to reflux for 3h while stirring. The mixture was evaporated to remove solvents and the residue was purified by silica gel column chromatography (PE/EtOAc = 25: 1) to give Methyl 3-oxodtetraecanoate (S-**2**) as a colorless solid (1.34 g, 5.23 mmol, 78% over 2 steps from Meldrum's acid).

**TLC**: $R_f$ = 0.18 (petroleum ether/EtOAc = 25:1).

**$^1$H-NMR** ($CDCl_3$, 250 MHz, 297 K): $\delta$ = 0.87 (t, $J$ = 6.5 Hz, 3 H), 1.25 (m, 16 H), 1.58 (m, 2 H), 2.52 (t, $J$ = 7.3 Hz, 2 H), 3.44 (s, 2 H), 3.74 (s, 3 H) ppm.

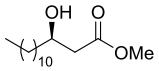
**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = 14.3, 22.8, 23.6, 29.1, 29.5, 29.5, 29.6, 29.7, 32.0, 43.2, 49.2, 52.5, 167.8, 203.0 ppm.

**HRMS** (ESI-TOF) calculated for $C_{15}H_{28}O_3$ $[M+H]^+$ 257.2111; found 257.2118.

**IR** (ATR): $\tilde{\nu}$ = 2955 (m), 2920 (s), 2851 (s), 1748 (s), 1713 (s), 1470 (w), 1408 (w), 1315 (w), 1258 (w), 1234 (w), 1161 (w) cm $^{-1}$.

**Melting Point**: $T_m$ = 30.5 °C.

### 3.1.2 Methyl (*R*)-3-hydroxytertradecanoate ((*R*)-S-3)



(*R*)-S-**3**

To a mixture of bis(2-methylallyl)(1,5-cyclooctadiene)ruthenium(II) (34.9 mg, 0.109 mmol, 0.02 eq.) and (*R*)-BINAP (81.6 mg, 0.131 mmol, 0.024 eq.) in dry acetone (5.7 mL) was added Methanoic HBr (1.54 mL of 180 µL of 48% HBr in dry MeOH 9 mL) at room temperature under argon. The mixture was stirred for 30 min and the solvent was carefully removed under high vacuum. Methyl 3-oxotetradecanoate (S-**2**) (1.40 g, 5.46 mmol, 1.0 eq.) in degassed MeOH (11.5 mL) was added by cannula and hydrogen gas was bubbled to the reaction mixture for 5 min. then the reaction mixture was heated up to 55 °C and stirred for 6h. The reaction was quenched by argon gas bubbling and the mixture was filtered through a pad of celite. The filtrate was evaporated and the remaining crude mixture was purified by flash column chromatography (160 mL silica, petroleum ether/EtOAc = 8:1) to give methyl (*R*)-3-hydroxytetradecanoate ((*R*)-S-**3**) as a colorless solid (1.40 g, 5.42 mmol, 99%).

**TLC**: $R_f$ = 0.27 (petroleum ether/EtOAc = 6:1).

**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.88 (t, $J$ = 7.1 Hz, 3 H), 1.23 -1.35 (m, 17 H), 1.43 (m, 2 H), 1.52 (m, 1 H), 2.41 (dd, $J$ = 16.5, 9.2 Hz, 1 H), 2.51 (dd, $J$ = 16.5, 3.0 Hz, 1 H), 2.84 (d, $J$ = 3.7 Hz, 1 H), 3.71 (s, 3 H), 4.00 (m, 1 H) ppm.

**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = 14.3, 22.8, 25.6, 29.5, 29.7, 29.7, 29.7, 29.8, 29.8, 32.1, 36.7, 41.2, 51.9, 68.2, 173.7 ppm.

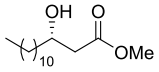
**HRMS** (ESI-TOF) calculated for $C_{15}H_{30}O_3$ 259.2268; found 259.2272.

$[\alpha]_D$ = -15.6 ($CHCl_3$, c = 1.0, 23.8 °C).

**IR** (ATR): $\tilde{\nu}$ = 3387 (w), 3306 (w), 2955 (m), 2916 (s), 2847 (s), 1740 (m), 1694 (m), 1466 (w), 1439 (w), 1304 (w), 1173 (m), 1096 (w), 1076 (w), 756 (m) cm $^{-1}$.

**Melting Point**: $T_m$ = 41.7 °C.

### 3.1.3 Methyl (*S*)-3-hydroxytertradecanoate ((*S*)-S-3)




(*S*)-S-**3**

(*S*)-S-**3** was prepared in the same procedure as (*R*)-S-**3** from β-keto ester (S-**2**) (510 mg, 1.97 mmol) and Methyl (*S*)-3-hydroxytertradecanoate ((*S*)-S-**3**) was obtained as a colorless solid (785 mg, 3.04 mmol, 78%).

**TLC**: $R_f$ = 0.27 (petroleum ether/EtOAc = 6:1).

**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.87 (t, $J$ = 7.1 Hz, 3 H), 1.23 - 1.35 (m, 17 H), 1.43 (m, 2 H), 1.52 (m, 1 H), 2.41 (dd, $J$ = 16.5, 9.2 Hz, 1 H), 2.51 (dd, $J$ = 16.4, 3.1 Hz, 1 H), 2.85 (d, $J$ = 3.5 Hz, 1 H), 3.71 (s, 3 H), 4.00 (m, 1 H) ppm.

**$^{13}$C-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = 14.3, 22.8, 25.6, 29.5, 29.7, 29.7, 29.7, 29.8, 29.8, 32.1, 36.7, 41.2, 51.9, 68.2, 173.7 ppm.

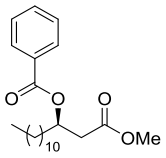
**HRMS** (ESI-TOF) calculated for $C_{15}H_{30}O_3$ $[M+Na]^+$ 281.2087; found 281.2089.

$[\alpha]_D$ = 15.4 ($CHCl_3$, c = 1.0, 23.6 °C).

**IR** (ATR): $\tilde{\nu}$ = 3387 (w), 3306 (w), 2955 (m), 2916 (s), 2847 (s), 1736 (m), 1694 (m), 1462 (w), 1439 (w), 1304 (w), 1173 (w), 733 (w) cm $^{-1}$.

**Melting Point**: $T_m$ = 42.6 °C.

### 3.1.4 Methyl (*R*)-3-benzoyloxytertradecanoate ((*R*)-S-4)




(*R*)-S-**4**

To a solution of β-hydroxy ester ((*R*)-S-**4**) (20.0 mg, 77.4 µmol, 1 eq.) in $CH_2Cl_2$ (0.4 mL) were added pyridine (31.3 µL, 387 µmol, mol, 5 eq.) and benzoyl chloride (18 µL, 155 µmol, 2 eq.) at 0 °C. The ice bath was removed and the mixture was stirred at room temperature. After stirring for 5 h, additional benzoyl chloride (9 µL, 77.5 µmol, 1 eq.) was added to the reaction mixture and stirred for 10 min. The reaction mixture was washed with saturated $NaHCO_3$, 1 M HCl (aq.) and brine, dried over $MgSO_4$ and filtered. The filtrate was evaporated and the remaining crude mixture was purified by flash column chromatography (36 mL silica, petroleum ether/EtOAc = 25:1) to give methyl (*R*)-3-benzoyloxytetradecanoate ((*R*)-S-**4**) as a colorless oil (22.0 mg, 60.7 mmol, 78%).

**TLC**: $R_f$ = 0.64 (petroleum ether/EtOAc = 6:1).

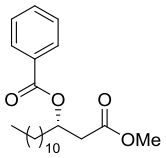
**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.87 (t, *J* = 7.1 Hz, 3 H), 1.24 - 1.44 (m, 18 H), 1.69 - 1.81 (m, 2 H), 2.67 (dd, *J* = 15.3, 5.5 Hz, 1 H), 2.76 (dd, *J* = 15.3, 7.4 Hz, 1 H), 3.66 (s, 3 H), 5.46 (tt, *J* = 7.4, 5.5 Hz, 1H), 7.43 (m, 2 H), 7.55 (m, 1 H), 8.02 (m, 2 H) ppm.

**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 126 MHz, 297 K): $\delta$ = 14.3, 22.8, 25.3, 29.5, 29.5, 29.6, 29.7, 29.8, 32.0, 34.3, 39.4, 52.0, 71.4, 128.5, 129.8, 130.5, 133.1, 166.0, 171.1 ppm.

**HRMS** (ESI-TOF) calculated for $C_{22}H_{34}O_4$ $[M+H]^+$ 363.2530; found 363.2532.

**IR** (ATR): $\tilde{\nu}$ = 2924 (s), 2855 (s), 1744 (s), 1721 (s), 1450 (w), 1439 (w), 1315 (w), 1269 (s), 1200 (w), 1173 (m), 1111 (m), 1069 (w), 1026 (w), 710 (m) cm $^{-1}$.

### 3.1.5 Methyl (*S*)-3-benzoyloxytertradecanoate ((*S*)-S-4)




(*S*)-S-**4**

To a solution of β-hydroxy ester ((*S*)-S-**3**) (20.0 mg, 77.4 µmol, 1 eq.) in $CH_2Cl_2$ (0.4 mL) was added pyridine (31.3 µL, 387 µmol, mol, 5 eq.) and benzoyl chloride (27 µL, 233 µmol, 3 eq.) at 0 °C. The ice bath was removed and the mixture was stirred at room temperature for 5 h. The reaction mixture was washed with saturated $NaHCO_3$, 1 M HCl (aq.) and brine, dried over $MgSO_4$ and filtered. The filtrate was evaporated and the residue was purified by flash column chromatography (36 mL silica, petroleum ether/EtOAc = 25:1) to give methyl (*S*)-3-benzoyloxytetradecanoate ((*S*)-S-**4**) as a colorless oil (28.0 mg, 60.7 mmol, 99%).

**TLC**: $R_f$ = 0.66 (petroleum ether/EtOAc = 6:1).

**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.87 (t, $J$ = 7.1 Hz, 3 H), 1.24 - 1.44 (m, 18 H), 1.69 - 1.81 (m, 2 H), 2.67 (dd, $J$ = 15.3, 5.5 Hz, 1 H), 2.76 (dd, $J$ = 15.3, 7.4 Hz, 1 H), 3.66 (s, 3 H), 5.46 (tt, $J$=7.4, 5.4 Hz, 1 H), 7.43 (m, 2 H), 7.55 (m, 1 H), 8.02 (m, 2 H) ppm.

**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = 14.3, 22.8, 25.3, 29.5, 29.5, 29.6, 29.7, 29.8, 32.0, 34.3, 39.4, 51.9, 71.4, 128.5, 129.8, 130.5, 133.1, 166.0, 171.0 ppm.

**HRMS** (ESI-TOF) calculated for $C_{22}H_{34}O_4$ $[M+H]^+$ 363.2530; found 363.2535.

**IR** (ATR): $\tilde{\nu}$ = 2924 (s), 2855 (s), 1744 (s), 1721 (s), 1450 (w), 1439(w), 1315 (w), 1273 (s), 1200 (w), 1173 (m), 1111 (m), 1069 (w), 1026(w), 710 (m) cm $^{-1}$.

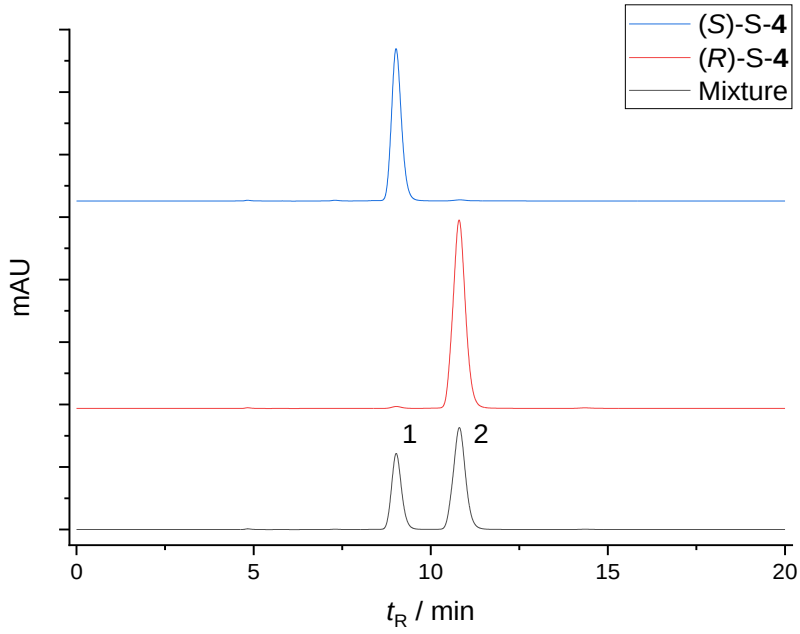
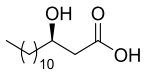



**Figure S1.** HPLC traces on chiral phase of (*R*)-S-**4** and (*S*)-S-**4**.
(Condition: NP-A, 220 nm); peak 1: $t_R$ = 9.02 min.; peak 2: $t_R$ = 10.8 min.

**Table S1.** Enantiomeric excess based on HPLC data.

| Compound Nr. | Peak 1[a] area (%) | Peak 2[b] area (%) | e.e. (%) |
|---|---|---|---|
| (*R*)-S-**4** | 0.741 | 99.26 | 98.5 |
| (*S*)-S-**4** | 99.18 | 0.822 | 98.4 |

### 3.1.6 (*R*)-3-hydroxytertradecanoic acid ((*R*)-S-5)




(*R*)-S-**5**

To a solution of β-hydroxy ester ((*R*)-S-**3**) (900 mg, 3.48 mmol, 1 eq.) in THF (7.7 mL) was added 1 M NaOH (aq.) (7.7 mL, 7.70 mmol, 2.2 eq.) at 0 °C. After stirring for 30 min, the ice bath was removed and the mixture was stirred at room temperature for another 30 min. The mixture was acidified with 1M HCl (aq.) at 0 °C and extracted with EtOAc (3 × 60 mL). The combined organic layers were washed with $H_2O$ (1 × 30 mL) and brine (1 × 30 mL), dried over $MgSO_4$ and filtered. The filtrate was evaporated to give the β-hydroxy acid (*R*)-S-**5** as colorless powder (850 mg, 3.48 mmol, quant.).

**$^1$H-NMR** ($CDCl_3$, 400 MHz, 297 K): $\delta$ = 0.87 (t, *J* = 6.7 Hz, 3 H), 1.19 - 1.37 (m, 17 H), 1.37-1.59 (m, 3 H), 2.47 (dd, *J* = 16.6, 9.0 Hz, 1 H), 2.57 (dd, *J* = 16.6, 3.1 Hz, 1 H), 4.03 (m, 1 H) ppm.

**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 101 MHz, 297 K): $\delta$ = 14.3, 22.8, 25.6, 29.5, 29.6, 29.7, 29.7, 29.8, 29.8, 32.1, 36.6, 41.2, 68.2, 178.2ppm.

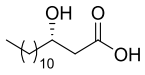
**HRMS** (ESI-TOF) calculated for $C_{14}H_{23}O_3$ $[M+H]^+$ 245.2111; found 245.2119.

$[\alpha]_D$ = –14.3 ($CHCl_3$, c = 1.0, 23.6 °C).

IR (ATR): $\tilde{\nu}$ = 3557 (w), 3534 (w), 3028 (w), 2955 (m), 2916, 2847, 1713 (w), 1678 (m), 1470 (w), 1296 (w), 1281 (w), 1227 (w) cm $^{-1}$.

**Melting Point**: $T_m$ = 73.2 °C.

### 3.1.7 (*S*)-3-hydroxytertradecanoic acid ((*S*)-S-5)




(*S*)-S-**5**

(*S*)-S-**5** was prepared in the same procedure as (*R*)-S-**5** from β-hydroxy ester ((*S*)-S-**3**) (510 mg, 1.97 mmol) and was obtained as colorless powder (482 mg, 1.47 mmol, quant.).

**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.88 (t, *J* = 7.0 Hz, 3 H), 1.19 - 1.38 (m, 17 H), 1.45 (m, 2 H), 1.55 (m, 1 H), 2.48 (dd, *J* = 16.6, 9.0 Hz, 1 H), 2.57 (dd, *J* = 16.6, 3.1 Hz, 1 H), 4.03 (m, 1 H) ppm.

**$^{13}C\{^1H\}$-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = 14.3, 22.8, 25.6, 29.5, 29.6, 29.7, 29.7, 29.8, 29.8, 32.1, 36.6, 41.2, 68.2, 178.0 ppm.

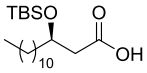
**HRMS** (ESI-TOF) calculated for $C_{14}H_{23}O_3$ $[M+H]^+$ 245.2111; found 245.2113.

$[\alpha]_D$ = 15.2 ($CHCl_3$, c = 1.0, 23.6 °C).

**IR** (ATR): $\tilde{\nu}$ = 3557 (w), 3534 (w), 3028 (w), 2955 (m), 2916, 2847, 1713 (w), 1678,1470 (w), 1296 (w), 1281 (w), 1227 (w) $cm^{-1}$.

**Melting Point**: $T_m$ = 73.1 °C.

### 3.1.8 (*R*)-3-((tert-butyldimethylsilyl)oxy)tetradecanoic acid ((*R*)-S-6)




(*R*)-S-**6**

To a solution of *tert*-butyldimethylsilylchloride (TBSCl) (1.24 g, 8.23 mmol, 4 eq.) in DMF (3.5 mL) was added imidazole (1.40 g, 20.6 mmol, 10 eq.) at 0 °C and stirred for 15 min. Then hydroxy acid ((*R*)-S-**5**) (500 mg, 2.05 mmol, 1 eq.) in DMF was added to the reaction mixture at 0 °C and the temperature was brought up to room temperature gradually. After stirring for 5.5 h, the reaction mixture was acidified with 1M HCl (aq.) at 0 °C and extracted with petroleum ether/$Et_2O$ (2:1) (5 × 20 mL). The combined organic layers were washed with $H_2O$ and brine, dried over $MgSO_4$ and filtered. The filtrate was evaporated to dryness. The resulting colorless oil was dissolved in THF (28 mL) and MeOH (55 mL), and $K_2CO_3$ (693 mg, 5.01 mmol, 2.4 eq.) in $H_2O$ (9.2 mL) was added at 0 °C. After stirring for 1h, the reaction mixture was acidified with 1 M HCl (aq.) at 0 °C and extracted with petroleum ether/$Et_2O$ (2:1) (5 × 60 mL). The combined organic layers were washed with $H_2O$ and brine, dried over $MgSO_4$ and filtered. The filtrate was evaporated to dryness. The resulting colorless oil was purified by flash column chromatography (125 mL silica, $CH_2Cl_2$/MeOH = 30:1) to give (*R*)-S-**6** as a colorless oil (701 mg, 1.95 mmol, 95%).

**TLC**: $R_f$ = 0.67 ($CH_2Cl_2$/MeOH = 20:1).

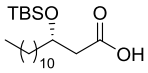
**$^1H$-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.08 (s, 3 H), 0.09 (s, 3 H), 0.87 - 0.89 (m, 12 H), 1.22-1.32 (m, 18 H), 1.53 (m, 2 H), 2.48 (dd, *J* = 15.3, 6.0 Hz, 1 H), 2.53 (dd, *J* = 15.3, 5.1 Hz, 1 H), 4.09 (m, 1 H) ppm.

**$^{13}C\{^1H\}$-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = -4.7, -4.4, 14.3, 18.1, 22.8, 25.3, 25.9, 29.5, 29.7, 29.7, 29.7, 29.8, 32.1, 37.3, 41.9, 69.6, 175.9 ppm.

**HRMS** (ESI-TOF) calculated for $C_{20}H_{42}O_3Si$ $[M+H]^+$ 359.2976; found 359.2977.

**IR** (ATR): $\tilde{\nu}$ = 2955 (m), 2924 (s), 2855 (s), 1713 (s), 1462 (w), 1254 (w), 1096 (w), 833 (w), 775 (w) $cm^{-1}$.

### 3.1.9 (*S*)-3-((tert-butyldimethylsilyl)oxy)tetradecanoic acid ((*S*)-S-6)




(*S*)-S-**6**

(*S*)-S-**6** was prepared in the same procedure as (*R*)-S-**6** from β-hydroxy acid ((*S*)-S-**5**) (284 mg, 1.16 mmol) and was obtained as a colorless oil (384 mg, 1.07 mmol, 92%).

**TLC**: $R_f$ = 0.66 ($CH_2Cl_2$/MeOH = 20:1).

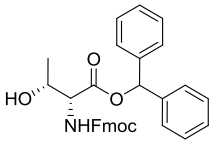
**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.08 (s, 3 H), 0.09 (s, 3 H), 0.87 - 0.89 (m, 12 H), 1.22 (s, 1 H), 1.22 - 1.32 (m, 18 H), 1.53 (m, 2 H), 2.48 (dd, *J* = 15.3, 6.1 Hz, 1 H), 2.53 (dd, *J* = 15.3, 5.1 Hz, 1 H), 4.09 (m, 1 H) ppm.

**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = -4.7, -4.4, 14.3, 18.1, 22.8, 25.3, 25.9, 29.5, 29.7, 29.7, 29.7, 29.8, 32.1, 37.3, 41.9, 69.6, 175.9 ppm.

**HRMS** (ESI-TOF) calculated for $C_{20}H_{42}O_3Si$ $[M+H]^+$ 359.2976; found 359.2976.

**IR** (ATR): $\tilde{\nu}$ = 2955 (m), 2924 (s), 2855 (s), 1713 (s), 1462 (w), 1254 (w), 1092 (w), 833 (m), 775 (w) $cm^{-1}$.

### 3.1.10 Fmoc-D-*allo*-Thr-ODpm (12)




**12**

To a mixture of Fmoc-D-*allo*-threonine (**11**) (2.00 g, 5.86 mmol, 1 eq.) and benzophenone hydrazone (2.30 g, 11.7 mmol, 2 eq.) in dry $CH_2Cl_2$ (20 mL) was added dropwise 1% (w/v) solution of $I_2$ in dry $CH_2Cl_2$ (1.2 mL) at 0 °C. (Diacetoxyiodo)benzene (3.77 g, 11.7 mmol, 2 eq) was added to the reaction mixture by portions (divided into 5 times) over 1.3 h and the mixture was stirred further for 1h at 0 °C. Solvents were removed by evaporation and the remaining mixture was dissolved in EtOAc (100 mL). The organic layer was washed with water (1 × 30 mL), saturated $NaHCO_3$ solution (3 × 30 mL), water (1 × 30 mL) and brine (1 × 30 mL), dried over $MgSO_4$ and evaporated to dryness after filtration. The crude mixture was dry loaded on silica gel and purified by flash column chromatography (300 mL silica, petroleum ether/EtOAc = 8:1 → $CH_2Cl_2$/MeOH = 30:1) once roughly and further by flash column chromatography (300 mL silica, $CH_2Cl_2$/EtOAc = 25:1) to give Fmoc-D-*allo*-Thr-ODpm (**12**) as a pale yellow solid (2.75 g, 5.42 mmol, 92%).

**TLC**: $R_f$ = 0.43 (petroleum ether/EtOAc = 2:1).

**$^1$H-NMR** ($CDCl_3$, 400 MHz, 297 K): $\delta$ = 1.09 (d, *J* = 6.4 Hz, 3 H; Thr C$\underline{H}_3$), 2.74 (br d, *J* = 6.4 Hz, 1 H; O$\underline{H}$),

4.16 - 4.29 (m, 2 H; Thr C$\underline{H}_{\beta}$, Fmoc C$\underline{H}_{Bn}$), 4.42 (m, 2 H; Fmoc C$\underline{H}_2$), 4.59 (m, 1 H; Thr C$\underline{H}_{\alpha}$), 5.69 (br d, $J$ = 7.0 Hz, 1 H; N$\underline{H}$), 6.94 (s, 1 H; Dpm C$\underline{H}_{Bn}$), 7.27 - 7.34 (m, 12 H; Dpm C$\underline{H}_{Ar}$, Fmoc C$\underline{H}_{Ar}$), 7.40 (t, $J$ = 7.5 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$), 7.58 (d, $J$ = 7.0 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$), 7.76 (d, $J$ = 7.5 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$) ppm.

**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 101 MHz, 297 K): $\delta$ = 18.8, 47.3, 59.7, 67.5, 69.3, 78.8, 120.1, 120.2, 125.2, 125.2, 127.1, 127.2, 127.4, 127.9, 128.4, 128.5, 128.8, 139.3, 139.4, 141.4, 141.5, 143.8, 143.9, 156.9, 169.5 ppm.

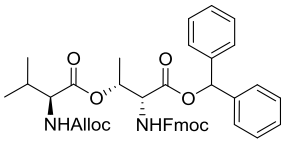
**HRMS** (ESI-TOF) calculated for $C_{32}H_{29}NO_5$ $[M+H]^+$ 508.2118; found 508.2119.

$[\alpha]_D$ = 11.4 ($CH_2Cl_2$, c = 1.0, 23.4 °C).

**IR** (ATR): $\tilde{\nu}$ = 3426 (m), 3383 (s), 1732 (s), 1709 (s), 1531 (s), 1497 (m), 1450 (m), 1300 (m), 1273 (s), 1258 (s), 1231 (s), 1184 (m), 1096 (w), 1069 (m), 999 (s), 741 (m), 702 (m) $cm^{-1}$.

**Melting Point**: $T_m$ = 168 °C.

### 3.1.11 Fmoc-D-*allo*-Thr-(O-Alloc-L-Val)-ODpm (13)




**13**

To a mixture of Fmoc-D-*allo*-Thr-ODpm (**12**) (1.00 g, 1.97 mmol, 1.00 eq) and DMAP (24.1 mg, 0.197 mmol, 0.1 eq) in dry $CH_2Cl_2$ (15 mL), Alloc-L-Valine (475 mg, 2.36 mmol, 1.2 eq.) in $CH_2Cl_2$ (4 mL) and EDC·HCl (136 mg, 0.709 mmol, 1.2 eq.) were added subsequently at 0 °C. Additional $CH_2Cl_2$ (1 mL) was used to wash down the reagents. The reaction mixture was warmed up to room temperature gradually stirred for overnight. The mixture was washed with 1M HCl (2 × 20 mL), water (1 × 20 mL) and brine (1 × 10 mL), dried over $MgSO_4$ and evaporated to dryness after filtration. The crude mixture was purified by flash column chromatography (712 mL silica, petroleum ether/EtOAc = 5:1) to give **13** as a colorless amorphous (1.31 g, 1.90 mmol, 96%).

**TLC**: $R_f$ = 0.71 (petroleum ether/EtOAc = 2:1).

**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.84 (d, $J$ = 6.9 Hz, 3 H; Val C$\underline{H}_3$), 0.90 (d, $J$ = 6.9 Hz, 3 H; Val C$\underline{H}_3$), 1.25 (d, $J$ = 6.7 Hz, 3 H; Thr C$\underline{H}_3$), 2.01 (m, 1 H; Val C$\underline{H}_{\beta}$) 4.12 (m, 1 H; Val C$\underline{H}_{\alpha}$), 4.22 (t, $J$ = 7.3 Hz, 1 H; Fmoc C$\underline{H}_{Bn}$), 4.36 (d, $J$ = 7.3 Hz, 2 H; Fmoc C$\underline{H}_2$), 4.49 (dd, $J$ = 13.4, 5.6 Hz, 1 H; C$\underline{H}_2$-CH=$CH_2$), 4.55 (dd, $J$ = 13.4, 5.6 Hz, 1 H; C$\underline{H}_2$-CH=$CH_2$), 4.77 (dd, $J$ = 8.5, 3.1 Hz, 1 H; Thr C$\underline{H}_{\alpha}$), 5.13 (d, $J$ = 10.7 Hz, 1 H; $CH_2$-CH=C$\underline{H}_2$), 5.23 (d, $J$ = 17.1 Hz, 1 H; $CH_2$-CH=C$\underline{H}_2$), 5.29 (d, $J$ = 8.4 Hz, 1 H; Val N$\underline{H}$) 5.36 (m, 1 H; Thr C$\underline{H}_{\beta}$), 5.84 (ddt, $J$ = 17.1, 10.7, 5.7 Hz, 1 H; $CH_2$-C$\underline{H}$=$CH_2$) 5.90 (d, $J$ = 8.5 Hz, 1 H; Thr N$\underline{H}$), 6.99 (s, 1 H; Dpm C$\underline{H}_{Bn}$) 7.28 - 7.41 (m, 14 H; Fmoc C$\underline{H}_{Ar}$, Dpm C$\underline{H}_{Ar}$) 7.60 (t, $J$ = 8.2 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$) 7.76 (d, $J$ = 7.5 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$) ppm.

**$^{13}C\{^1H\}$-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = 16.0 (Thr $\underline{C}H_3$), 17.7 (Val $\underline{C}H_3$), 19.1 (Val $\underline{C}H_3$), 31.0 (Val $\underline{C}H_\beta$), 47.2 (Fmoc $\underline{C}H_{Bn}$), 57.4 (Thr $\underline{C}H_\alpha$), 59.3 (Val $\underline{C}H_\alpha$), 66.0 ($\underline{C}H_2$-CH=$CH_2$), 67.7 (Fmoc C$\underline{H}_2$), 72.1 (Thr $\underline{C}H_\beta$), 78.9 (Dpm $\underline{C}H_{Bn}$), 118.0 ($CH_2$-CH=$\underline{C}H_2$), 120.1 (Fmoc $\underline{C}H_{Ar}$), 125.3 (Fmoc $\underline{C}H_{Ar}$), 125.4 (Fmoc $\underline{C}H_{Ar}$), 127.1 ($\underline{C}H_{Ar}$), 127.2 ($\underline{C}H_{Ar}$), 127.6 ($\underline{C}H_{Ar}$), 127.9 ($\underline{C}H_{Ar}$), 128.4 ($\underline{C}H_{Ar}$), 128.5 ($\underline{C}H_{Ar}$), 128.8 ($\underline{C}H_{Ar}$), 128.8 ($\underline{C}H_{Ar}$), 132.7 ($CH_2$-$\underline{C}$H=$CH_2$), 139.2 (Dpm $\underline{C}_q$), 139.3 (Dpm $\underline{C}_q$), 141.4 (Fmoc $\underline{C}_q$), 143.9 (Fmoc $\underline{C}_q$), 156.2 ($\underline{C}$ONH), 156.4 ($\underline{C}$ONH), 168.1 (Thr $\underline{C}$OODpm), 171.5 (Val $\underline{C}$OO) ppm.

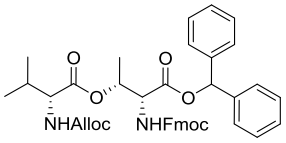
**HRMS** (ESI-TOF) calculated for $C_{41}H_{42}N_2O_8$ $[M+H]^+$ 691.3014; found 691.3020.

$[\alpha]_D$ = 9.0 ($CHCl_3$, c = 1.0, 23.9 °C).

**IR** (ATR): $\tilde{\nu}$ = 3348 (m), 3333 (m), 3063 (m), 3032 (m), 2967 (m), 2936 (m), 2878 (w), 1724 (s), 1524 (m), 1450 (w), 1389 (w), 1308 (w), 1246 (m), 1192 (w), 760 (w), 741(w) $cm^{-1}$.

**Melting Point**: $T_m$ = 50 - 57 °C.

### 3.1.12 Fmoc-D-*allo*-Thr-(O-Alloc-D-Val)-ODpm (13')




**13'**

To a mixture of Fmoc-D-*allo*-Thr-ODpm (**12**) (300 mg, 0.591 mmol, 1.00 eq) and DMAP (7.2 mg, 0.0589 mmol, 0.1 eq) in dry $CH_2Cl_2$ (3 mL), Alloc-D-Valine (143 mg, 0.711 mmol, 1.2 eq.) in $CH_2Cl_2$ (2.5 mL) and EDC•HCl (136 mg, 0.709 mmol, 1.2 eq.) were added subsequently at 0 °C. Additional $CH_2Cl_2$ (0.5 mL) was used to wash down the reagents. The reaction mixture was warmed up to room temperature gradually stirred for 7h. The mixture was washed with 1M HCl (2 × 10 mL), water (1 × 10 mL) and brine (1 × 10 mL), dried over $MgSO_4$ and evaporated to dryness after filtration. The crude mixture was purified by flash column chromatography (200 mL silica, petroleum ether/EtOAc = 5:1) to give **13'** as a colorless amorphous (389 mg, 0.563 mmol, 95%).

**TLC**: $R_f$ = 0.61 (petroleum ether/EtOAc = 2:1).

**$^1H$-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.79 (d, $J$ = 6.9 Hz, 3 H; Val C$\underline{H}_3$), 0.87 (d, $J$ = 6.9 Hz, 3 H; Val C$\underline{H}_3$), 1.31 (d, $J$ = 6.7 Hz, 3 H; Thr C$\underline{H}_3$), 1.97 (m, 1 H; Val C$\underline{H}_\beta$), 4.14 (m, 1 H; Val C$\underline{H}_\alpha$), 4.22 (t, $J$ = 7.3 Hz, 1 H; Fmoc C$\underline{H}_{Bn}$), 4.37 (m, 2 H; Fmoc C$\underline{H}_2$), 4.59 (qd, $J$ = 13.4, 5.6 Hz, 2 H; C$\underline{H}_2$-CH=$CH_2$), 4.72 (dd, $J$ = 8.4, 2.7 Hz, 1 H; Thr C$\underline{H}_\alpha$), 5.13 (d, $J$ = 8.3 Hz, 1 H; Val N$\underline{H}$), 5.21 (d, $J$ = 10.8 Hz, 1 H; $CH_2$-CH=C$\underline{H}_2$), 5.31 (m, 1 H; $CH_2$-CH=C$\underline{H}_2$), 5.36 (m, 1 H; Thr C$\underline{H}_\beta$), 5.74 (d, $J$ = 8.4 Hz, 1 H; Thr N$\underline{H}$), 5.92 (ddt, $J$ = 16.8, 10.8, 5.6 Hz, 1 H; $CH_2$-C$\underline{H}$=$CH_2$), 6.96 (s, 1 H; Dpm C$\underline{H}_{Bn}$), 7.28 - 7.37 (m, 12 H; Dpm C$\underline{H}_{Ar,}$ Fmoc C$\underline{H}_{Ar}$), 7.40 (t, $J$ = 7.5 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$), 7.59 (d, $J$ = 7.3 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$), 7.76 (d, $J$ = 7.5 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$) ppm.

**$^{13}C\{^1H\}$-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = 16.6 (Thr $\underline{C}H_3$), 17.3 (Val $\underline{C}H_3$), 19.3 (Val $\underline{C}H_3$), 30.8 (Val $\underline{C}H_\beta$), 47.2 (Fmoc $\underline{C}H_{Bn}$), 57.6 (Thr $\underline{C}H_\alpha$), 59.4 (Val $\underline{C}H_\alpha$), 66.1 ($\underline{C}H_2$-CH=$CH_2$), 67.5 (Fmoc $\underline{C}H_2$), 72.2 (Thr $\underline{C}H_\beta$), 79.1 (Dpm $\underline{C}H_{Bn}$), 118.0 ($CH_2$-CH=$\underline{C}H_2$), 120.1 (Fmoc $\underline{C}H_{Ar}$), 125.3 (Fmoc $\underline{C}H_{Ar}$), 127.1 ($\underline{C}H_{Ar}$), 127.2 ($\underline{C}H_{Ar}$), 127.7 ($\underline{C}H_{Ar}$), 127.9 ($\underline{C}H_{Ar}$), 128.4 ($\underline{C}H_{Ar}$), 128.6 ($\underline{C}H_{Ar}$), 128.8 ($\underline{C}H_{Ar}$), 132.8 ($CH_2$-$\underline{C}H$=$CH_2$), 139.2 ($\underline{C}_{Arq}$), 139.3 ($\underline{C}_{Arq}$), 141.4 ($\underline{C}_{Arq}$), 141.4 ($\underline{C}_{Arq}$), 143.8 ($\underline{C}_{Arq}$), 143.9 ($\underline{C}_{Arq}$), 155.9 ($\underline{C}$ONH), 156.5 ($\underline{C}$ONH), 168.3 (Thr $\underline{C}$OODpm), 171.1 (Val $\underline{C}$OO) ppm.

**HRMS** (ESI-TOF) calculated for $C_{41}H_{42}N_2O_8$ $[M+H]^+$ 691.3014; found 691.3019.

$[\alpha]_D$ = 13.7 ($CHCl_3$, c = 1.0, 23.9 °C).

**IR** (ATR): $\tilde{\nu}$ = 3348 (w), 3337(w), 3067 (w), 3032 (w), 2967 (w), 2940 (w), 1721 (s), 1512 (m), 1450 (m), 1389 (w), 1373 (w), 1312 (w), 1273 (m), 1238 (m), 1200 (m), 1107 (w), 1088 (w), 1061 (w), 1034 (w), 991 (w), 760 (w), 741(w) $cm^{-1}$.

**Melting Point**: $T_m$ = 58.8 °C.

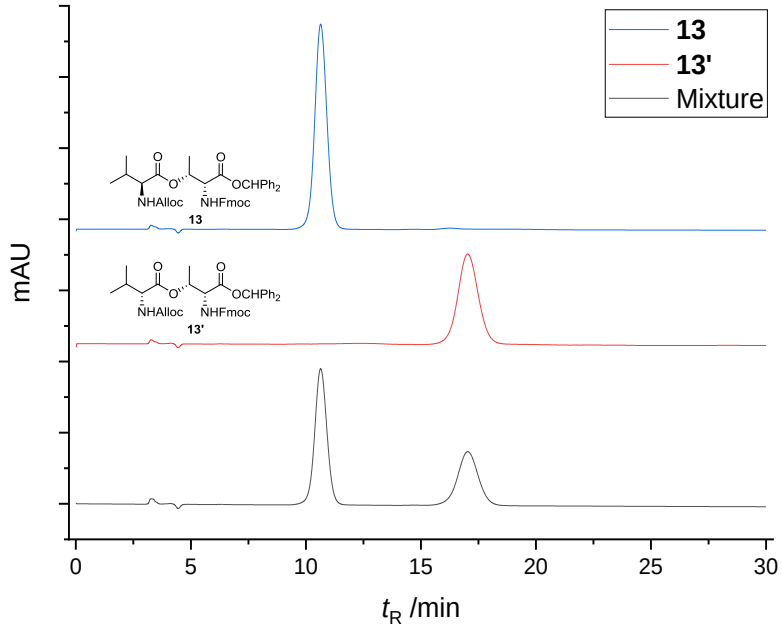
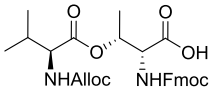



**Figure S2**. HPLC traces of dipeptides **13** and **13'** on chiral stationary phase (Condition: NP-B, 220 nm).

### 3.1.13 Fmoc-D-*allo*-Thr-(O-Alloc-L-Val)-OH (**14**)




**14**

To a solution of Fmoc-D-*allo*-Thr-(O-Alloc-L-Val)-ODpm (**13**) (0.963 g, 1.39 mmol, 1 eq.) in $CH_2Cl_2$ (7 mL) were added TIS (1.4 mL, 6.95 mmol, 5 eq.) and TFA (0.7 mL) at 0 °C. The reaction mixture was stirred at room temperature for 4h. After addition of toluene the mixture was evaporated to remove volatiles and the

crude mixture was purified by flash column chromatography (712 mL silica, $CH_2Cl_2$/MeOH = 30:1 → 20:1). The compound was purified further by trituration from petroleum ether/$CH_2Cl_2$ at 5 °C to give Fmoc-D-*allo*-Thr-(O-Alloc-L-Val)-OH (**14**) as a colorless solid (662 mg, 1.26 mmol, 91%).

**RP-HPLC**: $t_R$ = 8.89 min (RP-C).

**TLC**: $R_f$ = 0.52 ($CH_2Cl_2$/MeOH = 20:1 + 1% HCOOH).

**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.90 (d, *J* = 6.9 Hz, 3 H; Val C$\underline{H}_3$), 0.97 (d, *J* = 6.9 Hz, 3 H; Val C$\underline{H}_3$), 1.48 (d, *J* = 6.7 Hz, 3 H; Thr C$\underline{H}_3$), 2.18 (m, 1 H; Val C$\underline{H}_\beta$), 4.17 (t, *J* = 7.3 Hz, 1 H; Fmoc C$\underline{H}_{Bn}$), 4.24 (m, 1 H; Fmoc C$\underline{H}_2$), 4.38 (dd, *J* = 10.5, 7.6 Hz, 1 H; Fmoc C$\underline{H}_2$), 4.46 (dd, *J* = 9.3, 4.5 Hz, 1 H; Val C$\underline{H}_\alpha$), 4.52 (dd, *J* = 13.2, 5.7 Hz, 1 H; C$\underline{H}_2$-CH=CH$_2$), 4.59 (dd, *J* = 13.2, 5.7 Hz, 1 H; C$\underline{H}_2$-CH=CH$_2$), 4.67 (dd, *J* = 8.9, 3.1 Hz, 1 H; Thr C$\underline{H}_\alpha$), 5.19 (dd, *J* = 23.4, 10.7 Hz, 1 H; CH$_2$-CH=C$\underline{H}_2$), 5.28 (m, 1 H; CH$_2$-CH=C$\underline{H}_2$), 5.39 (qd, *J* = 6.7, 3.1 Hz, 1 H; Thr C$\underline{H}_\beta$), 5.52 (d, *J* = 9.3 Hz, 1 H; Val N$\underline{H}$), 5.85 (ddt, *J* = 17.1, 10.7, 5.7 Hz, 1 H; CH$_2$-C$\underline{H}$=CH$_2$), 6.59 (d, *J* = 8.9 Hz, 1 H; Thr N$\underline{H}$), 7.28 (m, 2 H; Fmoc C$\underline{H}_{Ar}$) 7.37 (t, *J* = 7.6 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$), 7.53 (d, *J* = 7.6 Hz, 1 H; Fmoc C$\underline{H}_{Ar}$), 7.57 (d, *J* = 7.6 Hz, 1 H; Fmoc C$\underline{H}_{Ar}$), 7.76 (d, *J* = 7.6 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$) ppm.

**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 151 MHz, 297 K): $\delta$ = 17.0 (Thr $\underline{C}H_3$), 17.3 (Val $\underline{C}H_3$), 19.2 (Val $\underline{C}H_3$), 31.2 (Val $\underline{C}H_\beta$), 47.2 (Fmoc $\underline{C}H_{Bn}$), 57.4 (Thr $\underline{C}H_\alpha$), 59.0 (Val $\underline{C}H_\alpha$), 66.5 ($\underline{C}H_2$-CH=CH$_2$), 67.5 (Fmoc $\underline{C}H_2$), 73.0 (Thr $\underline{C}H_\beta$), 118.4 (CH$_2$-CH=$\underline{C}H_2$), 120.0 (Fmoc $\underline{C}H_{Ar}$), 125.4 (Fmoc $\underline{C}H_{Ar}$), 125.4 (Fmoc $\underline{C}H_{Ar}$), 127.2 (Fmoc $\underline{C}H_{Ar}$), 127.8 (Fmoc $\underline{C}H_{Ar}$), 132.3 (CH$_2$-$\underline{C}$H=CH$_2$), 141.4 (Fmoc $\underline{C}q$), 143.9 (Fmoc $\underline{C}q$), 144.0 (Fmoc $\underline{C}q$), 156.2 (Fmoc $\underline{C}$ONH), 157.1 (Alloc $\underline{C}$ONH), 171.5 ($\underline{C}$OO), 171.6 ($\underline{C}$OO) ppm.

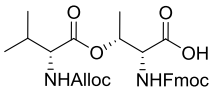
**HRMS** (ESI-TOF) calculated for $C_{28}H_{32}N_2O_8$ $[M+H]^+$ 525.2231; found 525.2235.

$[\alpha]_D$ = - 20.4 ($CHCl_3$, c = 1.0, 21.7 °C).

**IR** (ATR): $\tilde{\nu}$ = 3329 (s), 3213 (m), 3167 (m), 3121 (m), 3067 (m), 3044 (m), 3021 (m), 2967 (s), 2878 (m), 1717 (s), 1528 (m), 1450 (w), 1308 (w), 1246 (m), 1204 (w) cm$^{-1}$.

**Melting Point**: $T_m$ = 82.6 °C.

### 3.1.14 Fmoc-D-*allo*-Thr-(O-Alloc-D-Val)-OH (**14'**)




**14'**

To a solution of Fmoc-D-*allo*-Thr-(O-Alloc-D-Val)-ODpm (**13'**) (231 mg, 334 µmol, 1 eq.) in $CH_2Cl_2$ (1.7 mL) were added TIS (340 µL, 1.66 mmol, 5 eq.) and TFA (170 µL) at 0 °C. The reaction mixture was stirred at room temperature for 4h. After addition of toluene the mixture was evaporated to remove volatiles and the crude mixture was purified by flash column chromatography (712 mL silica, $CH_2Cl_2$/MeOH = 30:1 → 10:1).

The compound was purified further by trituration from petroleum ether/$CH_2Cl_2$ at 5 °C to give Fmoc-D-*allo*-Thr-(O-Alloc-D-Val)-OH (**14'**) as a colorless solid (136 mg, 259 µmol, 78%).

**RP-HPLC**: $t_R$ = 8.75 min (RP-C).

**TLC**: $R_f$ = 0.7 ($CH_2Cl_2$/MeOH = 20:1 + 1% HCOOH).

**$^1$H-NMR** ($CDCl_3$, 600 MHz, 297 K): $\delta$ = 0.89 (d, $J$ = 6.5 Hz, 3 H; Val C$\underline{H}_3$), 0.97 (d, $J$ = 6.5 Hz, 3 H; Val C$\underline{H}_3$), 1.42 (d, $J$ = 5.4 Hz, 3 H; Thr C$\underline{H}_3$), 2.18 (m, 1 H; Val C$\underline{H}_\beta$), 4.22 (m, 2 H; Val C$\underline{H}_\alpha$; Fmoc C$\underline{H}_{Bn}$), 4.38 (m, 2 H; Fmoc C$\underline{H}_2$), 4.48 (br s, 1 H), 4.52 - 4.67 (m, 3 H; Thr C$\underline{H}_\alpha$, C$\underline{H}_2$-CH=$CH_2$), 5.20 (m, 1 H; $CH_2$-CH=C$\underline{H}_2$), 5.30 (m, 1 H; $CH_2$-CH=C$\underline{H}_2$), 5.34 (m, 2 H; Thr C$\underline{H}_\beta$; Val N$\underline{H}$), 5.91 (m, 2 H; $CH_2$-C$\underline{H}$=$CH_2$, Thr N$\underline{H}$), 7.30 (m, 2 H; Fmoc C$\underline{H}_{Ar}$), 7.39 (t, $J$ = 7.5 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$), 7.55 (m,1 H; Fmoc C$\underline{H}_{Ar}$), 7.60 (m, 1 H; Fmoc C$\underline{H}_{Ar}$), 7.76 (d, $J$ = 7.5 Hz, 2 H; Fmoc C$\underline{H}_{Ar}$), 8.09 (br s, 1 H; COO$\underline{H}$) ppm.

**$^{13}$C{$^1$H}-NMR** ($CDCl_3$, 126 MHz, 297 K): $\delta$ = 16.7 (Thr C$\underline{H}_3$), 17.5 (Val C$\underline{H}_3$), 19.3 (Val C$\underline{H}_3$), 30.8 (Val C$\underline{H}_\beta$), 47.2 (Fmoc C$\underline{H}_{Bn}$), 57.5 (Thr C$\underline{H}_\alpha$), 59.6 (Val C$\underline{H}_\alpha$), 66.3 (C$\underline{H}_2$-CH=$CH_2$), 67.6 (Fmoc C$\underline{H}_2$), 72.1 (Thr C$\underline{H}_\beta$), 118.3 ($CH_2$-CH=C$\underline{H}_2$), 120.1 (Fmoc C$\underline{H}_{Ar}$), 125.2 (Fmoc C$\underline{H}_{Ar}$), 125.3 (Fmoc C$\underline{H}_{Ar}$), 127.2 (Fmoc C$\underline{H}_{Ar}$), 127.3 (Fmoc C$\underline{H}_{Ar}$), 127.9 (Fmoc C$\underline{H}_{Ar}$), 132.6 ($CH_2$-C$\underline{H}$=$CH_2$), 141.4 (Fmoc C$\underline{_q}$), 141.4 (Fmoc C$\underline{_q}$), 143.8 (Fmoc C$\underline{_q}$), 143.9 (Fmoc C$\underline{_q}$), 156.3 (Fmoc C$\underline{O}$NH), 156.8 (Alloc C$\underline{O}$NH), 171.3 (Val C$\underline{O}$O), 172.3 (Thr C$\underline{O}$OH) ppm.

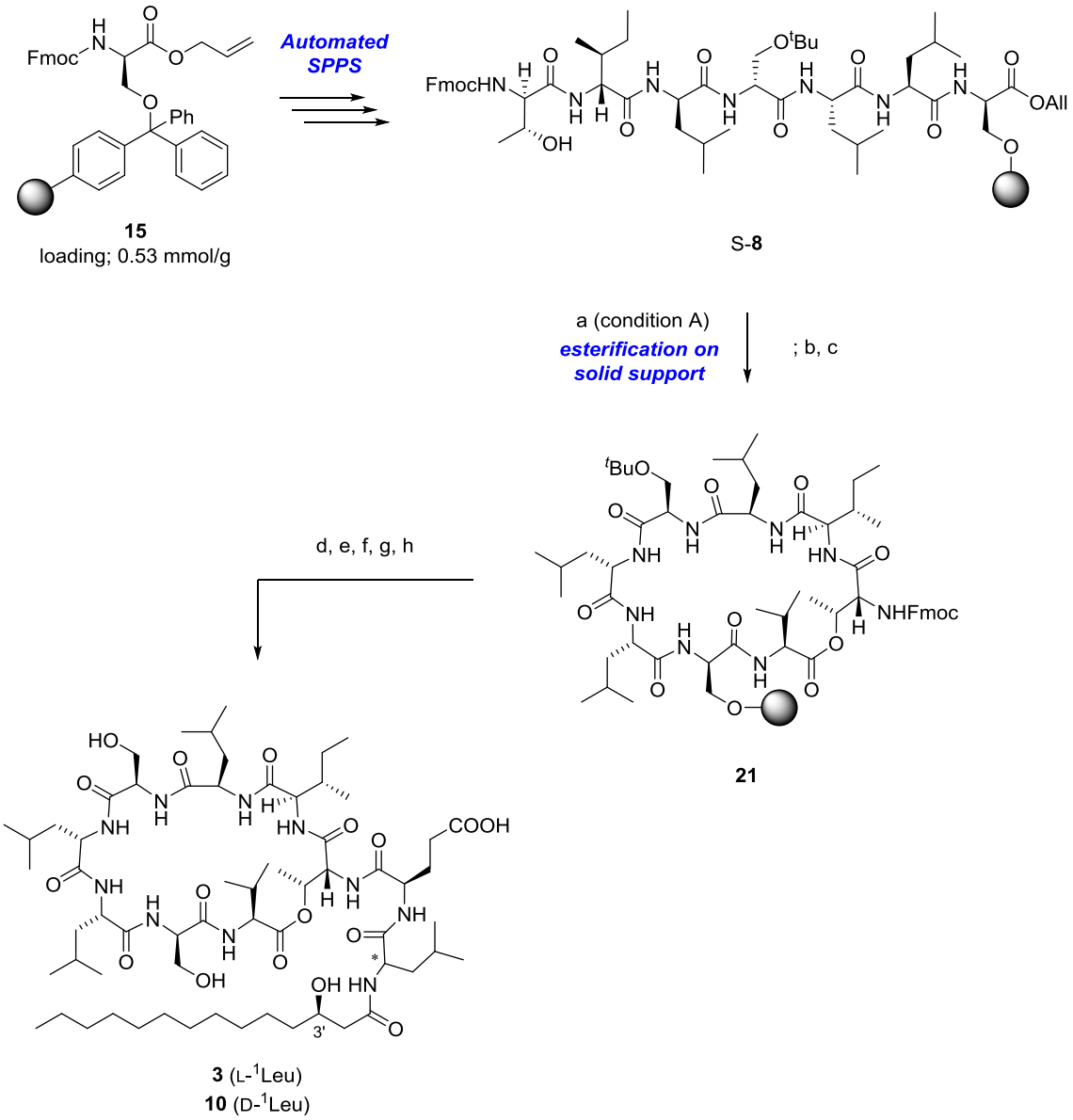
**HRMS** (ESI-TOF) calculated for $C_{28}H_{32}N_2O_8$ $[M+H]^+$ 525.2231; found 525.2234.

$[\alpha]_D$ = -2.3 ($CHCl_3$, c = 1.0, 20.8 °C).

**IR** (ATR): $\tilde{\nu}$ = 3329 (m), 3294 (m), 3213 (w), 3163 (w), 3067 (m), 3040 (m), 3013 (m), 2970 (m), 2897 (w), 1717 (s), 1524 (m), 1450 (m), 1242 (m), 1211 (m), 1092 (w), 1057 (w) $cm^{-1}$.

**Melting Point**: $T_m$ = 95.5 °C.

## 3.2 Peptide synthesis

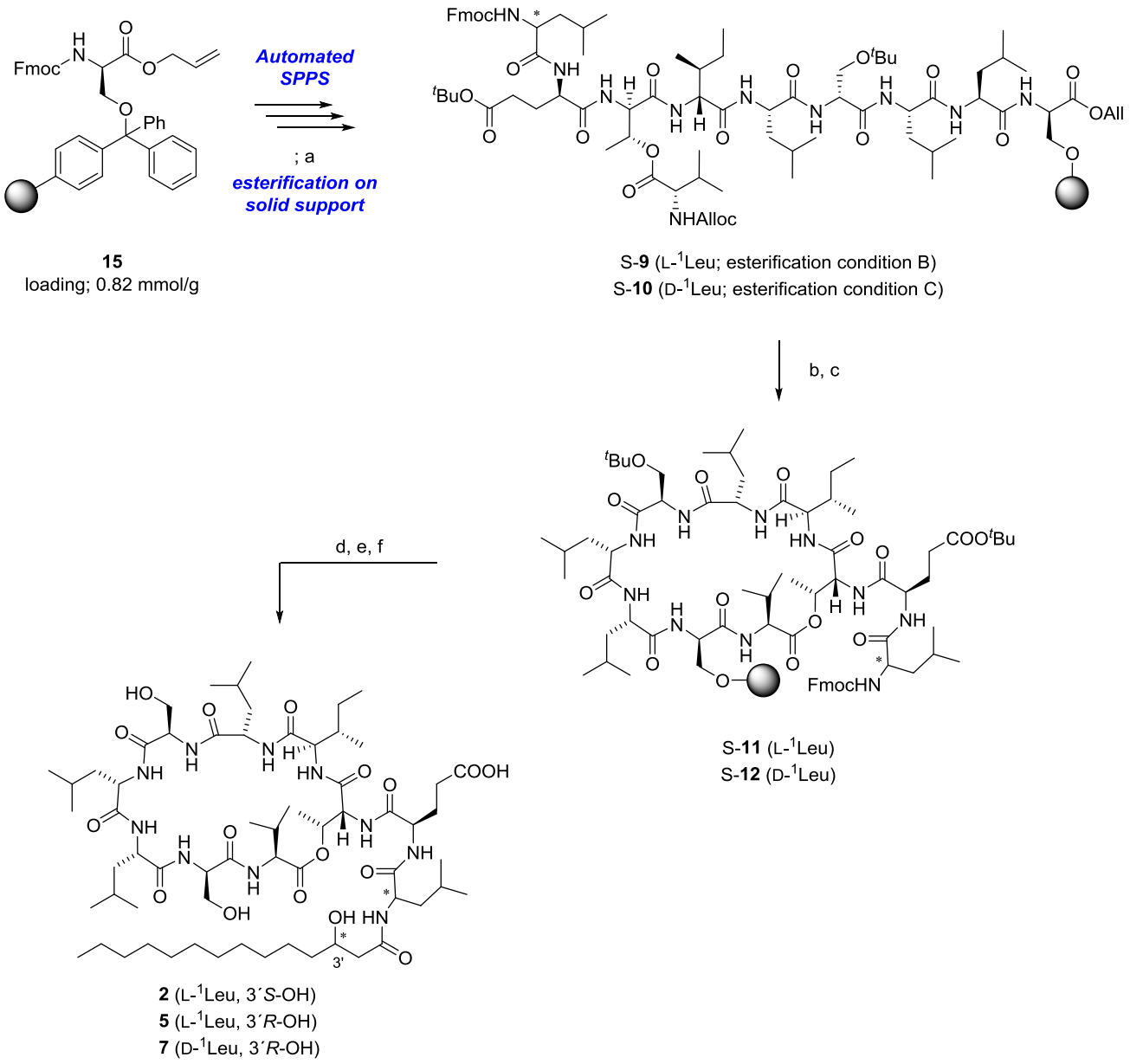



Reagents and conditions: a) Alloc-L-Valine, DIC, DMAP, DMF, 0 °C to rt; b) *cat.* $Pd(PPh_3)_4$, $PhSiH_3$, $CH_2Cl_2$, rt; c) HATU, HOAt, collidine, DMF, rt (Cyclization condition A); d) 2% DBU 2% piperidine/DMF, 30 sec. x 2; e) Fmoc-A.A., HBTU, HOBt, DIEA, DMF, rt; f) 20% piperidine/DMF; g) (*R*)-3-TBSoxytetradecanoic acid, HBTU, HOBt, DIEA, DMF, rt; h) 0.1 N HCl/HFIP + 1% TIS, rt.

**Scheme S2**. On resin esterification on heptapeptide S-**8** followed by cyclization and side chain elongation.

**Table S2.** Syntheses of orfamide A derivatives via on-resin esterification of heptapeptide S-8.

| Compound Nr. | A.A. residue 1 | A.A. residue 5 | 3'-OH | Yield |
|---|---|---|---|---|
| **3** | L-Leu | D-Leu | *R* | 6.9% |
| **10** | D-Leu | D-Leu | *R* | 11.7% |




Reagents and conditions: a) Alloc-L-Valine, DIC, DMAP, THF, 40 °C or Alloc-L-Valine, DIC, DMAP, DMF, rt; b) *cat.* $Pd(PPh_3)_4$, $PhSiH_3$, $CH_2Cl_2$, rt; c) HATU, HOAt, DIEA, DMF, rt (Cyclization condition B); d) 20% piperidine/DMF, rt; e) (*R*) or (*S*)-3-hydroxytetradecanoic acid, HBTU, HOBt, DIEA, DMF, rt; f) 0.1 N HCl/HFIP, rt.

**Scheme S3.** On resin esterification of nonapeptide S-**9** and S-**10** followed by cyclization and terminal acylation.

**Table S3.** Syntheses of orfamide A derivatives via on-resin esterification on nonapeptides **S-9** and **S-10**.

| Compound Nr. | A.A. residue 1 | A.A. residue 5 | 3'-OH | Yield |
|---|---|---|---|---|
| **2** | L-Leu | L-Leu | *S* | 2.2% |
| **5** | L-Leu | L-Leu | *R* | 2.0% |
| **7** | D-Leu | L-Leu | *R* | 6.4% |

### 3.2.1 General Procedures for Solid Phase Peptide Synthesis

All the peptide syntheses were performed on solid support and reactions were controlled by small scale cleavage of the peptidyl resin in following conditions and subjected to LC/MS and/or RP-HPLC analyses. Amounts of reagents and solvents used were calculated based on the initial amino acid loading on resin. Yields were calculated based on resin loading.

Cleavage condition A: 5% TFA in $CH_2Cl_2/H_2O$ (2.5:1; v/v), rt, 15 min.

Cleavage condition B: 0.1 N HCl/HFIP (10 µL 37% aq. HCl / 990 µL HFIP) + 1% TIS, rt, 30 - 60 min.[3, 5]

### 3.2.2 Fmoc-D-Ser(OH)-OAll loading on trityl chloride resin

The mixture of Fmoc-D-Ser(OH)-OAll (**15**) (0.5 - 1.5 eq.), trityl chloride resin (1.64 mmol/g; 1 eq.) and pyridine (3 eq.) in dry THF (5 - 10 mL/g dry resin) was stirred at 70 °C for 7 h. The resin was washed with $CH_2Cl_2$ (6 ×) and then treated with a solution of $CH_2Cl_2$/MeOH/DIEA (17:2:1, v/v/v; 20 mL) for 20 min. After the removal of solvents the resin was washed with $CH_2Cl_2$ (6 ×) and MeOH (1 ×), and dried in vacuo for overnight. The loading was determined by a small scale Fmoc cleavage with 20% piperidine/DMF (v/v) and measurement of the absorption of the cleaved piperidine-dibenzofulvene adduct.

### 3.2.3 SPPS using an automated peptide synthesizer

The fully automated parallel peptide synthesizer (MultiSyn Tech, Syro ll) was programmed to couple amino acids sequentially from C-terminus to N-terminus. A single coupling cycle consisted of Fmoc deprotection, amino acid coupling and washing with DMF (3 ×) after each step. All steps were performed at room temperature in open air. Detailed protocol used to program Syro ll is shown in table S4.

**Table S4.** Single cycle of the SPPS using an automated peptide synthesizer (Syro ll)

| Step | Operation | Solvent/Reagent | Time (repetitions) |
|---|---|---|---|
| 1 | Swelling | DMF | 20 min 30 s (2 ×) [a, b] |
| 2 | Fmoc deprotection | 20 % piperidine in DMF (v/v) | 3 min (1 ×) [a, c]<br>7 min (2 ×) [a, c] |
| 3 | Wash | DMF | 3 min (3 ×) [a, c] |
| 4 | Coupling | Fmoc-A.A. solution in DMF (0.4 M, 4 eq.); add HBTU/HOBt in DMF (0.3 M each, 4 eq.); add DIPEA in NMP (1.3 M) | 2 h* (1 ×) [a, d] |
| 5 | Wash | DMF | 3 min (3 ×) [a, c] |

[a] Vortex = 20 sec.; [b] Break = 3 min; [c] Break = 1 min; [d] Break = 5 min.

* Couplings of Fmoc-D-*allo*-Isoleucine and Fmoc- D-*allo*-Threonine were performed for 8 h.

**Table S5.** LC/MS analyses of peptides synthesized on Syro II, test cleavage condition: A.

| Compound Nr. | Formula | Exact Mass | LC/MS method | Observed Mass |
|---|---|---|---|---|
| **16** | $C_{52}H_{78}N_6O_{11}$ | 962.6 | LC/MS-B | 963.7 / $[M+H]^+$ |
| **17** | $C_{52}H_{78}N_6O_{11}$ | 962.6 | LC/MS-C | 963.7 / $[M+H]^+$ |
| S-**8** | $C_{56}H_{85}N_7O_{13}$ | 1063.6 | LC/MS-A | 1064.9 / $[M+H]^+$ |
| S-**9** | $C_{71}H_{111}N_9O_{17}$ | 1361.8 | LC/MS-C | 1363.3 / $[M+H]^+$ |
| S-**10** | $C_{71}H_{111}N_9O_{17}$ | 1361.8 | LC/MS-C | 1362.9 / $[M+H]^+$ |

### 3.2.4 Manual SPPS

The resin was swollen prior to all steps.

#### 3.2.4.1 Fmoc deprotection conditions

Fmoc deprotection was performed under two conditions.

Condition A: 20% piperidine/DMF (v/v)

The deprotection solution (20% piperidine/DMF (v/v)) was added to the peptidyl resin in a syringe and the syringe was shaken for 5 min at room temperature. The Fmoc deprotection solution was removed, and the deprotection step was repeated once for another 15 min, and the resulting peptidyl resin was washed with DMF (6 ×) prior to the subsequent coupling.

Condition B: DBU/piperidine/DMF (2/2/96; v/v/v)

The deprotection solution (DBU/piperidine/DMF (2/2/96; v/v/v)) was added to the peptidyl resin in a syringe and the syringe was shaken for 30 seconds at room temperature. The Fmoc deprotection solution was removed and the resin was washed with DMF (1 ×). The deprotection step was repeated once for another 30 seconds, and the resulting peptidyl resin was washed with DMF (6 ×). All procedures were performed within 5 min prior to the next step.

#### 3.2.4.2 Amino acid/building block coupling conditions

All the couplings were performed at room temperature.

Coupling cocktail A:

Respective amino acid or building block (4 eq.), HBTU (4 eq.), HOBt (4 eq.), DIEA (8 eq.) in DMF (0.25 M; with respect to A.A. or building blocks).

Coupling cocktail B:

Respective amino acid or building block (5 eq.), HBTU (5 eq.), HOBt (5 eq.), DIEA (10 eq.) in DMF (0.4 M; with respect to A.A. or building blocks).

Coupling cocktail C:

Respective amino acid or building block (2 eq.), HBTU (2 eq.), HOBt (2 eq.), DIEA (4 eq.) in DMF (0.4 M; with respect to A.A. or building blocks).

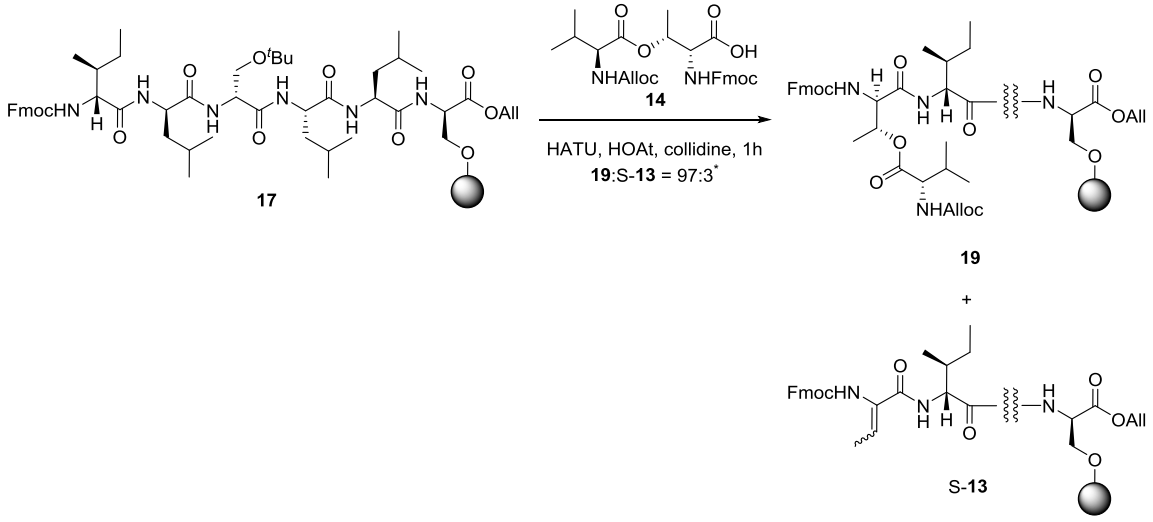
Coupling cocktail D:

Respective amino acid or building block (2 eq.), HATU (2 eq.), HOAt (2 eq.), collidine (4 eq.) in DMF (0.4 M; with respect to A.A. or building blocks).

### 3.2.4.3 Dipeptide coupling (Scheme 2)

After Fmoc deprotection (Condition A), the coupling cocktail D with dipeptide (**14**) was added to the syringe containing the peptidyl resin, and the syringe was shaken for 1 h at room temperature. The coupling mixture was removed from the syringe and the peptidyl resin was washed with DMF (6 x) and $CH_2Cl_2$ (3 ×). During the coupling process, a minor byproduct S-**13** resulting from the elimination of Alloc-L-Valine was observed by HPLC and LC/HRMS analysis, which was suppressed after optimization (Scheme S4).

**HRMS** (ESI-TOF) calculated for S-**13** $C_{56}H_{83}N_7O_{12}$ $[M+H]^+$ 1046.6172; found 1046.6173.




**Scheme S4**. Side reaction of Dipeptide **14** coupling. Product ratio* was determined by RP-HPLC

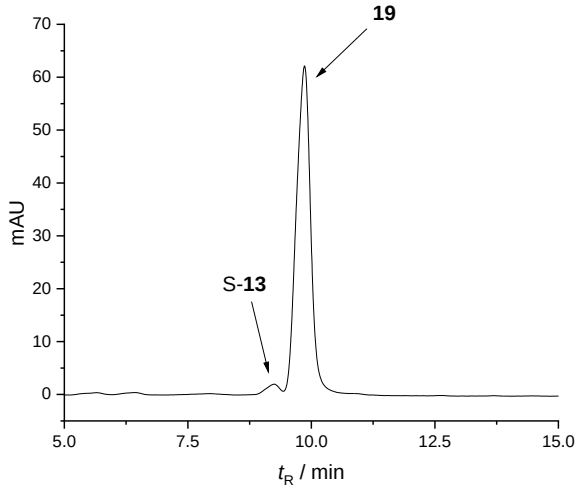



**Figure S3.** HPLC traces of the test cleavage after dipeptide coupling.
(Condition: RP-D, 280 nm).

#### 3.2.4.4 On-resin esterification

The on-resin esterification was performed under one of the conditions described below.

Condition A:

To the reaction vessel containing peptidyl resin and DMAP (10 eq.) was added Alloc-L-Valine (10 eq.) in DMF and DIC (10 eq.) at 0 °C. The mixture was stirred under $N_2$ for 3 h and the temperature was gradually warmed up to room temperature. The coupling mixture was removed and the peptidyl resin was washed with DMF (6 ×) and $CH_2Cl_2$ (3 x).

Condition B:

To the reaction vessel containing peptidyl resin was added Alloc-L-Valine (10 eq.), DIC (10 eq.) and DMAP (1 eq.) in THF and the mixture was stirred at 40 °C for 4 h. Then additional DIC (10 eq.) was added to the reaction mixture and stirred for another 4 h. The peptidyl resin was washed with $CH_2Cl_2$ (6 ×), DMF (3 ×) and $CH_2Cl_2$ (6 ×). This step was repeated if necessary.

Condition C:

To the syringe containing peptidyl resin was added Alloc-L-Valine (10 eq.), DIC (10 eq.) and DMAP (1 eq.) in DMF and the syringe was shaken for 4 h at room temperature. The coupling mixture was removed and the peptidyl resin was washed with DMF (6 ×) and $CH_2Cl_2$ (3 x). This step was repeated if necessary.

#### 3.2.4.5 Alloc/All deprotection

Peptidyl resin was dried under high vacuum prior to Alloc/All deprotection and the resin was swollen in $CH_2Cl_2$ under Ar atmosphere. A solution of $Pd(PPh_3)_4$ (0.2 eq.) and $PhSiH_3$ (20 eq.) in $CH_2Cl_2$ (4.05 mM as total, based on $Pd(PPh_3)_4$) was added to the reaction pot containing the resin and the mixture was stirred at room temperature for 2 h in the dark. The resin was washed with $CH_2Cl_2$ (× 3), DMF (× 3) and $CH_2Cl_2$ (× 3).

#### 3.2.4.6 On-resin cyclization

The on-resin cyclization was performed under one of the conditions described below.

Condition A: (Scheme 2)

To the syringe containing peptidyl resin was added HATU (2 eq.), HOAt (2 eq.) and collidine (4 eq.) in DMF (0.4 M with respect to HATU) and the syringe was shaken for 1 h. The reagents mixture was removed then the peptidyl resin was washed with DMF (6 ×) and $CH_2Cl_2$ (3 ×).

Condition B: (Scheme S2, S3)

To the syringe containing peptidyl resin was added HATU (5 eq.), HOAt (5 eq.) and DIEA (10 eq.) in DMF (0.25 M with respect to HATU) and the syringe was shaken for 3 - 18 h. The reagents mixture was removed then the peptidyl resin was washed with DMF (6 ×) and $CH_2Cl_2$ (3 ×).

### 3.2.4.7 Rapid Fmoc deprotection; Subsequent Amino acid coupling (Scheme 2, S-2)

After cyclization, the Fmoc group from the cyclic octadepsipeptide (**20**, **21**) was quickly removed (Fmoc cleavage condition B). The resulting peptidyl resin was immediately subjected to subsequent A.A. coupling (Coupling cocktail B). The mixture of Fmoc-D-Glu(O$^t$Bu)-OH, HBTU and HOBt in DMF was stirred for 20 min prior to the coupling, and DIEA was added just before the coupling cocktail was added to the syringe. The mixture was shaken for 1 h at room temperature.

### 3.2.4.8 Side chain elongation

The amino acid and/or β-hydroxy fatty acid were coupled to the peptidyl resin under the conditions described below.

| | |
|---|---|
| Fmoc deprotection: | Condition A. |
| Fmoc-A.A.: | Coupling cocktail A, 2 h. |
| β-hydroxy fatty acid: | Coupling cocktail C, 2 h. |

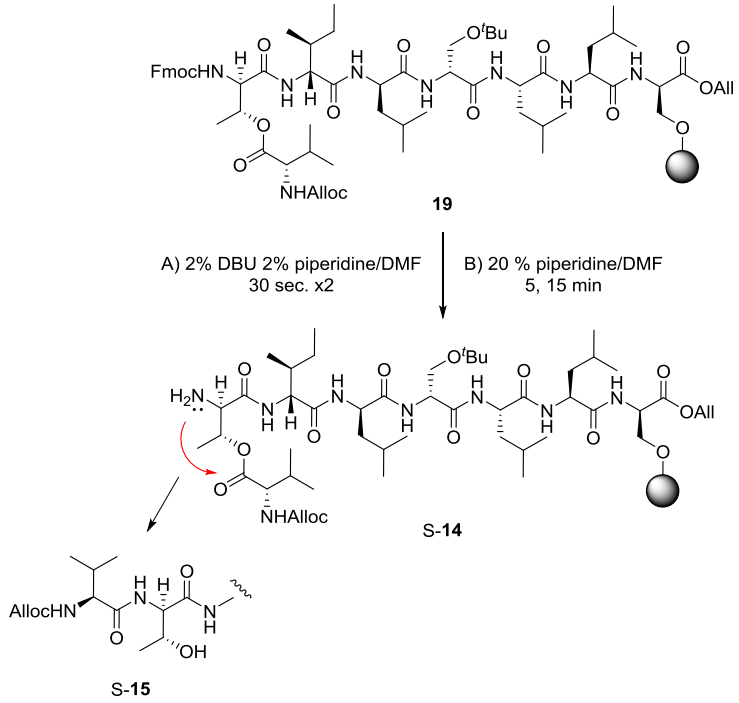
### 3.2.4.9 Cleavage and global deprotection of peptide from resin

Peptidyl resin was dried under high vacuum prior to peptide cleavage from resin. Peptides were cleaved from resin by cleavage condition B (approx. 100 mg peptidyl resin/1 mL cleavage cocktail). The syringe was shaken for 20 min at room temperature and the solution was collected in a round bottom flask. This procedure was repeated three times. The resulting mixture was stirred further at room temperature until the full deprotection was observed by LC/MS. Then the mixture evaporated with toluene and the crude mixture was purified by prep. RP-HPLC.

### 3.2.4.10 *O*→*N* acyl shift during Fmoc deprotection in linear and cyclic octadepsi-peptide

The *O*→*N* shift in two Fmoc deprotection conditions (section **3.2.4.1**) was monitored on resin for linear and cyclic resin-bound octadepsipeptides **19** and **21**. After the deprotection, the resin was quickly washed with $CH_2Cl_2$ (3 x). Resulting peptides were cleaved from resin (cleavage condition A) and monitored by RP-HPLC (Figure S3 and S4). Eluate corresponding to peaks for unchanged amine and *O*→*N* shifted amide was independently retrieved by RP-HPLC and subjected to LC/HRMS analysis.

**HRMS** (ESI-TOF) calculated for S-**14** $C_{50}H_{88}N_8O_{14}$ $[M+H]^+$ 1025.6493; found 1025.6489;
**HRMS** (ESI-TOF) calculated for S-**15** $C_{50}H_{88}N_8O_{14}$ $[M+H]^+$ 1025.6493; found 1025.6494;
**HRMS** (ESI-TOF) calculated for **23** $C_{43}H_{78}N_8O_{11}$ $[M+H]^+$ 883.5863; found 883.5863;
**HRMS** (ESI-TOF) calculated for **24** $C_{43}H_{78}N_8O_{11}$ $[M+H]^+$ 883.5863; found 883.5865.




**Scheme S5**. *O*→*N* acyl shift of Fmoc deprotection in linear depsipeptide **19**.

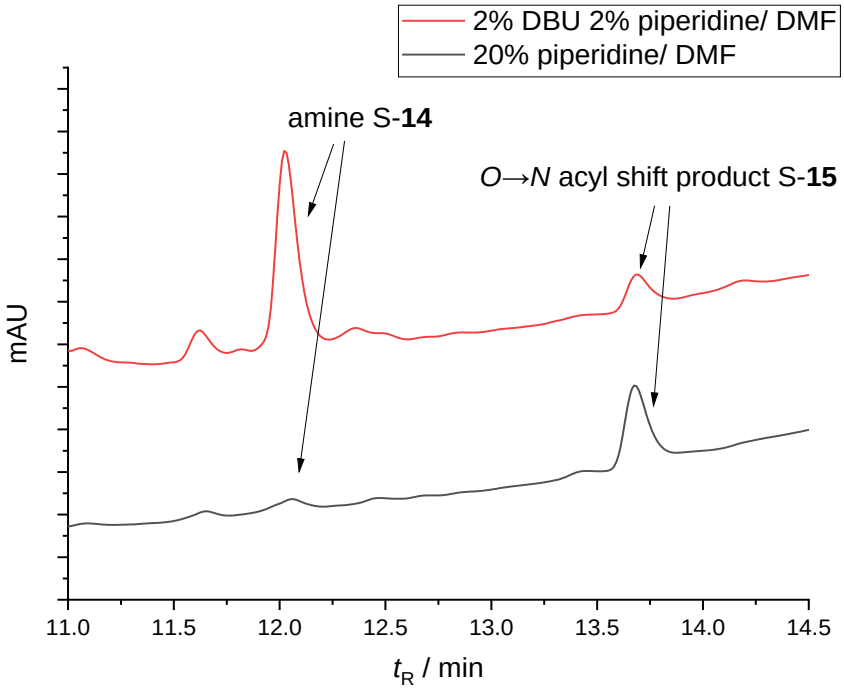



**Figure S4**. HPLC traces of Fmoc deprotection of linear octadepsipeptide **19**. (Condition: RP-A, 220 nm).

**Table S6.** Comparison of Fmoc deprotection conditions with linear octadepsipeptide **19**.

| Entry | Fmoc cleavage Conditions | Ratio (Amine S-**14** :*O*→*N* acyl Shift S-**15**) |
|---|---|---|
| 1 | 2% DBU 2% piperidine / DMF | 85:15 |
| 2 | 20% piperidine / DMF | 13:86 |

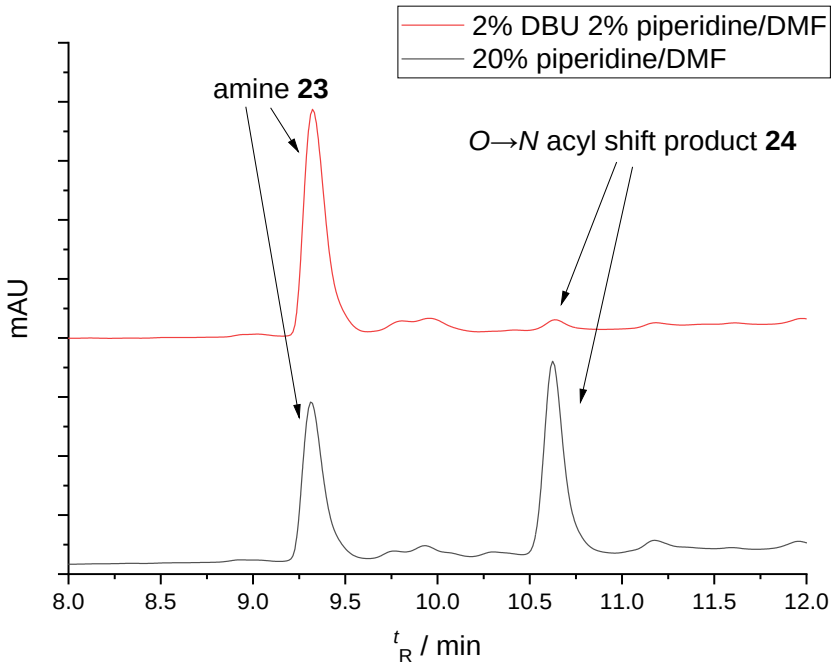



**Figure S5.** HPLC traces of Fmoc deprotection of cyclic octadepsipeptide **21**. (Condition: RP-B, 220 nm).

**Table S7.** Comparison of Fmoc deprotection conditions with cyclic depsipeptide **21**.

| Entry | Fmoc cleavage conditions | Ratio (Amine **23** :*O*→*N* acyl shift **24**) |
|---|---|---|
| 1 | 2% DBU 2% piperidine / DMF | 96:4 |
| 2 | 20% piperidine / DMF | 46:54 |

### 3.3 $^{1}$H NMR comparison of synthetic and natural orfamide A

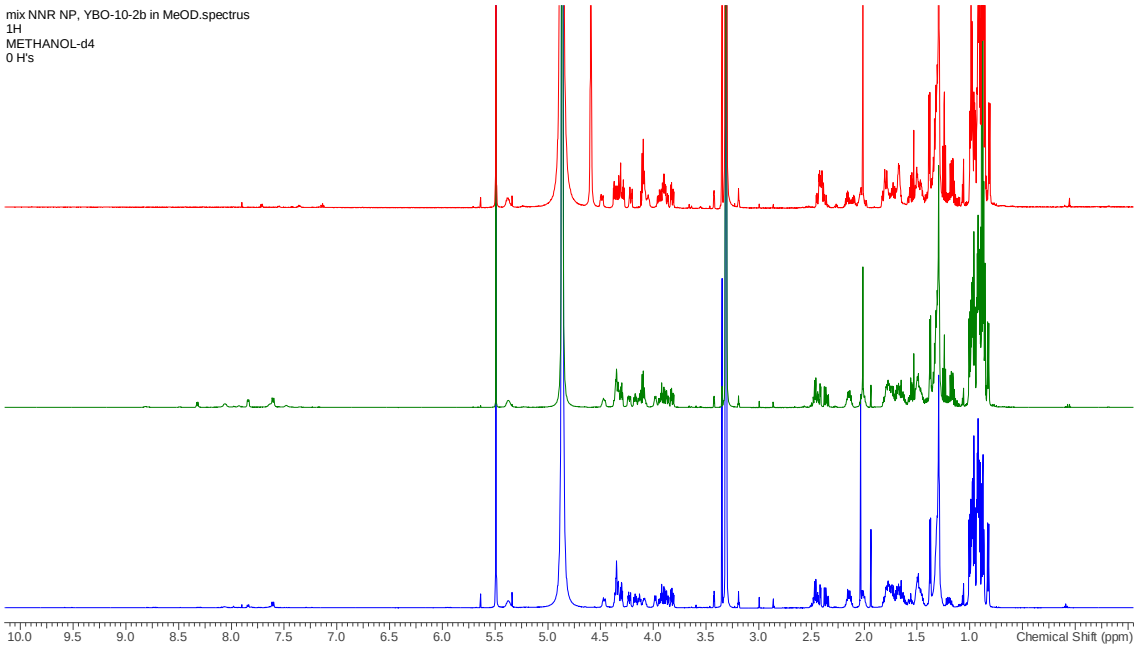



**Figure S6.** $^{1}$H NMR spectra of synthetic **3** and isolated orfamide A (commercial, Santa Cruz Biotechnology) in MeOH-$d_4$ (600 MHz, 297 K). (top: natural product 0.25 mg / center: mixture of the natural product and **3** / bottom: pure compound **3** 0.40 mg)

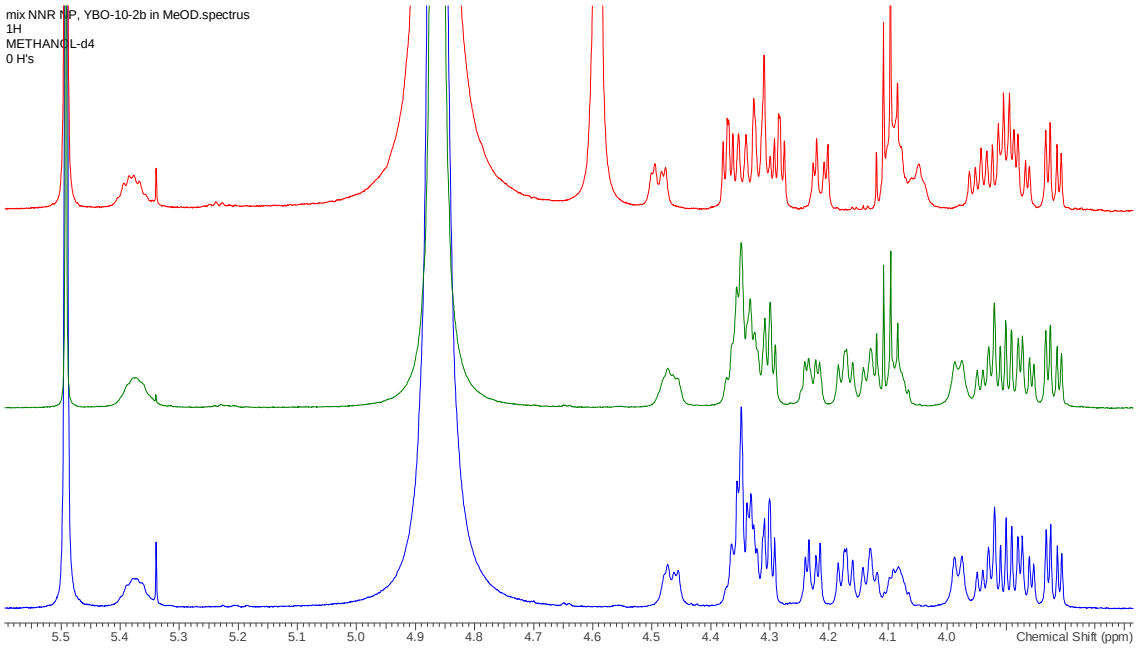



**Figure S7.** $^{1}$H NMR spectra of synthetic **3** and isolated orfamide A (commercial) in MeOH-$d_4$ (600 MHz, 3.7 – 5.5 ppm).

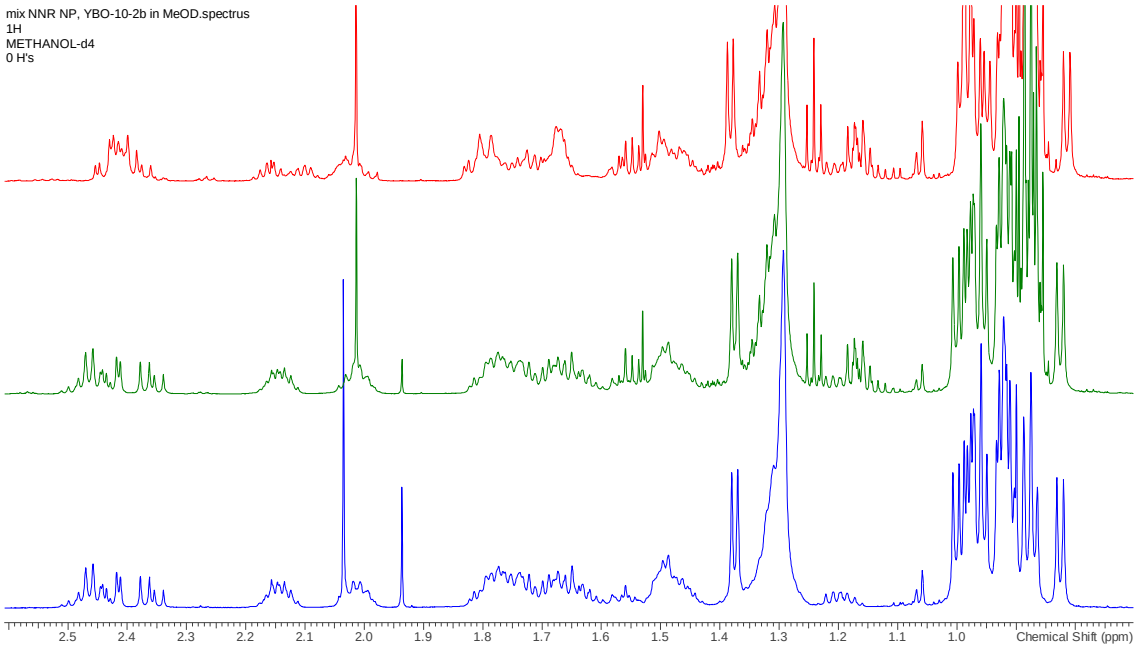



**Figure S8.** $^{1}$H NMR spectra of synthetic **3** and isolated orfamide A in MeOH-$d_4$ (600 MHz, 0.75 – 2.6 ppm).

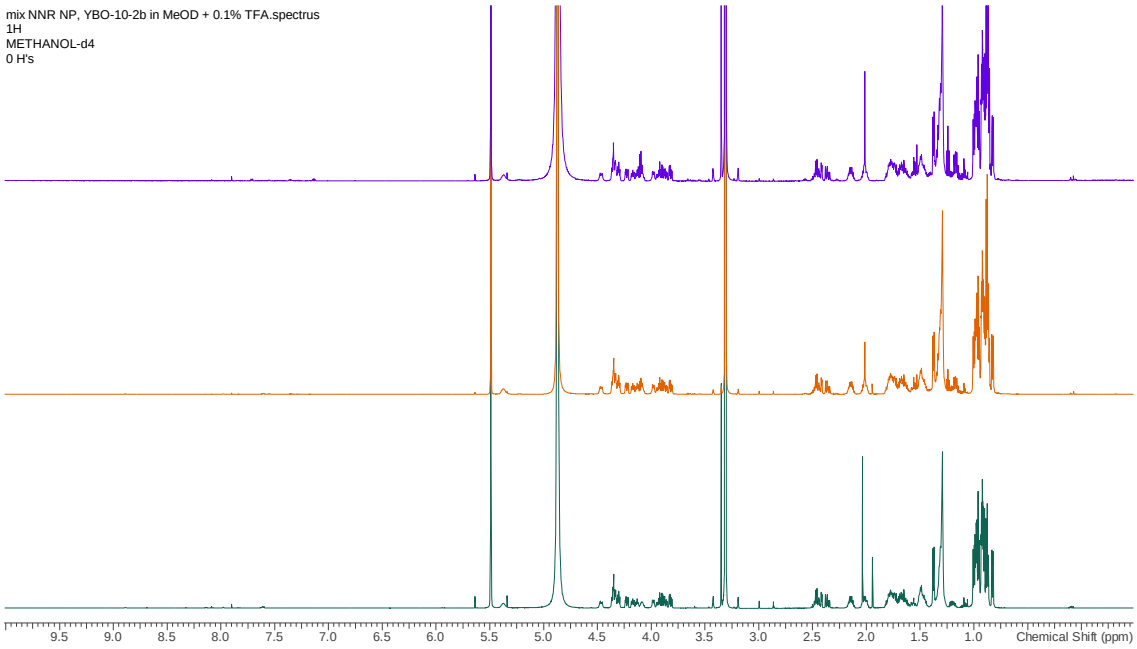



**Figure S9.** $^{1}$H NMR spectra of synthetic **3** and isolated orfamide A in MeOH-$d_4$ + 0.1% TFA (600 MHz, 297 K). (top: natural product / center: mixture of natural product and **3** / bottom: **3**).

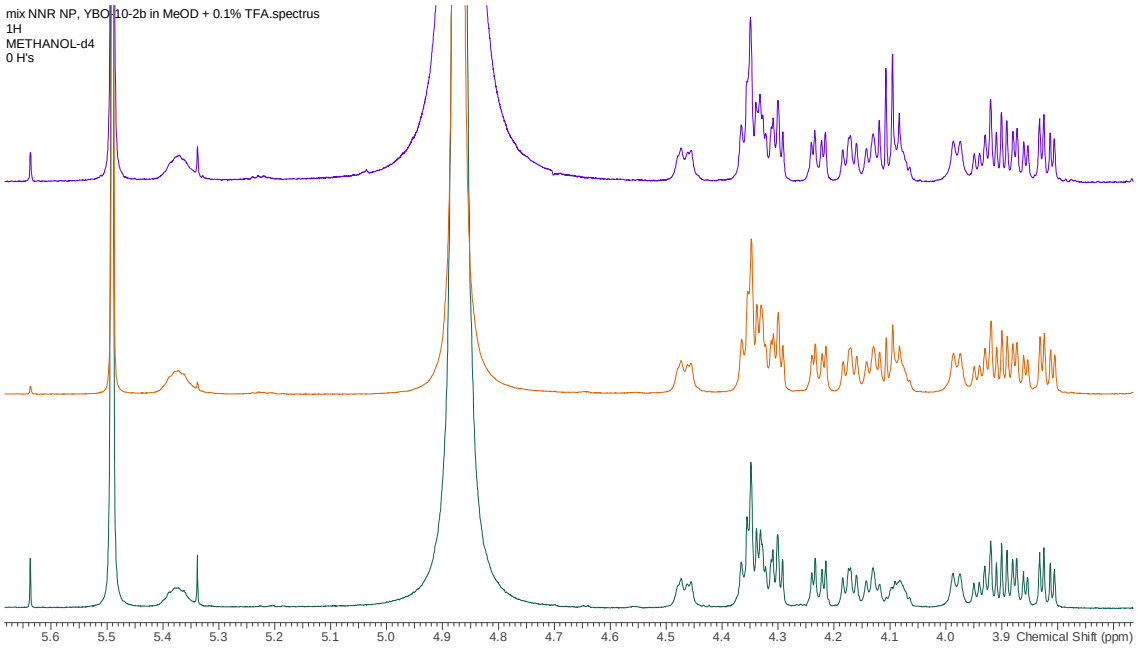



**Figure S10.** $^{1}$H NMR spectra of synthetic **3** and isolated orfamide A in MeOH-$d_4$ + 0.1% TFA. (600 MHz, 3.7 - 5.6 ppm.)

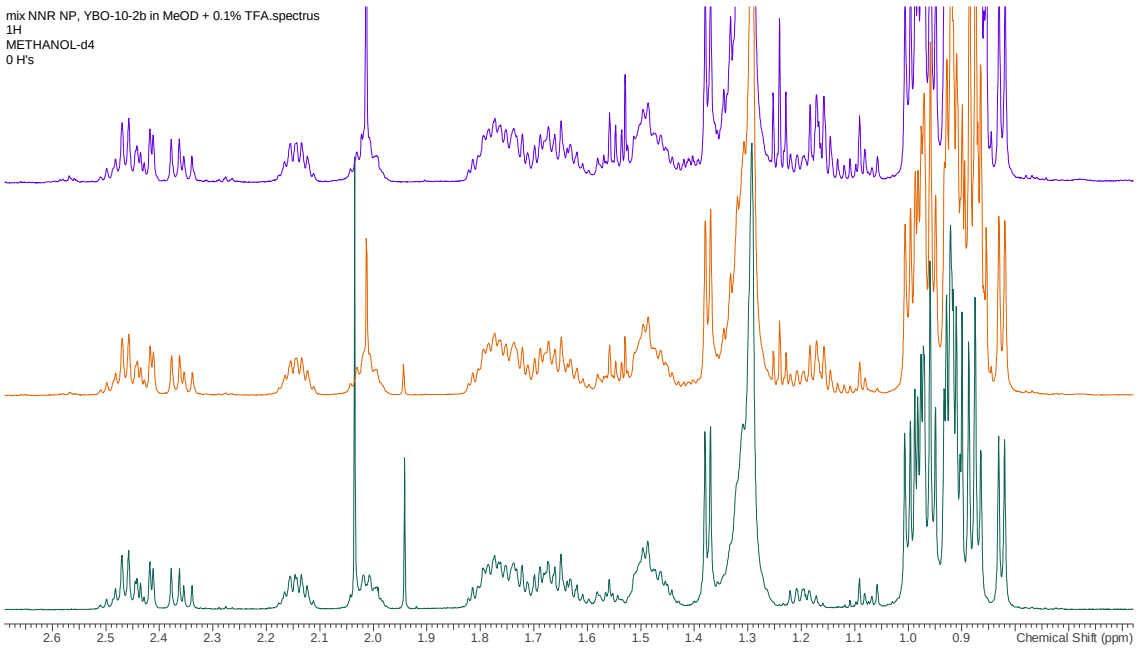
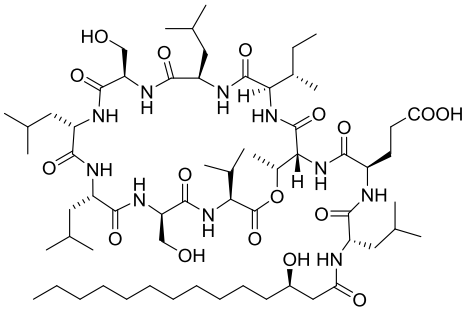



**Figure S11.** $^{1}$H NMR spectra of synthetic **3** and isolated orfamide A in MeOH-$d_4$ + 0.1% TFA. (600 MHz, 0.6 – 2.6 ppm.)

### 3.4 Analytical data of synthesized cyclic lipodepsipeptides

#### 3.4.1 Synthetic orfamide A (3)



orfamide A (revised structure)
**3**

Obtained as a colorless solid (7.0 – 36.3 mg, 5.40 – 28.0 µmol, 33 - 35% from resin loading 0.51 - 0.52 mmol/g (Scheme 2) or 1.7 mg, 1.31 µmol, 6.9% from resin loading 0.53 mmol/g (Scheme S2)).

**RP-HPLC**: $t_R$ = 14.4 min (RP-D).

**RP-prep.HPLC**: Prep.-D.

**$^1$H-NMR** (MeOH-$d_4$, 600 MHz, 297 K): $\delta$ = 0.82 - 1.01 (m, 39 H; Leu C$\underline{H}_3$ × 8, Ile C$\underline{H}_3$ × 2, Val C$\underline{H}_3$ × 2, F.A. C$\underline{H}_3$), 1.20 (m, 1 H; Ile C$\underline{H}_{2\gamma}$), 1.25 - 1.35 (m, 18 H; F.A. C$\underline{H}_2$, Leu C$\underline{H}_{2\beta}$), 1.38 (d, $J$ = 6.1 Hz, 3 H; Thr C$\underline{H}_3$), 1.42 - 1.83 (m, 15 H; Leu C$\underline{H}_\gamma$, Leu C$\underline{H}_{2\beta}$, Ile C$\underline{H}_{2\gamma}$, F.A. C$\underline{H}_2$), 2.02 (m, 2 H; Ile C$\underline{H}_\beta$, Glu C$\underline{H}_{2\beta}$), 2.15 (m, 2 H; Val C$\underline{H}_\beta$, Glu C$\underline{H}_{2\beta}$), 2.36 (dd, $J$ = 14.2, 9.1 Hz, 1 H; F.A. C$\underline{H}_{2\alpha}$), 2.46 (m, 3 H; F.A. C$\underline{H}_{2\alpha;}$ Glu C$\underline{H}_{2\gamma}$), 3.82 (dd, $J$ = 11.4, 4.5 Hz, 1 H; Ser C$\underline{H}_{2\beta}$), 3.87 (dd, $J$ = 11.6, 4.4 Hz, 1 H; Ser C$\underline{H}_{2\beta}$), 3.90 (dd, $J$ = 11.4, 5.8 Hz, 1 H; Ser C$\underline{H}_{2\beta}$), 3.94 (dd, $J$ = 11.6, 5.6 Hz, 1 H; Ser C$\underline{H}_{2\beta}$), 3.98 (d, $J$ = 7.7 Hz, 1 H; Ile C$\underline{H}_\alpha$), 4.08 (m, 1 H; F.A. C$\underline{H}_\beta$), 4.13 (t, $J$ = 7.3 Hz, 1 H; Leu C$\underline{H}_\alpha$), 4.17 (dd, $J$ = 8.7, 6.1 Hz, 1 H; Glu C$\underline{H}_\alpha$), 4.22 (dd, $J$ = 11.0, 4.0 Hz, 1 H; Leu C$\underline{H}_\alpha$), 4.31 (m, 1H; Ser C$\underline{H}_\alpha$), 4.32 (m, 1 H; Leu C$\underline{H}_\alpha$), 4.33 (m, 1H; Ser C$\underline{H}_\alpha$), 4.35 (m, 1H; Thr C$\underline{H}_\alpha$), 4.37 (m, 1 H; Val C$\underline{H}_\alpha$), 4.46 (dd, $J$ = 10.8, 4.3 Hz, 1 H; Leu C$\underline{H}_\alpha$), 5.37 (m, 1 H; Thr C$\underline{H}_\beta$) ppm. (COO$\underline{H}$, CON$\underline{H}$ and O$\underline{H}$ not assigned).

**$^{13}$C{$^1$H}-NMR** (MeOH-$d_4$, 151 MHz, 297 K): $\delta$ = 11.8, 14.5, 16.3, 18.6, 18.9, 19.8, 21.2, 21.6, 22.0, 22.6, 23.1, 23.4, 23.7, 23.7, 23.8, 25.6, 25.8, 25.8, 25.9, 26.7, 27.1, 27.1, 30.5, 30.7, 30.8, 30.8, 30.9, 31.0, 31.5, 31.5, 33.1, 37.1, 38.6, 40.8, 40.9, 40.9, 41.4, 41.8, 44.5, 53.8, 54.3, 54.9, 55.0 , 55.1, 56.0, 57.8, 57.9, 58.3, 58.4, 59.6, 59.7, 60.7, 61.7, 62.6, 62.8, 69.9, 70.7, 171.0, 172.3, 172.4, 173.0, 174.9, 175.0, 175.2, 175.5, 175.6, 175.9, 176.3 ppm.

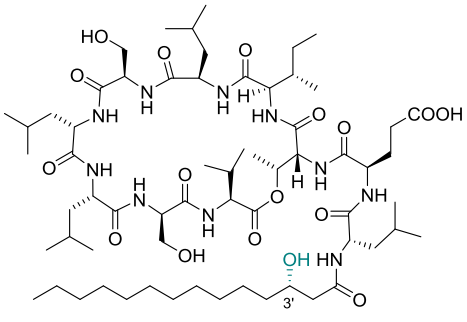
**$^1$H-NMR** (DMSO-$d_6$, 600 MHz, 297 K): $\delta$ = 0.66 (d, J = 6.4 Hz, 3 H; Leu C$\underline{H}_3$), 0.74 (d, $J$ = 6.8 Hz, 3 H; Val C$\underline{H}_3$), 0.77 - 0.92 (m, 33 H; Leu C$\underline{H}_3$ × 7, Val C$\underline{H}_{3,}$ Ile C$\underline{H}_3$ × 2, F.A. C$\underline{H}_3$), 1.05 (m, 1 H; Ile C$\underline{H}_2$), 1.14 (d, $J$ = 6.1 Hz, 3 H; Thr C$\underline{H}_3$), 1.16 - 1.27 (m, 17 H; F.A. C$\underline{H}_2$), 1.27 - 1.37 (m, 5 H; Leu C$\underline{H}_2$, Ile C$\underline{H}_2$, F.A. C$\underline{H}_2$), 1.43 (t, $J$ = 7.3 Hz, 2 H; Leu C$\underline{H}_2$), 1.47 (m, 1 H; Leu C$\underline{H}_2$), 1.50 - 1.75 (m, 9 H; Leu C$\underline{H}_2$, Leu C$\underline{H}_\gamma$, Glu C$\underline{H}_{2\beta}$), 1.86 (m, 2 H; Ile C$\underline{H}_\beta$, Glu C$\underline{H}_{2\beta}$), 2.12 (m, 1 H; Val C$\underline{H}_\beta$), 2.16 - 2.24 (m, 4 H; F.A. C$\underline{H}_{2\alpha}$, Glu C$\underline{H}_{2\gamma}$), 3.51 (m, 2 H; Ser C$\underline{H}_{2\beta}$), 3.59 (dd, $J$ = 10.9, 6.1 Hz, 1H; Ser C$\underline{H}_{2\beta}$), 3.64 (dd, $J$ = 10.9, 6.0 Hz, 1H; Ser C$\underline{H}_{2\beta}$), 3.78 (m, 1 H; F.A.

C$\underline{H}_{\beta}$), 4.05 (m, 1 H; Leu C$\underline{H}_{\alpha}$), 4.09 (m, 1 H; Leu C$\underline{H}_{\alpha}$), 4.13 (m, 2 H; Leu C$\underline{H}_{\alpha}$, Ile C$\underline{H}_{\alpha}$), 4.19 (q, *J* = 6.6 Hz, 1 H; Ser C$\underline{H}_{\alpha}$), 4.23 (m, 1 H; Glu C$\underline{H}_{\alpha}$), 4.30 (m, 2 H; Leu C$\underline{H}_{\alpha}$, Ser C$\underline{H}_{\alpha}$), 4.48 (m, 2 H; Val C$\underline{H}_{\alpha}$, Thr C$\underline{H}_{\alpha}$), 4.76 (br s, 1 H; O$\underline{H}$), 4.84 (br s, 1 H; O$\underline{H}$), 4.89 (m, 1 H; Thr C$\underline{H}_{\beta}$), 7.19 (br s, 1 H; Ser CON$\underline{H}$), 7.66 (d, *J* = 6.9 Hz, 1 H; Leu CON$\underline{H}$), 7.84 (m, 4 H; Ser, Leu, Val, Ile CON$\underline{H}$), 8.00 (d, *J* = 7.7 Hz, 1 H; Leu CON$\underline{H}$), 8.15 (d, *J* = 8.1 Hz, 2 H; Thr, Glu CON$\underline{H}$), 8.15 (d, *J* = 3.1 Hz, 1 H; Leu CON$\underline{H}$) 12.04 (br s, 1 H; Glu COO$\underline{H}$) ppm.

**$^{13}$C{$^{1}$H}-NMR** (DMSO-$d_6$, 126 MHz, 297 K): $\delta$ = 11.4, 14.0, 14.8, 17.0, 17.4, 19.0, 21.0, 21.0, 21.3, 21.7, 22.9, 22.9, 23.0, 23.2, 23.9, 24.2, 24.3, 25.1, 25.6, 27.0, 28.7, 29.0, 29.1, 29.1, 29.9, 30.0, 31.3, 35.7, 37.0, 39.8$^{*}$, 40.8, 43.4, 51.1, 51.9, 52.0, 52.2, 52.6, 55.1, 55.8, 56.3, 56.8, 56.9, 61.0, 61.9, 67.5, 70.1, 169.4, 170.2, 170.4, 171.1, 171.4, 171.7, 172.4, 172.5, 173.2, 173.8 ppm. ($^{*}$ From HSQC-DEPT.)

**HRMS** (ESI-TOF) calculated for $C_{64}H_{114}N_{10}O_{17}$ $[M+H]^+$ 1295.8436; found 1295.8438.

### 3.4.2 3'-*epi*-orfamide A (8)




Obtained as a colorless solid (5.6 mg, 4.32 µmol, 36% from resin loading 0.51 mmol/g).

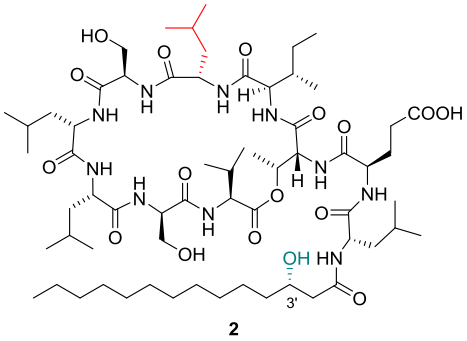
**RP-HPLC**: $t_R$ = 11.4 min (RP-D).

**RP-prep.HPLC**: Prep.-A.

**$^1$H-NMR** (MeOH-$d_4$, 500 MHz, 297 K): $\delta$ = 0.83 - 1.01 (m, 39 H), 1.19 (m, 1 H), 1.25 - 1.41 (m, 18 H), 1.34 (d, *J* = 6.1 Hz, 3 H), 1.41 - 1.85 (m, 15 H), 1.99 (m, 2 H), 2.17 (m, 2 H), 2.37-2.48 (m, 4 H), 3.82 - 3.89 (m , 3 H), 3.91 (dd, *J* = 11.3, 5.5 Hz, 1 H), 4.03 (m, 1 H), 4.10 (d, *J* = 7.2 Hz, 1 H), 4.16 - 4.27 (m, 3 H), 4.33 (m, 3 H), 4.40 (m, 3 H), 5.32 (m, 1H) ppm. (COOH, CONH and OH not assigned).

**HRMS** (ESI-TOF) calculated for $C_{64}H_{114}N_{10}O_{17}$ $[M+H]^+$ 1295.8436; found 1295.8437.

### 3.4.3 Formerly proposed[6] structure of orfamide A (2)



**2**

Obtained as a colorless solid (2.4 mg, 1.85 µmol, 2.2% from resin loading 0.82 mmol/g (Scheme S3), or 10.0 mg, 7.72 µmol, 36% from resin loading 0.52 mmol/g (Scheme 2)).

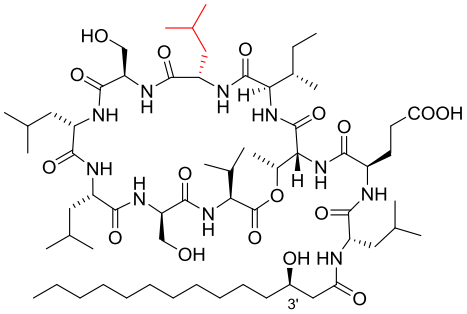
**RP-HPLC**: $t_R$ = 9.15 min (RP-D).

**RP-prep. HPLC**: Prep.-A.

**$^1$H-NMR** (MeOH-$d_4$, 600 MHz, 297 K): $\delta$ = 0.85 - 1.01 (m, 39 H), 1.15 (m, 1 H), 1.23 - 1.40 (m, 21 H), 1.56 - 1.41 (m, 5 H), 1.56 - 1.74 (m, 11 H), 1.87 (m, 1 H), 1.92 (m, 1 H), 2.30 (m, 1 H), 2.35 (m, 2 H), 2.40 (m, 2 H), 3.77 (dd, *J* = 11.0, 5.3 Hz, 1 H), 3.82 (dd, *J* = 10.9, 4.8 Hz, 1 H), 3.88 (dd, *J* = 10.9, 4.8 Hz, 1 H), 3.95 (dd, *J* = 11.0, 5.3 Hz, 1 H), 4.03 (m, 1 H), 4.32 - 4.37 (m, 3 H), 4.38 - 4.47 (m, 7 H), 4.50 (t, *J* = 6.6 Hz, 1 H), 4.58 (t, *J* = 5.2 Hz, 1 H), 4.72 (d, *J* = 7.5 Hz, 1 H), 5.19 (m, 1 H) ppm. (COO<u>H</u>, CON<u>H</u> and O<u>H</u> not assigned).

**HRMS** (ESI-TOF) calculated for $C_{64}H_{114}N_{10}O_{17}$ $[M+H]^+$ 1295.8436; found 1295.8436.

### 3.4.4 Compound 5 (L-$^{1}$Leu derivative)



**5**

Obtained as a colorless solid (2.2 mg, 1.70 µmol, 2.0% from resin loading 0.82 mmol/g (Scheme S3), or 11.8 mg, 9.11 µmol, 38% from resin loading 0.52 mmol/g (Scheme 2)).

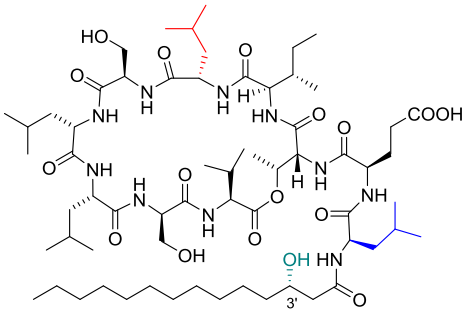
**RP-HPLC**: $t_R$ = 12.0 min (RP-D).

**RP-prep. HPLC**: Prep.-A.

**$^{1}$H-NMR** (MeOH-$d_4$, 600 MHz, 297 K): $\delta$ = 0.85 - 1.03 (m. 39 H), 1.15 (m, 1 H), 1.25 - 1.39 (18 H), 1.31 (d, *J* = 6.3 Hz, 3 H), 1.41 - 1.52 (m, 4 H), 1.52 - 1.75 (m, 12 H), 1.87 (m, 1 H), 1.93 (m, 1 H), 2.14 (m, 1 H), 2.29 (m, 1 H), 2.36 (m, 3 H), 2.44 (dd, *J* = 14.2, 4.2 Hz, 1 H), 3.79 (dd, *J* = 11.0, 4.9 Hz, 1 H), 3.82 (dd, *J* = 11.1, 5.0 Hz, 1 H), 3.89 (m, 1 H), 3.93 (dd, *J* = 11.1, 5.6 Hz, 1 H), 4.32 - 4.36 (m, 2 H), 4.37 – 4.41 (m, 2 H), 4.43 – 4.47 (m, 3 H), 4.54 (t, *J* = 5.3 Hz, 1 H), 4.67 (d, *J* = 7.7 Hz, 1 H), 5.21 (m, 1 H) ppm. (COO<u>H</u>, CON<u>H</u> and O<u>H</u> not assigned).

**HRMS** (ESI-TOF) calculated for $C_{64}H_{114}N_{10}O_{17}$ $[M+H]^+$ 1295.8436; found 1295.8434.

### 3.4.5 Compound 6 (3´-*S*-OH, D-$^{1}$Leu, L-$^{5}$Leu derivative)



**6**

Obtained as a colorless solid (8.7 mg, 6.71 µmol, 18% from resin loading 0.52 mmol/g (Scheme 2)).

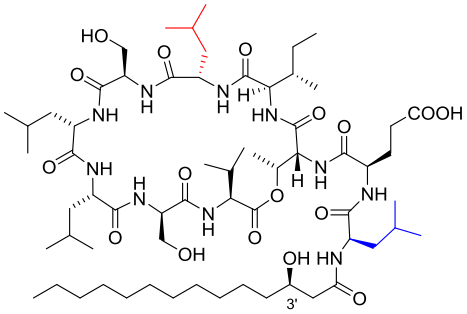
**RP-HPLC**: $t_R$ = 11.6 min (RP-D).

**RP-prep. HPLC**: Prep.-A.

**$^{1}$H-NMR** (MeOH-$d_4$, 600 MHz, 297 K): $\delta$ = 0.89 – 0.97 (m, 39 H), 1.16 (m, 1 H), 1.28 (d, *J* = 6.2 Hz, 3 H), 1.27 - 1.33 (m, 18 H), 1.44 - 1.51 (m, 4 H), 1.58 - 1.75 (m, 11 H), 1.88 (m, 1 H), 1.97 (m, 1 H), 2.08 (m, 1 H), 2.30 (m, 1 H), 2.33 - 2.45 (m, 3 H), 3.78 (dd, *J* = 11.0, 5.1 Hz, 1 H), 3.81 - 3.91 (m, 3 H), 4.00 (m, 1 H), 4.28 (d, *J* = 6.4 Hz, 1 H), 4.31 - 4.39 (m, 3 H), 4.39 - 4.44 (m, 2 H), 4.45 (m, 1 H), 4.48 (m, 1 H), 4.53 (t, *J* = 5.6 Hz, 1 H), 4.66 (d, *J* = 8.4 Hz, 1 H), 5.15 (m, 1 H) ppm. (CONH and OH not assigned).

**HRMS** (ESI-TOF) calculated for $C_{64}H_{114}N_{10}O_{17}$ $[M+H]^+$ 1295.8436; found 1295.8437.

### 3.4.6 Compound 7 (D-$^{1}$Leu, L-$^{5}$Leu derivative)




**7**

Obtained as a colorless solid (2.0 mg, 1.54 µmol, 6.4% from resin loading 0.80 mmol/g (Scheme S3), or 8.7 mg, 6.71 µmol, 40% from resin loading 0.52 mmol/g (Scheme 2)).

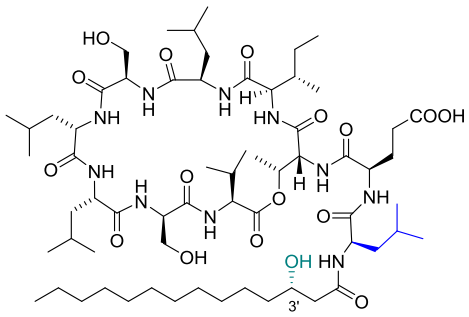
**RP-HPLC**: $t_R$ = 14.6 min (RP-D).

**RP-prep. HPLC**: Prep.-B or Prep.-D.

**$^{1}$H-NMR** (MeOH-$d_4$, 600 MHz, 297 K): $\delta$ = 0.84 - 1.02 (m, 39 H), 1.16 (m, 1 H), 1.25 - 1.43 (m, 21 H), 1.44 - 1.69 (m, 15 H), 1.88 (m, 1 H), 1.96 (m, 1 H), 2.07 (m, 1 H), 2.29 (m, 1 H), 2.40 (m, 4 H), 3.80 (dd, *J* = 11.1, 5.0 Hz, 1 H), 3.82 – 3.91 (m, 3 H), 4.03 (m, 1 H), 4.18 (d, *J* = 6.8 Hz, 1 H), 4.29 - 4.37 (m, 3 H), 4.40 - 4.45 (m, 4 H), 4.49 (t, *J* = 5.6 Hz, 1 H), 4.60 (d, *J* = 8.8 Hz, 1 H), 5.18 (m, 1H) ppm. (COOH, CONH and OH not assigned).

**HRMS** (ESI-TOF) calculated for $C_{64}H_{114}N_{10}O_{17}$ $[M+H]^+$ 1295.8436; found 1295.8437.

### 3.4.7 Compound 9 (3´-*S*-OH, D-$^{1}$Leu derivative)



**9**

Obtained as a colorless solid (7.0 mg, 5.40 µmol, 44% from resin loading 0.51 mmol/g (Scheme 2)).

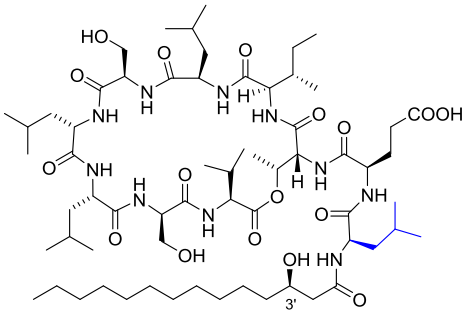
**RP-HPLC**: $t_R$ = 14.0 min (RP-D).

**RP-prep.HPLC**: Prep.-D.

**$^{1}$H-NMR** (MeOH-$d_4$, 500 MHz, 297 K): $\delta$ = 0.82 (d, *J* = 6.6 Hz, 3 H), 0.84 - 1.03 (m, 36 H), 1.17 (m, 1 H), 1.24 - 1.39 (m, 18 H), 1.32 (d, *J* = 6.0 Hz, 3 H), 1.42 - 1.90 (15 H), 2.04 (m, 3 H), 2.17 (m ,1 H), 2.39 (dd, *J* = 13.8, 9.3 Hz, 1 H), 2.45 (t, *J* = 7.1 Hz, 2 H), 2.56 (dd, *J* = 13.8, 4.0 Hz, 1 H), 3.81 - 3.85 (m, 3 H), 3.90 - 3.94 (m, 2 H), 4.10 - 4.18 (m, 3 H), 4.20 - 4.26 (m, 2 H), 4.29 (dd, *J* = 5.8, 4.2 Hz, 1 H), 4.34 (m, 2 H), 4.45 (m, 2 H), 5.37 (m, 1 H) ppm. (COO<u>H</u>, CON<u>H</u> and O<u>H</u> not assigned).

**HRMS** (ESI-TOF) calculated for $C_{64}H_{114}N_{10}O_{17}$ $[M+H]^+$ 1295.8436; found 1295.8436.

### 3.4.8 Compound 10 (D-$^{1}$Leu derivative)



**10**

Obtained as a colorless solid (6.7 mg, 5.17 µmol, 44% from resin loading 0.51 mmol/g (Scheme 2) or 2.1 mg, 1.62 µmol, 12% from resin loading 0.53 mmol/g (Scheme S2)).

**RP-HPLC**: $t_R$ = 15.5 min (RP-D).

**RP-prep.HPLC**: Prep.-C or Prep.-D.

**$^{1}$H-NMR** (MeOH-$d_4$, 600 MHz, 297 K): $\delta$ = 0.80 (d, J = 6.7 Hz, 3 H), 0.84 - 1.02 (m, 36 H), 1.17 (m, 1 H), 1.25 - 1.39 (m, 18 H), 1.35 (d, *J* = 6.0 Hz, 3 H), 1.45 - 1.93 (m, 15 H), 2.03 (m, 3 H), 2.17 (m, 1 H), 2.44 (m, 3 H), 2.53 (dd, *J* = 14.0, 4.1 Hz, 1 H), 3.73 (d, *J* = 9.1 Hz, 1 H), 3.844 (m, 2 H), 3.93 (m, 2 H), 4.06 (q, *J* = 8.1 Hz, 2 H), 4.17 (m, 1 H), 4.21 (m, 2 H), 4.27 (m, 1 H), 4.32 (m, 2 H), 4.48 (m, 2 H), 5.37 (m, 1 H) ppm. (CONH and OH not assigned).

**HRMS** (ESI-TOF) calculated for $C_{64}H_{114}N_{10}O_{17}$ $[M+H]^+$ 1295.8436; found 1295.8438.

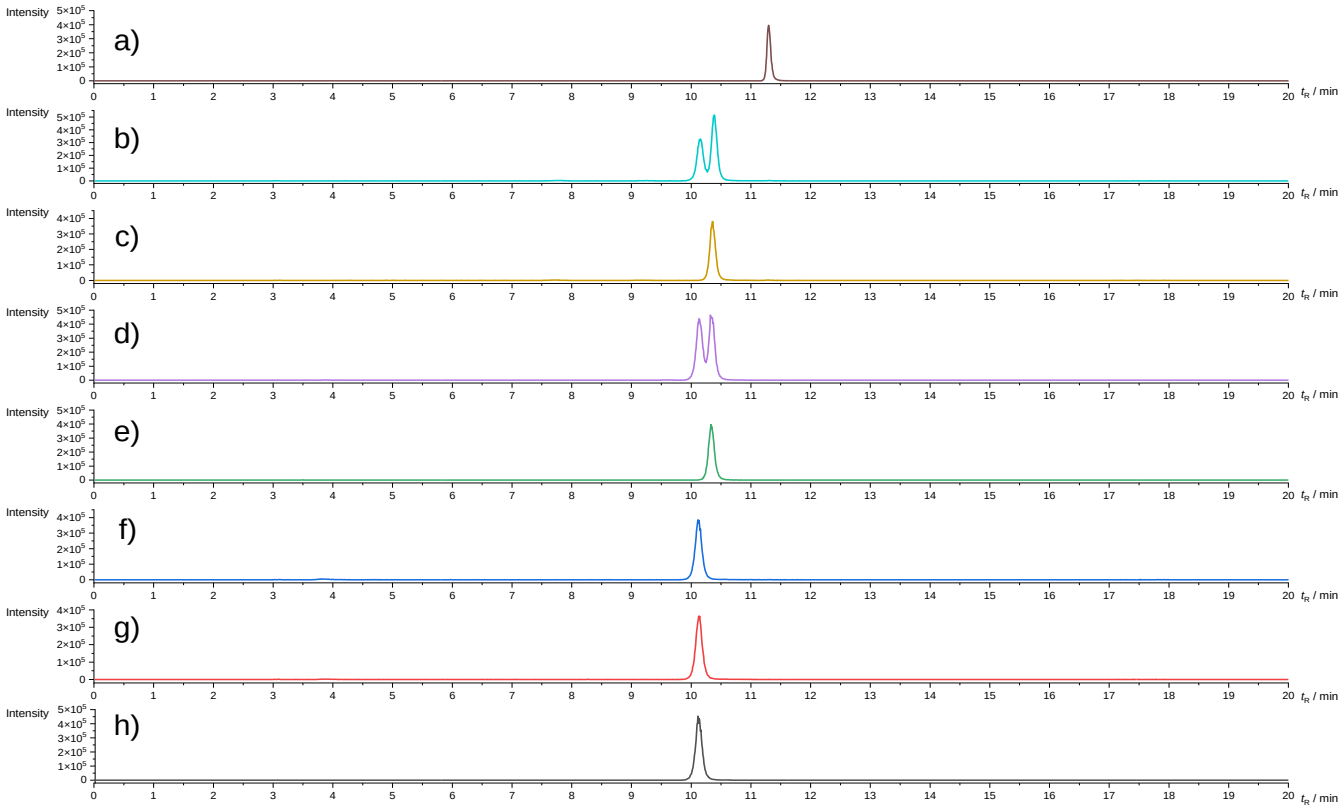
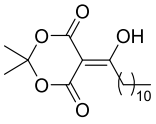
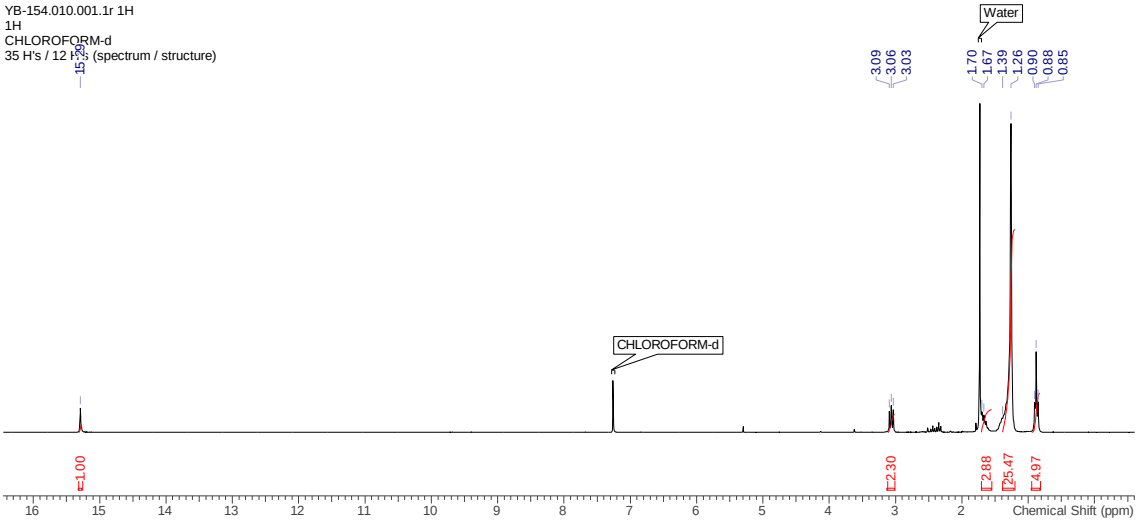


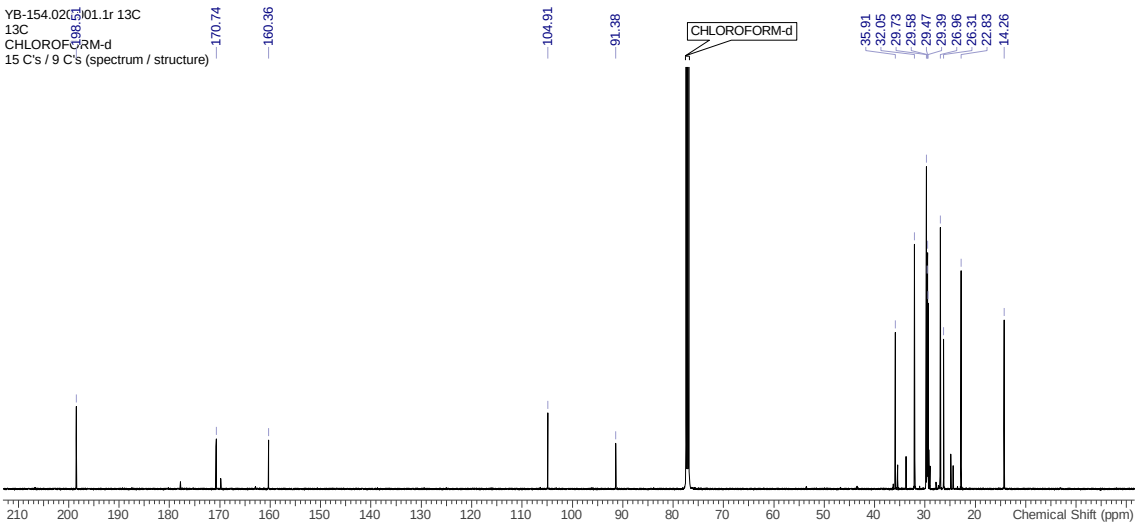

**Figure S12.** Extracted ion chromatogram (*m*/*Z*= 1295.8 ± 0.5 D) of the natural product (NP) and selected orfamide A derivatives (compare to Table 1 and Figure 4 in the main text): a) **10**; b) mixture of **9** and NP; c) **9**; d) mixture of **7** and NP; e) **7**; f) **3**; g) mixture of **3** and NP; h) NP.

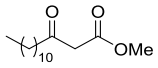
## 4 NMR Data


O
O
OH
O
O
10

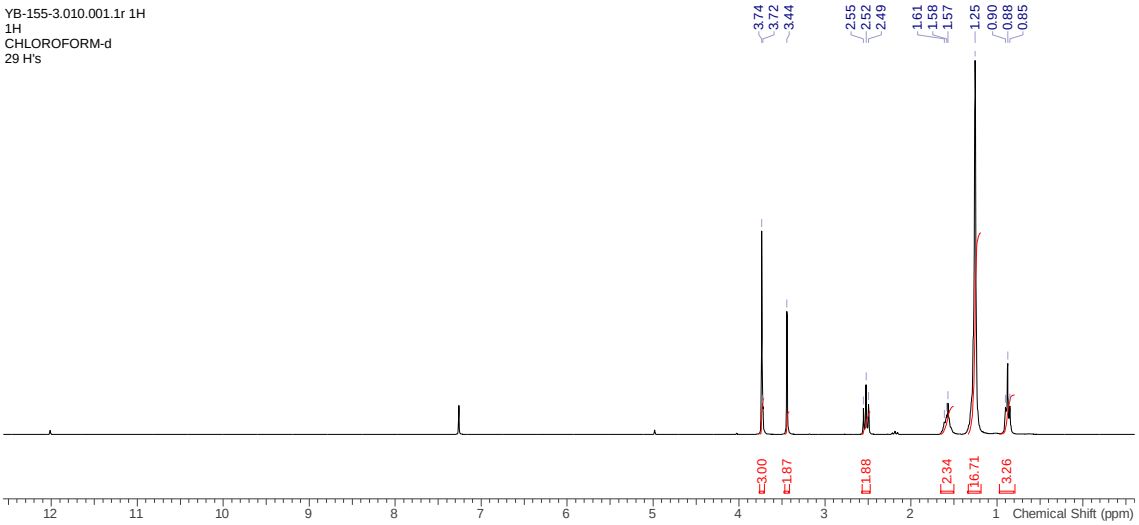
S-**7**

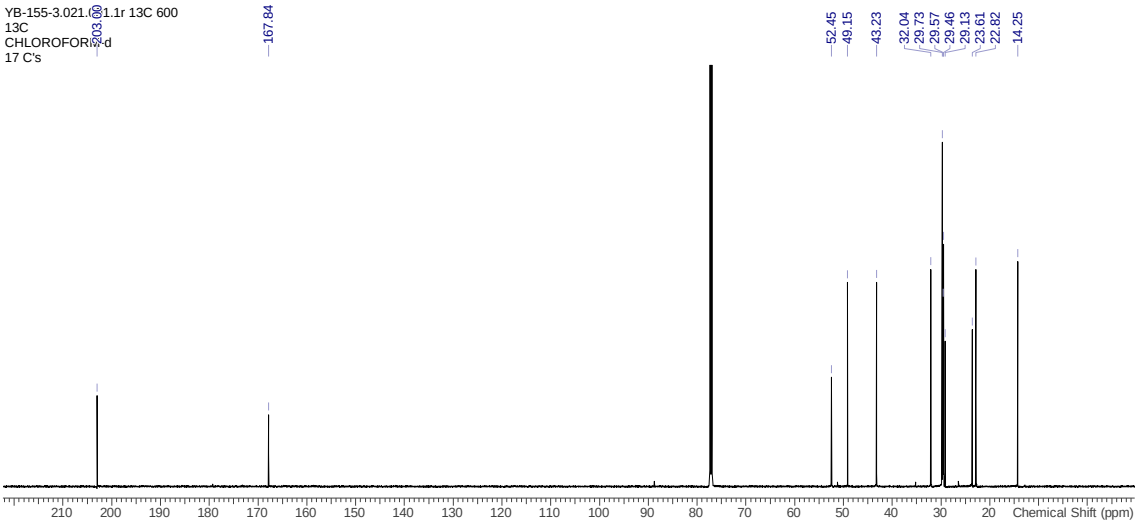

YB-154.010.001.1r 1H
1H
CHLOROFORM-d
35 H's / 12 H's (spectrum / structure)
15.29
3.09
3.06
3.03
1.70
1.67
1.39
1.26
0.90
0.88
0.85
Water
CHLOROFORM-d
1.00
2.30
2.88
25.47
4.97
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
Chemical Shift (ppm)

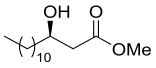

YB-154.020.001.1r 13C
13C
CHLOROFORM-d
15 C's / 9 C's (spectrum / structure)
96.51
170.74
160.36
104.91
91.38
CHLOROFORM-d
35.91
32.05
29.73
29.58
29.47
29.39
26.96
26.31
22.83
14.26
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
Chemical Shift (ppm)


O
O
OMe
10

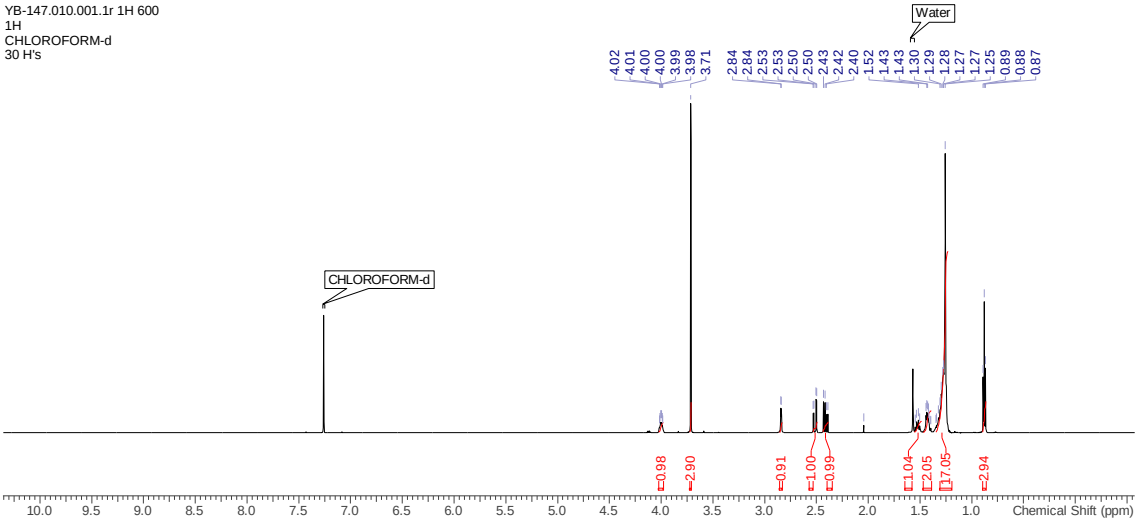
S-**2**

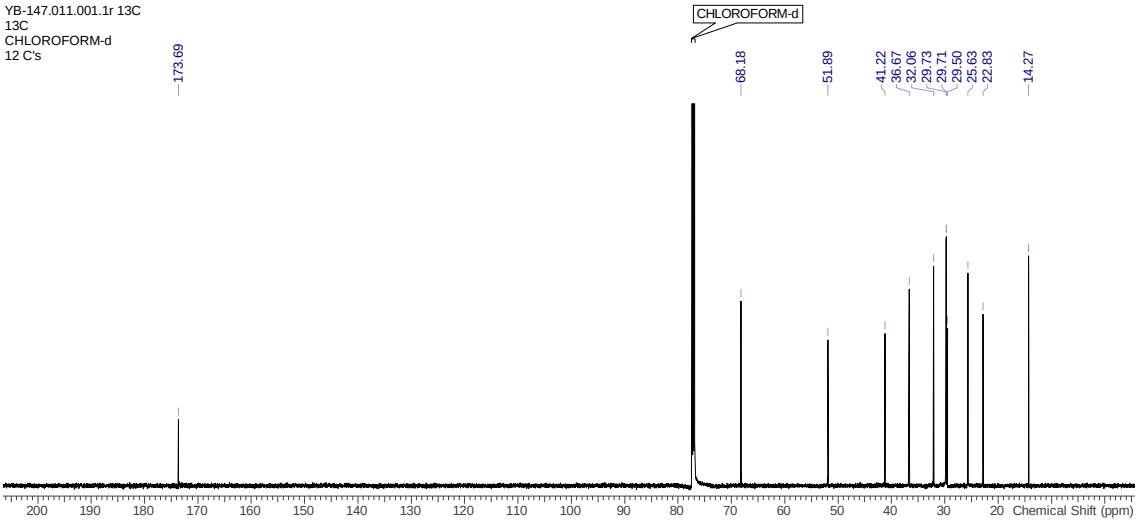

YB-155-3.010.001.1r 1H
1H
CHLOROFORM-d
29 H's
3.74
3.72
3.44
2.55
2.52
2.49
1.61
1.58
1.57
1.25
0.90
0.88
0.85
3.00
1.87
1.88
2.34
16.71
3.26
12
11
10
9
8
7
6
5
4
3
2
1
Chemical Shift (ppm)

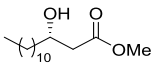

YB-155-3.021.001.1r 13C 600
13C
CHLOROFORM-d
17 C's
203.00
167.84
52.45
49.15
43.23
32.04
29.73
29.57
29.46
29.13
23.61
22.82
14.25
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
Chemical Shift (ppm)


OH
O
OMe
10

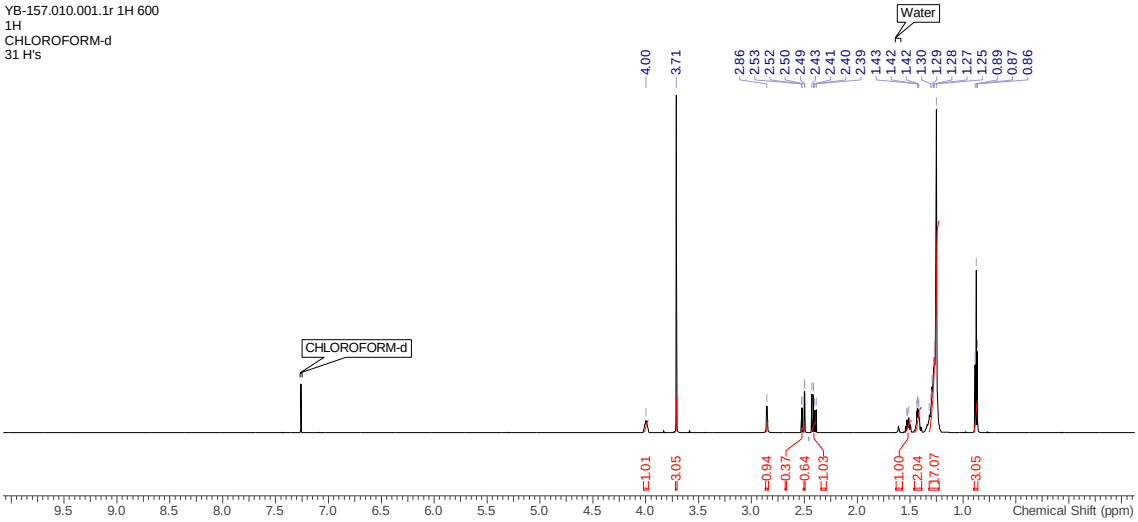
(*R*)-S-**3**

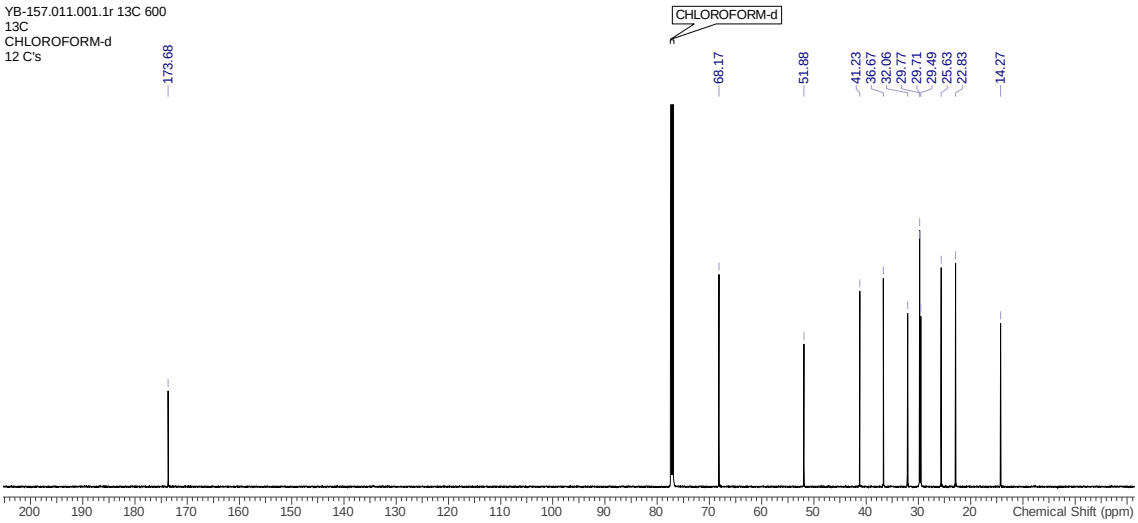

YB-147.010.001.1r 1H 600
1H
CHLOROFORM-d
30 H's
Water
CHLOROFORM-d
4.02
4.01
4.00
4.00
3.99
3.98
3.71
2.84
2.84
2.53
2.53
2.50
2.50
2.43
2.42
2.40
1.52
1.43
1.43
1.30
1.29
1.28
1.27
1.27
1.25
0.89
0.88
0.87
0.98
2.90
0.91
1.00
0.99
1.04
2.05
7.05
2.94
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

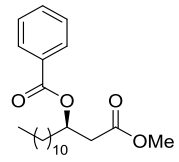

YB-147.011.001.1r 13C
13C
CHLOROFORM-d
12 C's
CHLOROFORM-d
173.69
68.18
51.89
41.22
36.67
32.06
29.73
29.71
29.50
25.63
22.83
14.27
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
Chemical Shift (ppm)


OH
O
OMe
10

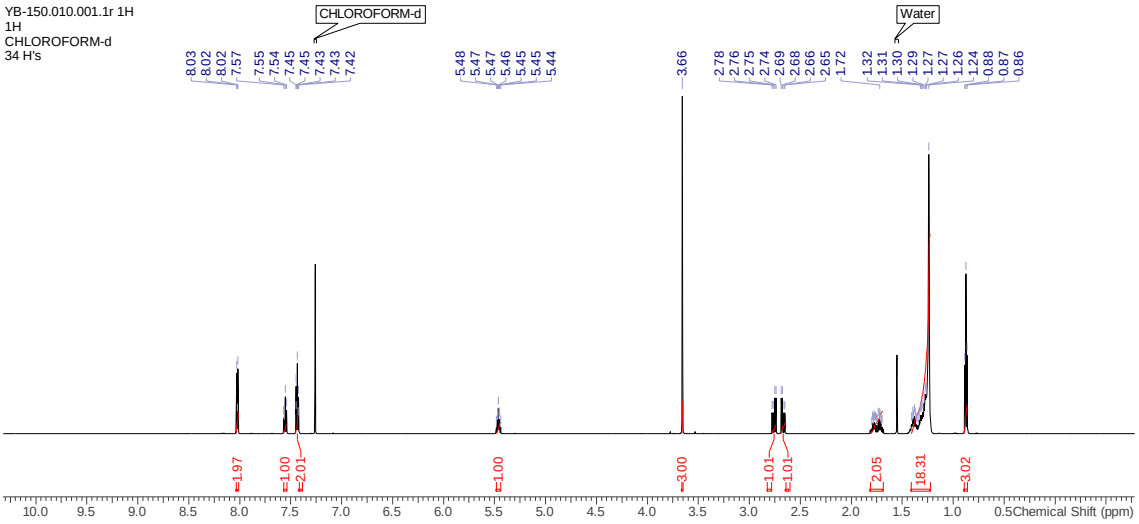
(*S*)-S-**3**

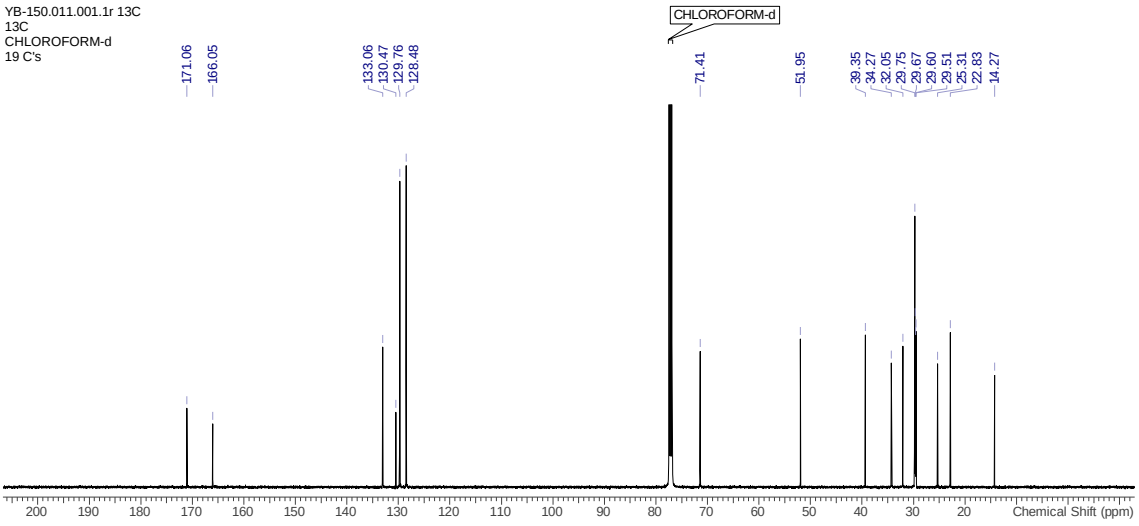

YB-157.010.001.1r 1H 600
1H
CHLOROFORM-d
31 H's
Water
CHLOROFORM-d
4.00
3.71
2.86
2.53
2.52
2.50
2.49
2.43
2.41
2.40
2.39
1.43
1.42
1.42
1.30
1.29
1.28
1.27
1.25
0.89
0.87
0.86
1.01
3.05
0.94
0.37
0.64
1.03
1.00
2.04
7.07
3.05
Chemical Shift (ppm)

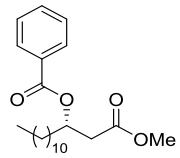

YB-157.011.001.1r 13C 600
13C
CHLOROFORM-d
12 C's
CHLOROFORM-d
173.68
68.17
51.88
41.23
36.67
32.06
29.77
29.71
29.49
25.63
22.83
14.27
Chemical Shift (ppm)


O
O
O
OMe
10

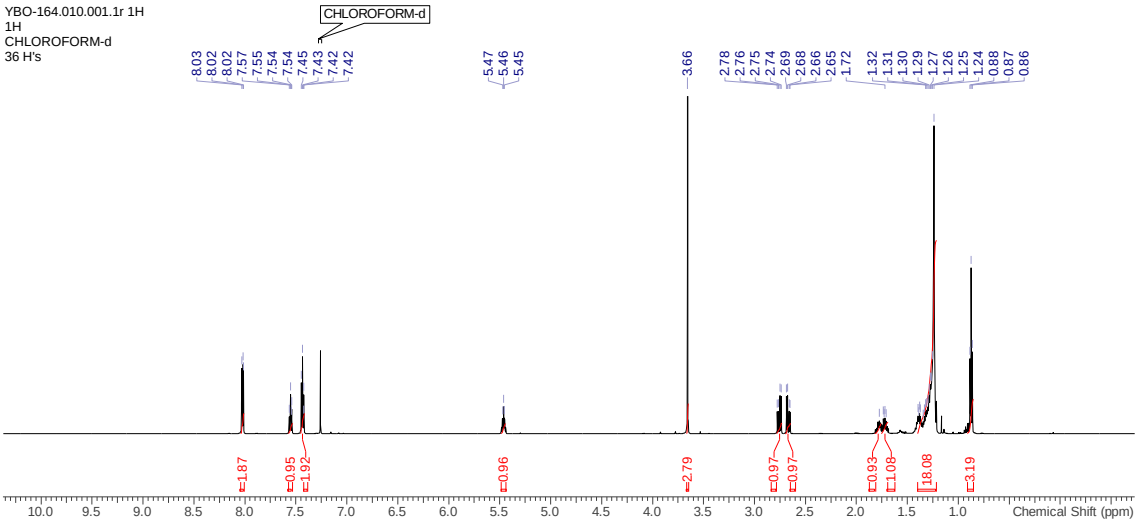
(*R*)-S-**4**

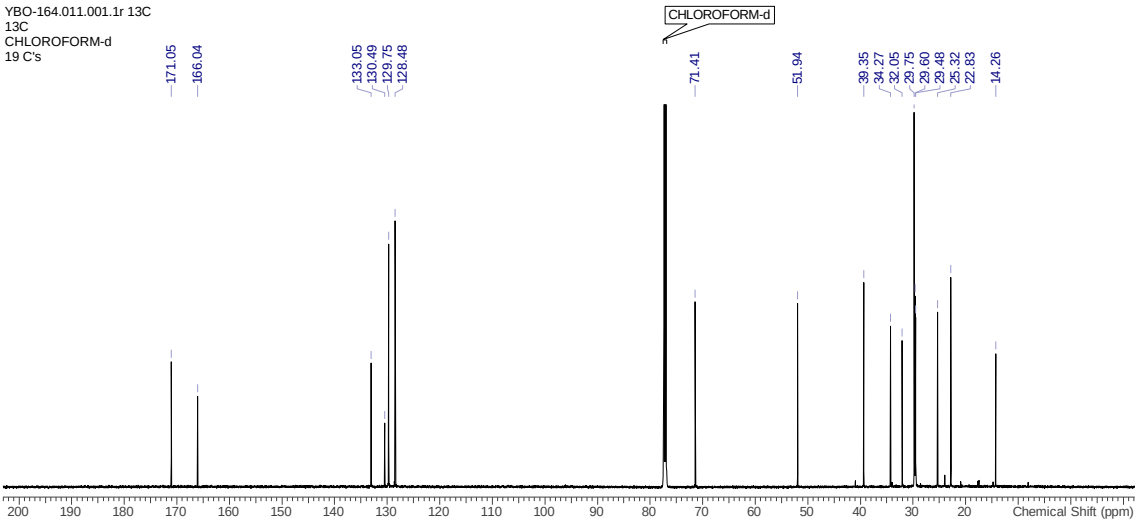

YB-150.010.001.1r 1H
1H
CHLOROFORM-d
34 H's
CHLOROFORM-d
Water
8.03
8.02
8.02
7.57
7.55
7.54
7.45
7.45
7.43
7.43
7.42
5.48
5.47
5.47
5.46
5.45
5.45
5.44
3.66
2.78
2.76
2.75
2.74
2.69
2.68
2.66
2.65
1.72
1.32
1.31
1.30
1.29
1.27
1.27
1.26
1.24
0.88
0.87
0.86
1.97
1.00
2.01
1.00
3.00
1.01
1.01
2.05
18.31
3.02
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Chemical Shift (ppm)

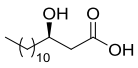

YB-150.011.001.1r 13C
13C
CHLOROFORM-d
19 C's
CHLOROFORM-d
171.06
166.05
133.06
130.47
129.76
128.48
77.41
51.95
39.35
34.27
32.05
29.75
29.67
29.60
29.51
25.31
22.83
14.27
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
Chemical Shift (ppm)


O
O
O
OMe
10

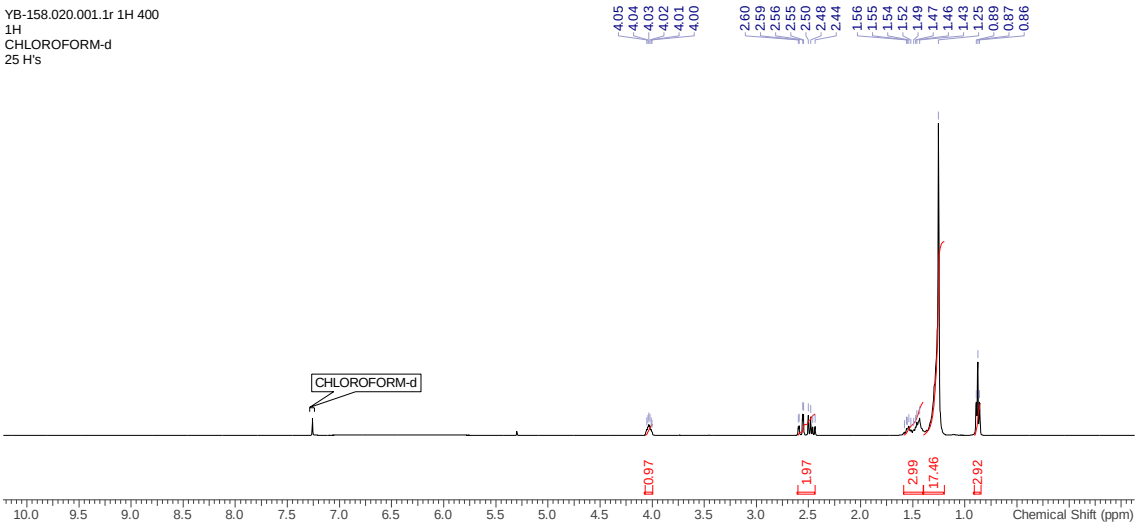
(*S*)-S-**4**

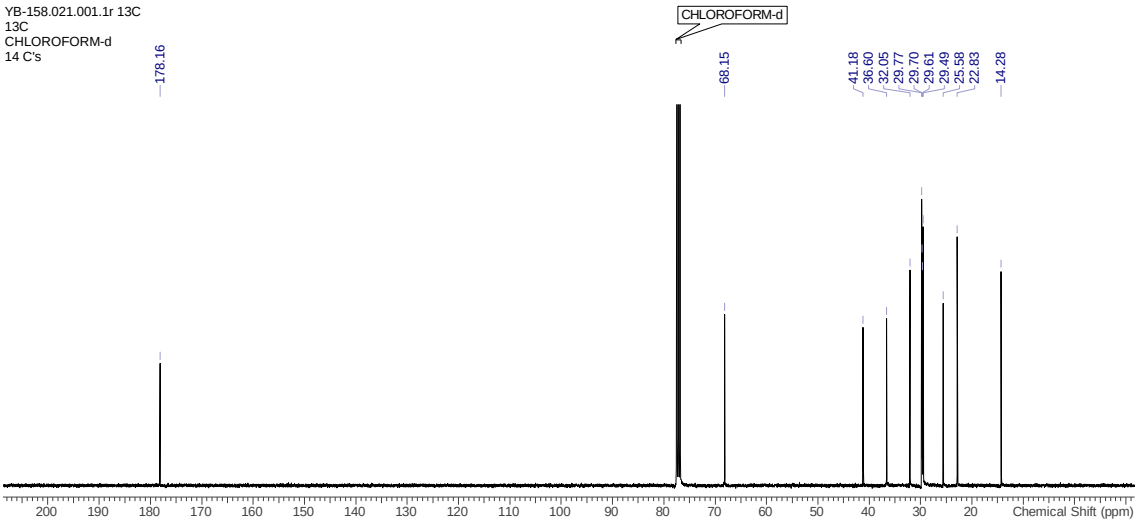

YBO-164.010.001.1r 1H
1H
CHLOROFORM-d
36 H's
CHLOROFORM-d
8.03
8.02
8.02
7.57
7.55
7.54
7.54
7.45
7.43
7.42
7.42
5.47
5.46
5.45
3.66
2.78
2.76
2.75
2.74
2.69
2.68
2.66
2.65
1.72
1.32
1.31
1.30
1.29
1.27
1.26
1.25
1.24
0.88
0.87
0.86
1.87
0.95
1.92
0.96
2.79
0.97
0.97
0.93
1.08
18.08
3.19
Chemical Shift (ppm)

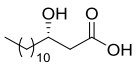

YBO-164.011.001.1r 13C
13C
CHLOROFORM-d
19 C's
CHLOROFORM-d
171.05
166.04
133.05
130.49
129.75
128.48
77.41
51.94
39.35
34.27
32.05
29.75
29.60
29.48
25.32
22.83
14.26
Chemical Shift (ppm)


OH
O
OH
10

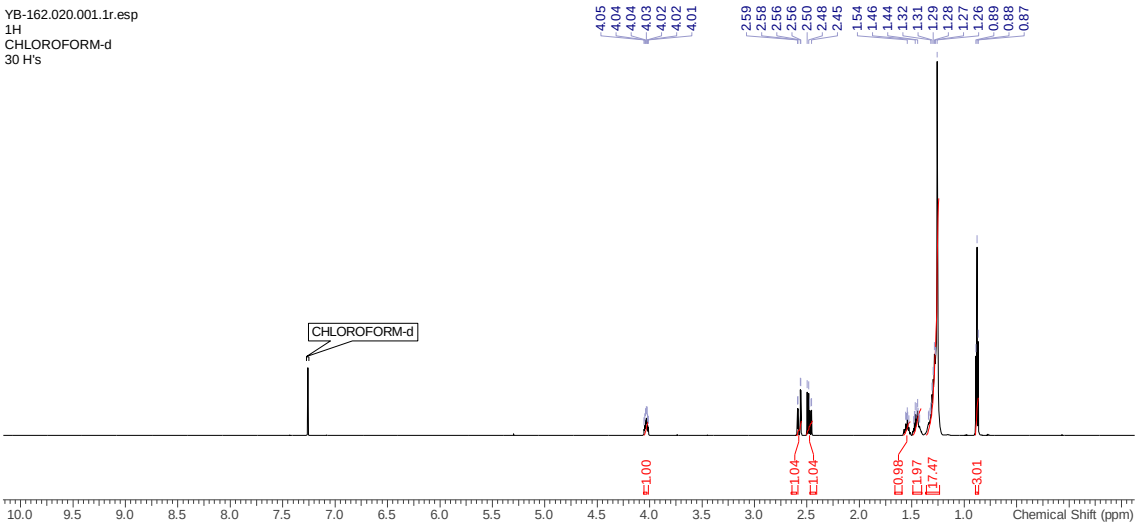
(*R*)-S-**5**

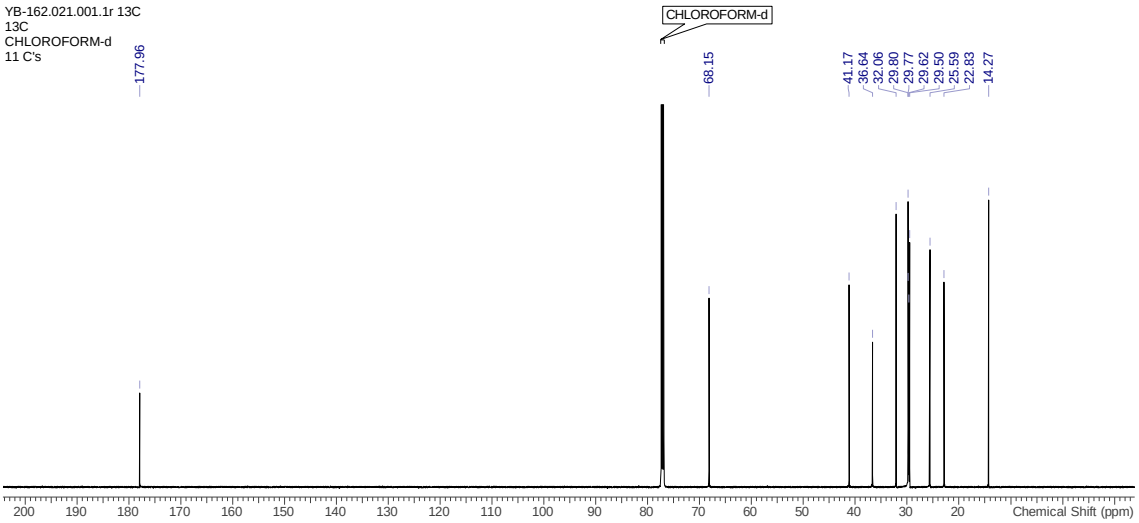

YB-158.020.001.1r 1H 400
1H
CHLOROFORM-d
25 H's
4.05
4.04
4.03
4.02
4.01
4.00
2.60
2.59
2.56
2.55
2.50
2.48
2.44
1.56
1.55
1.54
1.52
1.49
1.47
1.46
1.43
1.25
0.89
0.87
0.86
CHLOROFORM-d
0.97
1.97
2.99
17.46
2.92
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

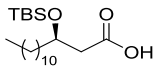

YB-158.021.001.1r 13C
13C
CHLOROFORM-d
14 C's
178.16
CHLOROFORM-d
68.15
41.18
36.60
32.05
29.77
29.70
29.61
29.49
25.58
22.83
14.28
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
Chemical Shift (ppm)


OH
O
OH
10

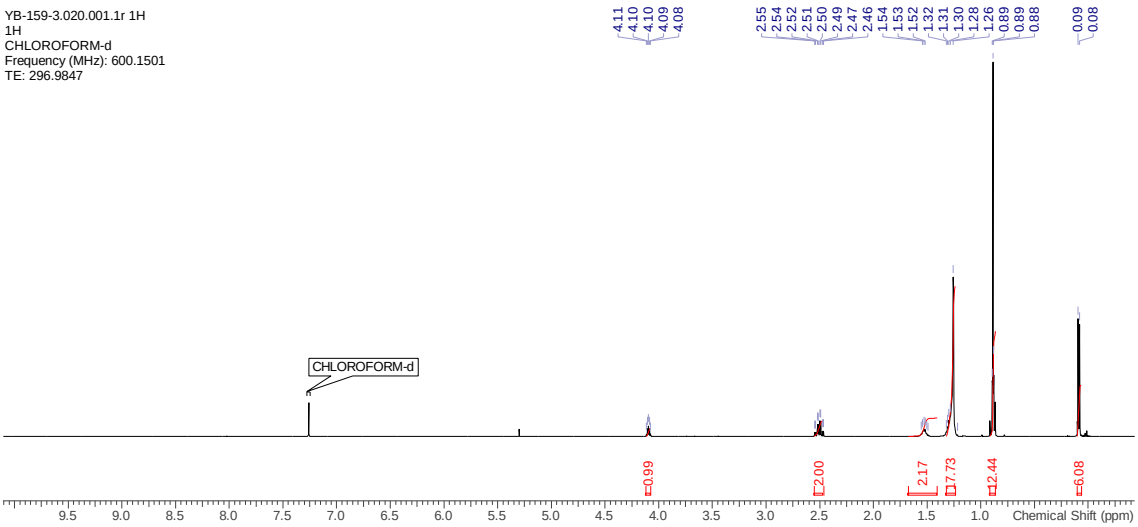
(*S*)-S-**5**

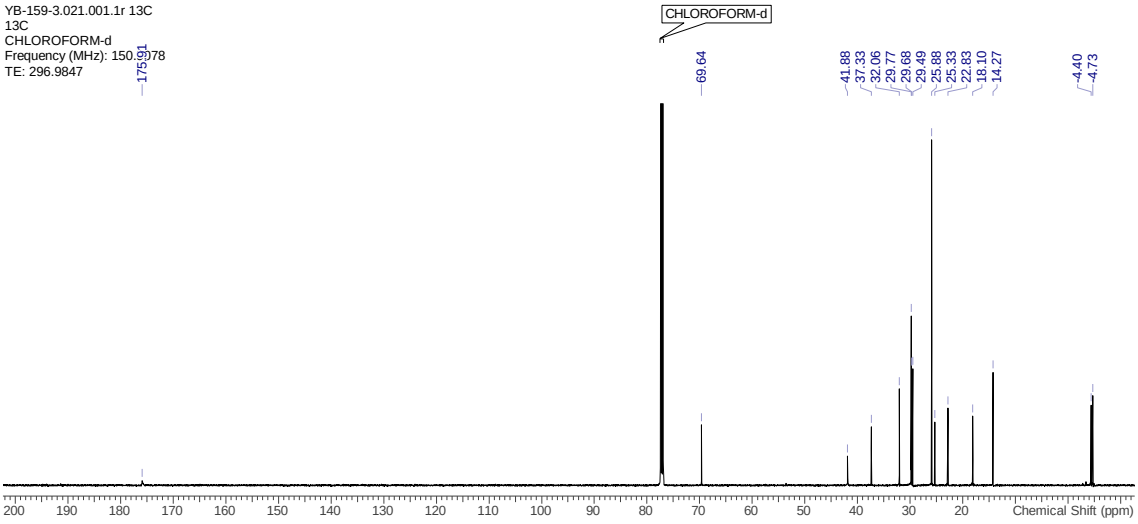

YB-162.020.001.1r.esp
1H
CHLOROFORM-d
30 H's
4.05
4.04
4.04
4.03
4.02
4.02
4.01
2.59
2.58
2.56
2.56
2.50
2.48
2.45
1.54
1.46
1.44
1.32
1.31
1.29
1.28
1.27
1.26
0.89
0.88
0.87
CHLOROFORM-d
1.00
1.04
1.04
0.98
1.97
7.47
3.01
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

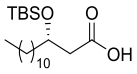

YB-162.021.001.1r 13C
13C
CHLOROFORM-d
11 C's
177.96
CHLOROFORM-d
68.15
41.17
36.64
32.06
29.80
29.77
29.62
29.50
25.59
22.83
14.27
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
Chemical Shift (ppm)


TBSO
O
OH
10

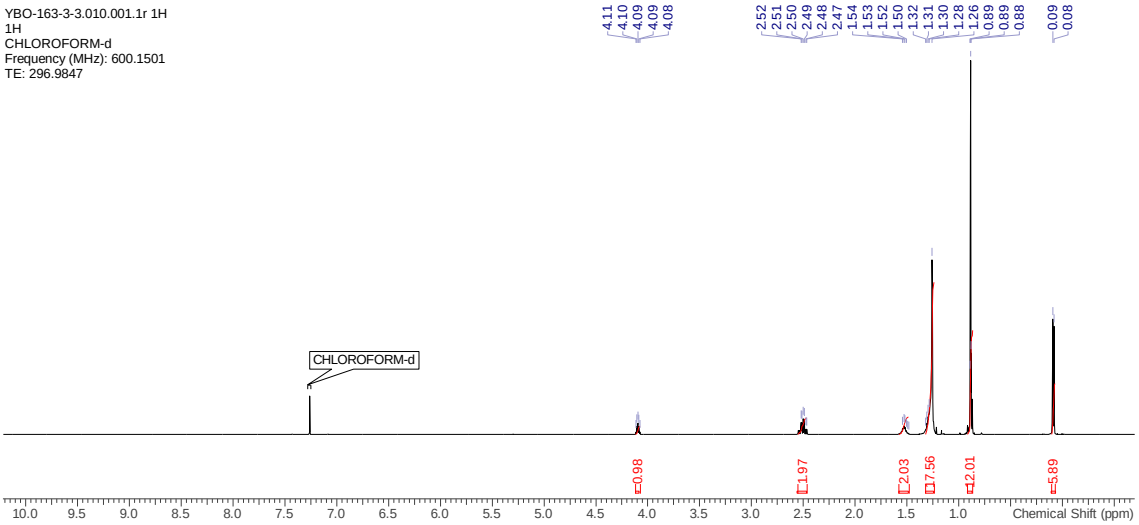
(*R*)-S-**6**

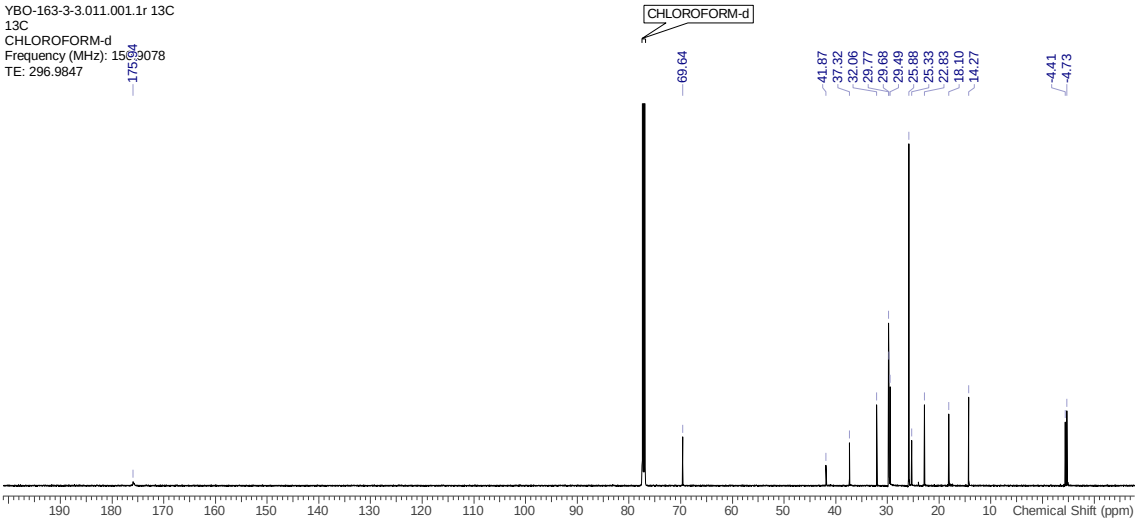

YB-159-3.020.001.1r 1H
1H
CHLOROFORM-d
Frequency (MHz): 600.1501
TE: 296.9847
4.11
4.10
4.10
4.09
4.08
2.55
2.54
2.52
2.51
2.50
2.49
2.47
2.46
1.54
1.53
1.52
1.32
1.31
1.30
1.28
1.26
0.89
0.89
0.88
0.09
0.08
CHLOROFORM-d
0.99
2.00
2.17
17.73
12.44
6.08
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

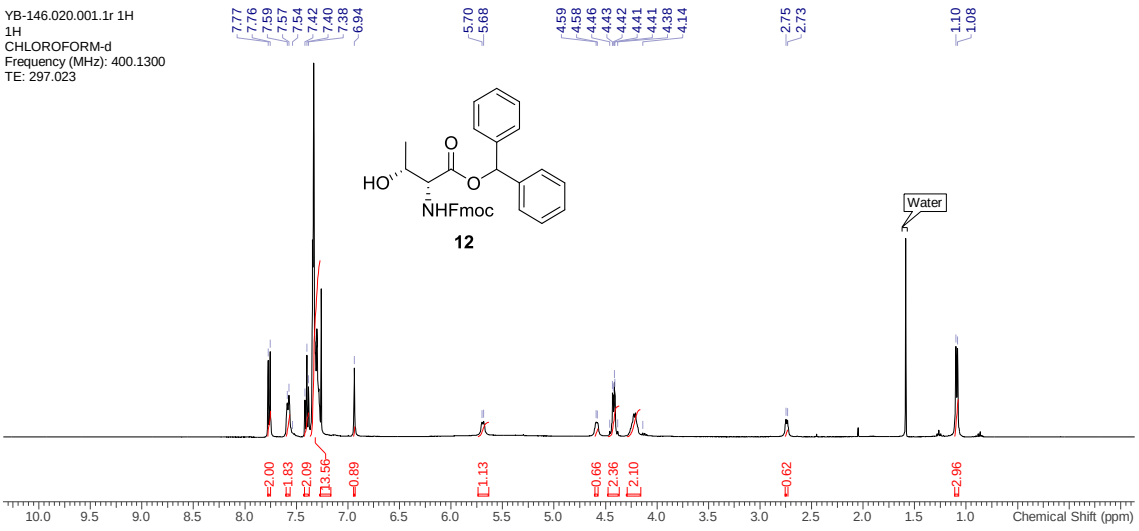

YB-159-3.021.001.1r 13C
13C
CHLOROFORM-d
Frequency (MHz): 150.9078
TE: 296.9847
175.91
CHLOROFORM-d
69.64
41.88
37.33
32.06
29.77
29.68
29.49
25.88
25.33
22.83
18.10
14.27
-4.40
-4.73
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
Chemical Shift (ppm)

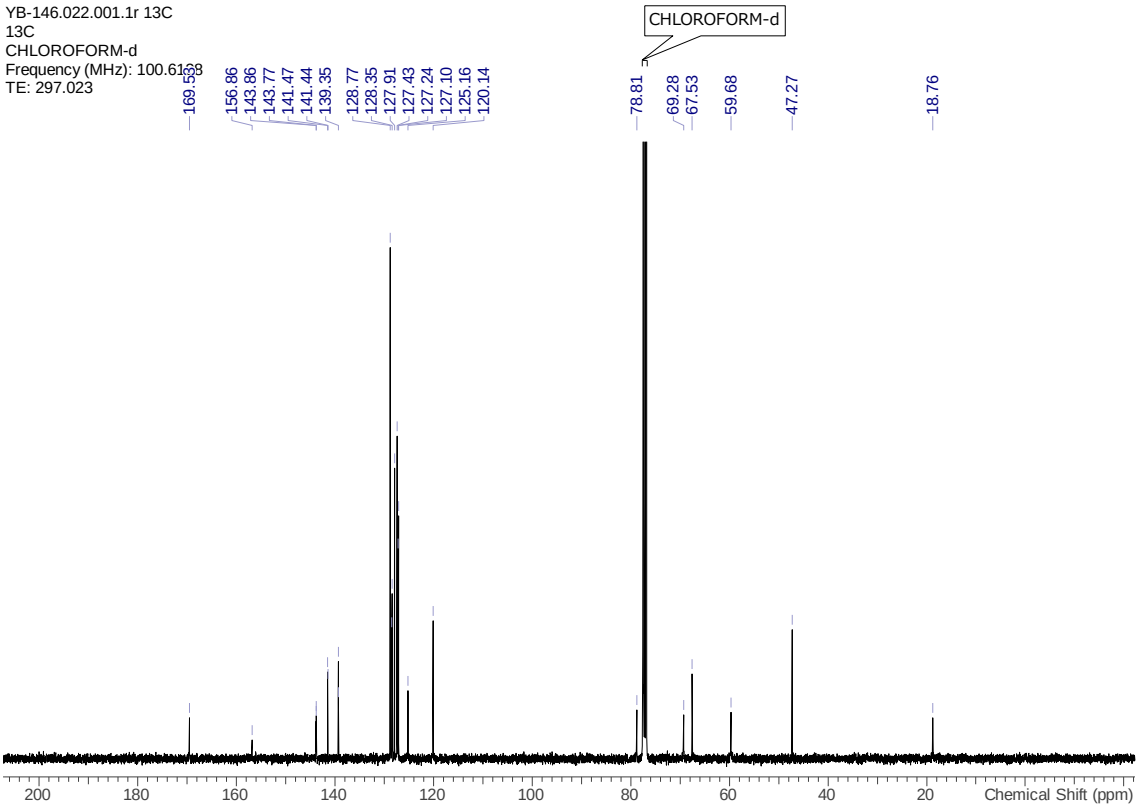

TBSO
O
OH
10

(*S*)-S-**6**

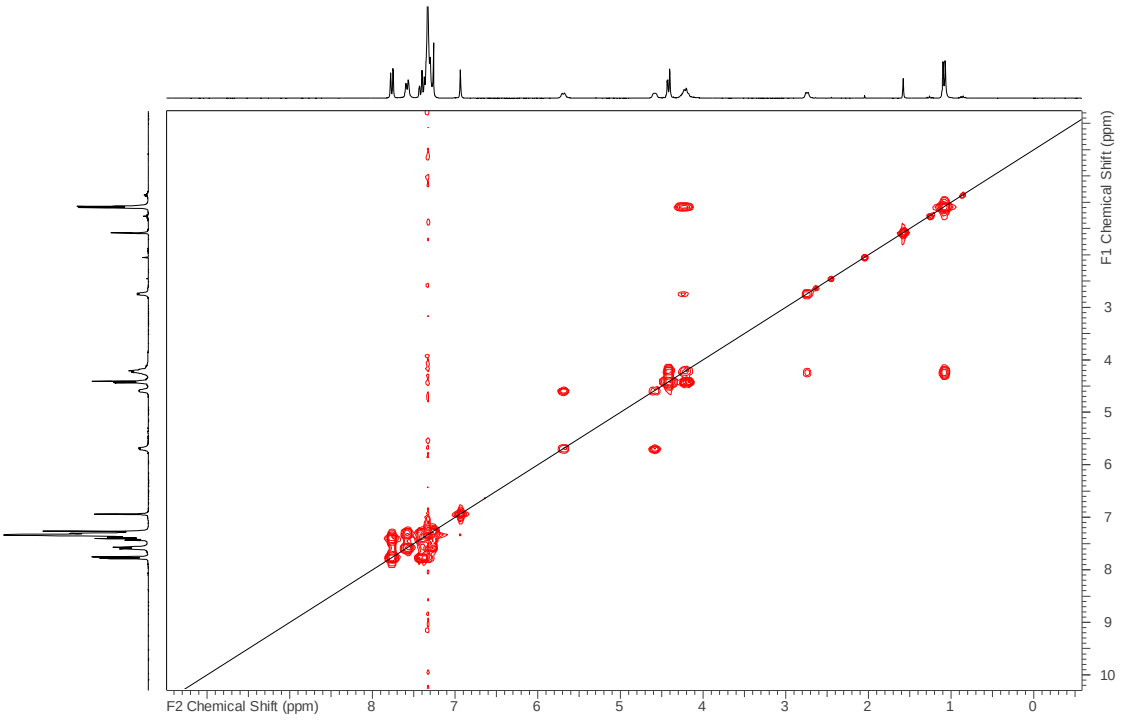

YBO-163-3-3.010.001.1r 1H
1H
CHLOROFORM-d
Frequency (MHz): 600.1501
TE: 296.9847
4.11
4.10
4.09
4.09
4.08
2.52
2.51
2.50
2.49
2.48
2.47
1.54
1.53
1.52
1.50
1.32
1.31
1.30
1.28
1.26
0.89
0.89
0.88
0.09
0.08
CHLOROFORM-d
0.98
1.97
2.03
7.56
12.01
5.89
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

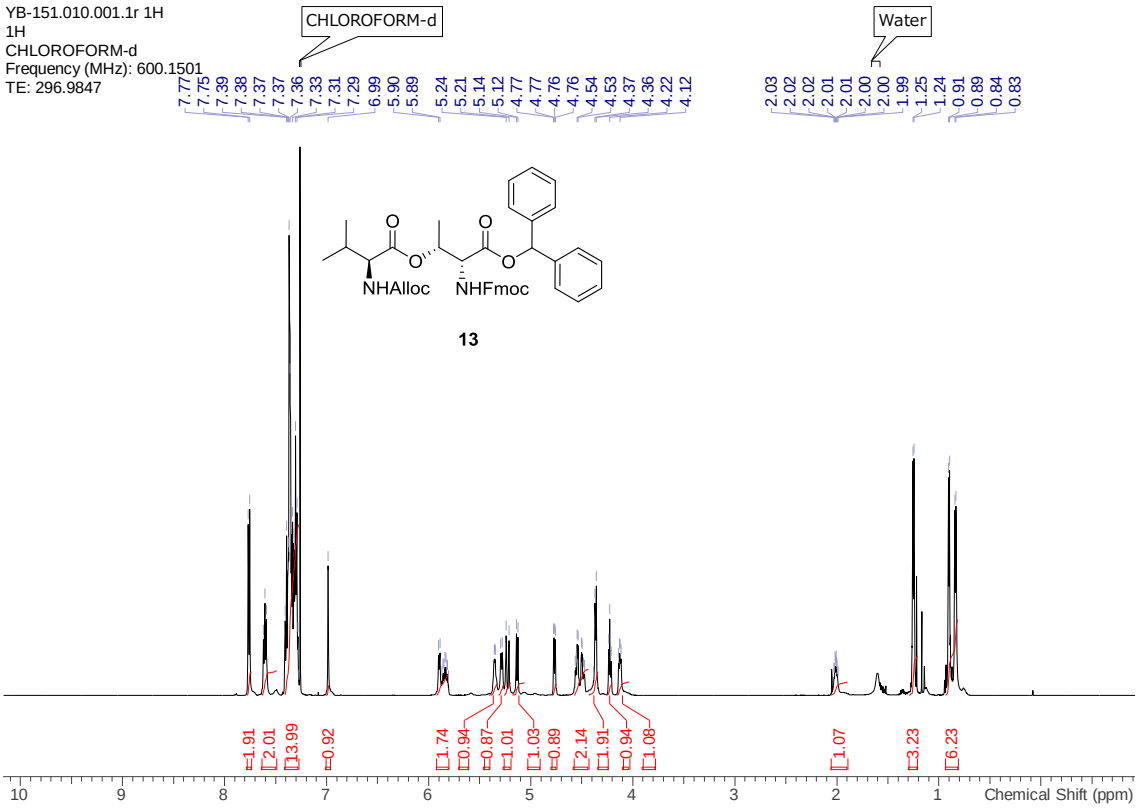

YBO-163-3-3.011.001.1r 13C
13C
CHLOROFORM-d
Frequency (MHz): 150.9078
TE: 296.9847
175.84
CHLOROFORM-d
69.64
41.87
37.32
32.06
29.77
29.68
29.49
25.88
25.33
22.83
18.10
14.27
-4.41
-4.73
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Chemical Shift (ppm)

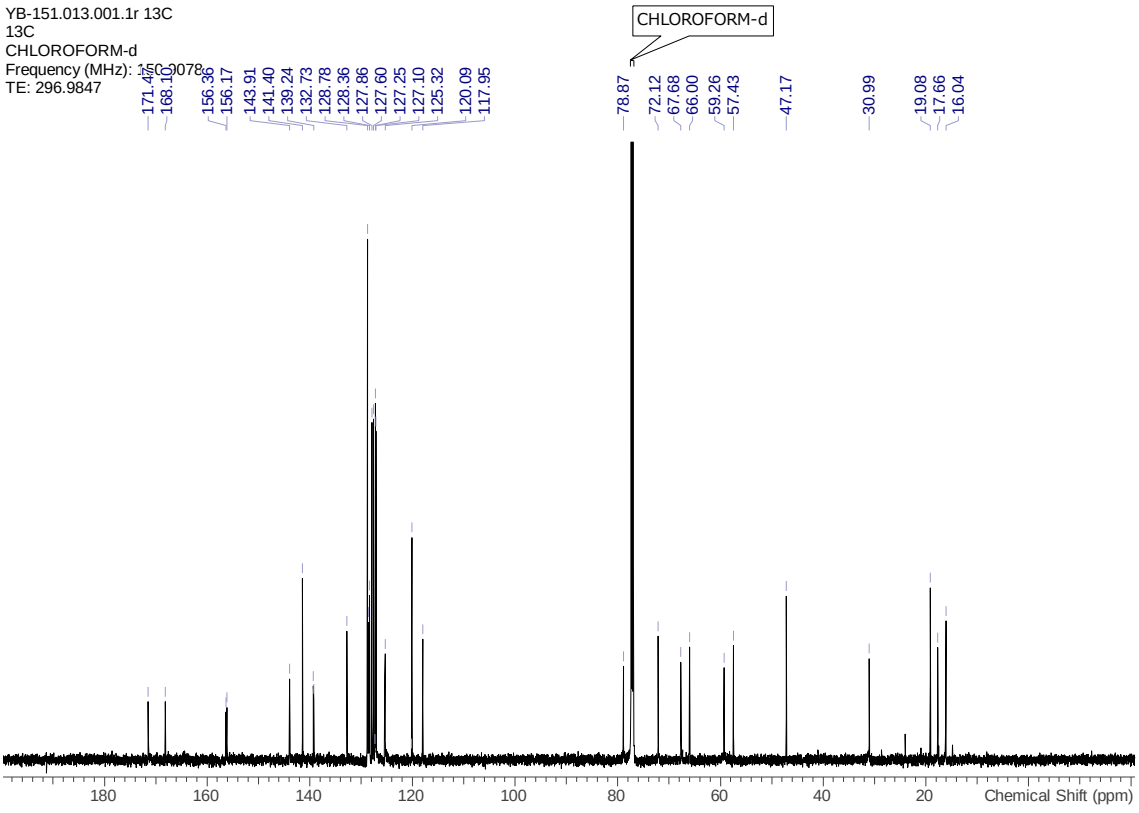

YB-146.020.001.1r 1H
1H
CHLOROFORM-d
Frequency (MHz): 400.1300
TE: 297.023
7.77
7.76
7.59
7.57
7.54
7.42
7.40
7.38
6.94
5.70
5.68
4.59
4.58
4.46
4.43
4.42
4.41
4.41
4.38
4.14
2.75
2.73
1.10
1.08
HO
O
O
NHFmoc
12
Water
2.00
1.83
2.09
3.56
0.89
1.13
0.66
2.36
2.10
0.62
2.96
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)


YB-146.022.001.1r 13C
13C
CHLOROFORM-d
Frequency (MHz): 100.6128
TE: 297.023
CHLOROFORM-d
169.53
156.86
143.86
143.77
141.47
141.44
139.35
128.77
128.35
127.91
127.43
127.24
127.10
125.16
120.14
78.81
69.28
67.53
59.68
47.27
18.76
200
180
160
140
120
100
80
60
40
20
Chemical Shift (ppm)

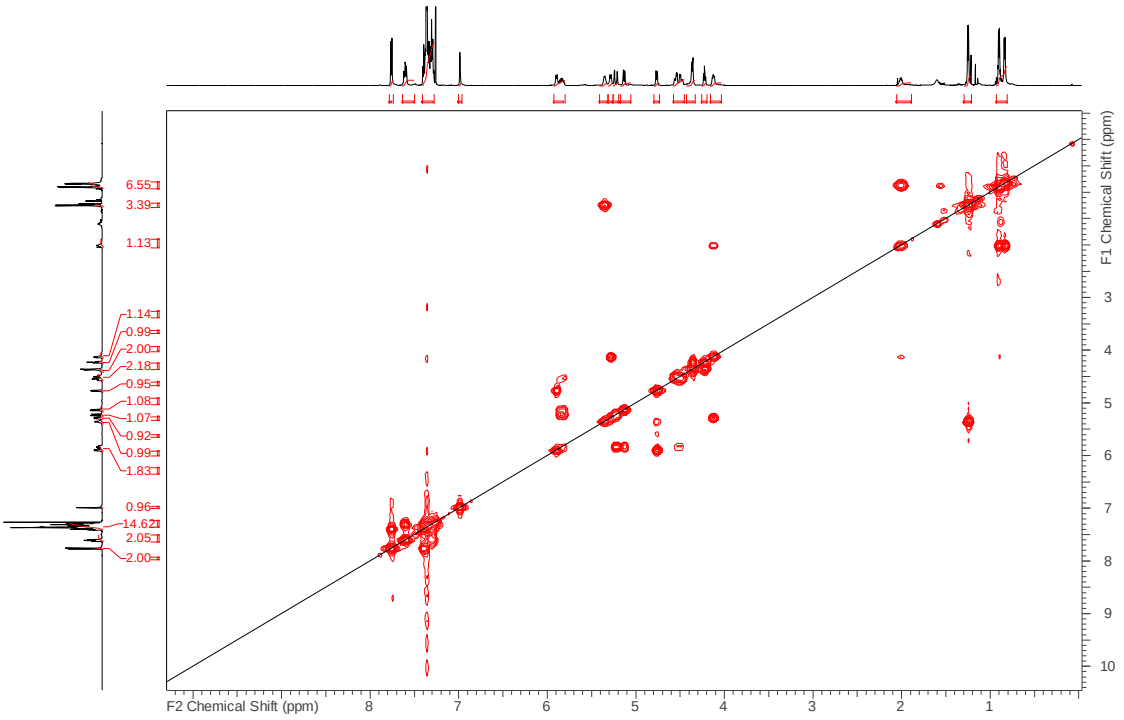
COSY-NMR ($CDCl_3$, 250 MHz, 297 K)



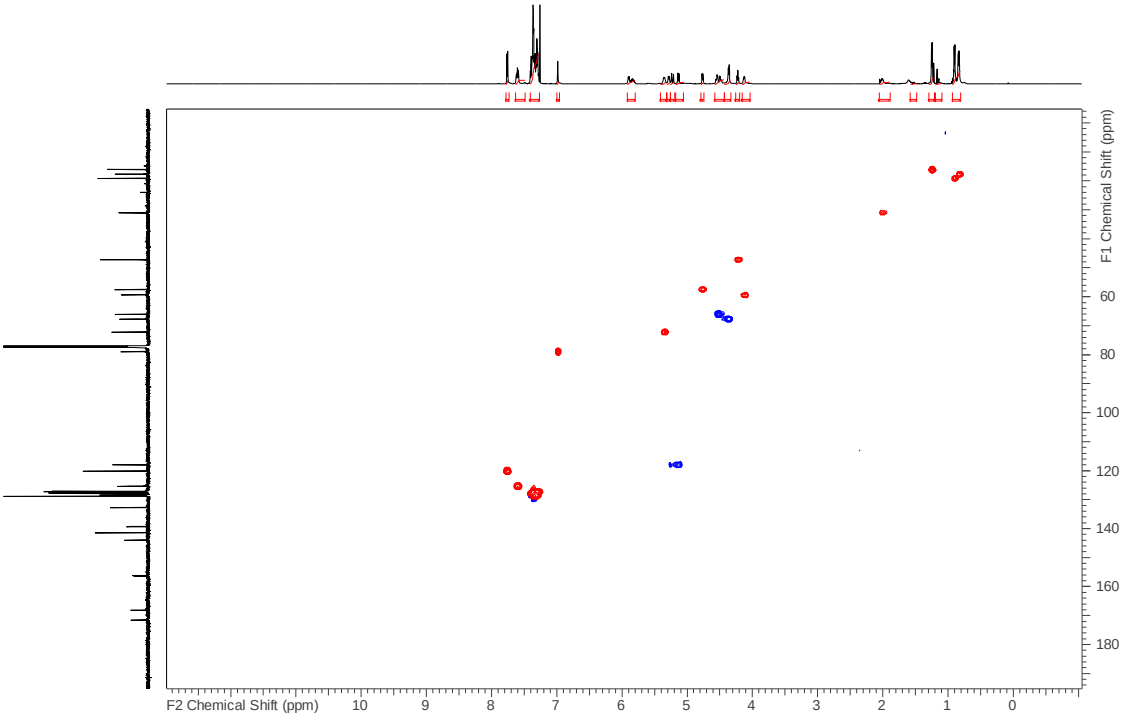

YB-151.010.001.1r 1H
1H
CHLOROFORM-d
Frequency (MHz): 600.1501
TE: 296.9847
CHLOROFORM-d
Water
7.77 7.75 7.39 7.38 7.37 7.37 7.36 7.33 7.31 7.29 6.99 5.90 5.89 5.24 5.21 5.14 5.12 4.77 4.77 4.76 4.76 4.54 4.53 4.37 4.36 4.22 4.12
2.03 2.02 2.02 2.01 2.01 2.00 2.00 1.99 1.25 1.24 0.91 0.89 0.84 0.83
O
O
O
O
NHAlloc
NHFmoc
13
1.91 2.01 3.99 0.92 1.74 0.94 0.87 1.01 1.03 0.89 2.14 1.91 0.94 1.08 1.07 3.23 6.23
10 9 8 7 6 5 4 3 2 1
Chemical Shift (ppm)


YB-151.013.001.1r 13C
13C
CHLOROFORM-d
Frequency (MHz): 150.9078
TE: 296.9847
CHLOROFORM-d
171.45 168.10 156.36 156.17 143.91 141.40 139.24 132.73 128.78 128.36 127.86 127.60 127.25 127.10 125.32 120.09 117.95
78.87 72.12 67.68 66.00 59.26 57.43 47.17 30.99 19.08 17.66 16.04
180 160 140 120 100 80 60 40 20
Chemical Shift (ppm)

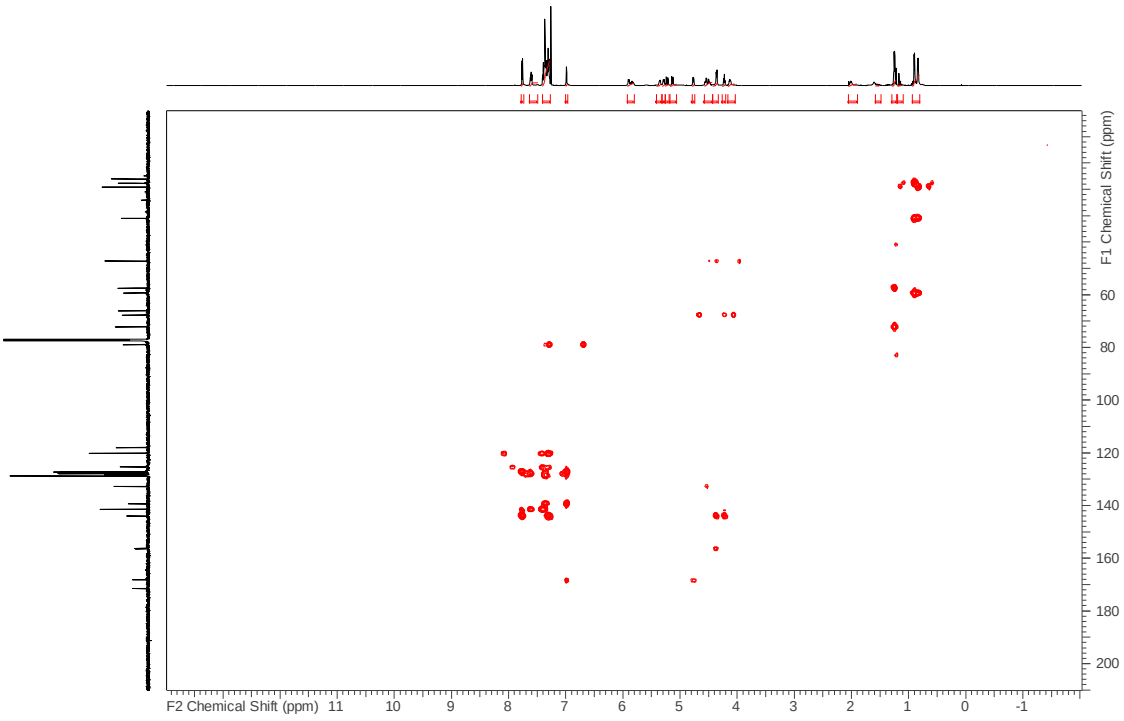
COSY ($CDCl_3$, 600 MHz, 297 K)

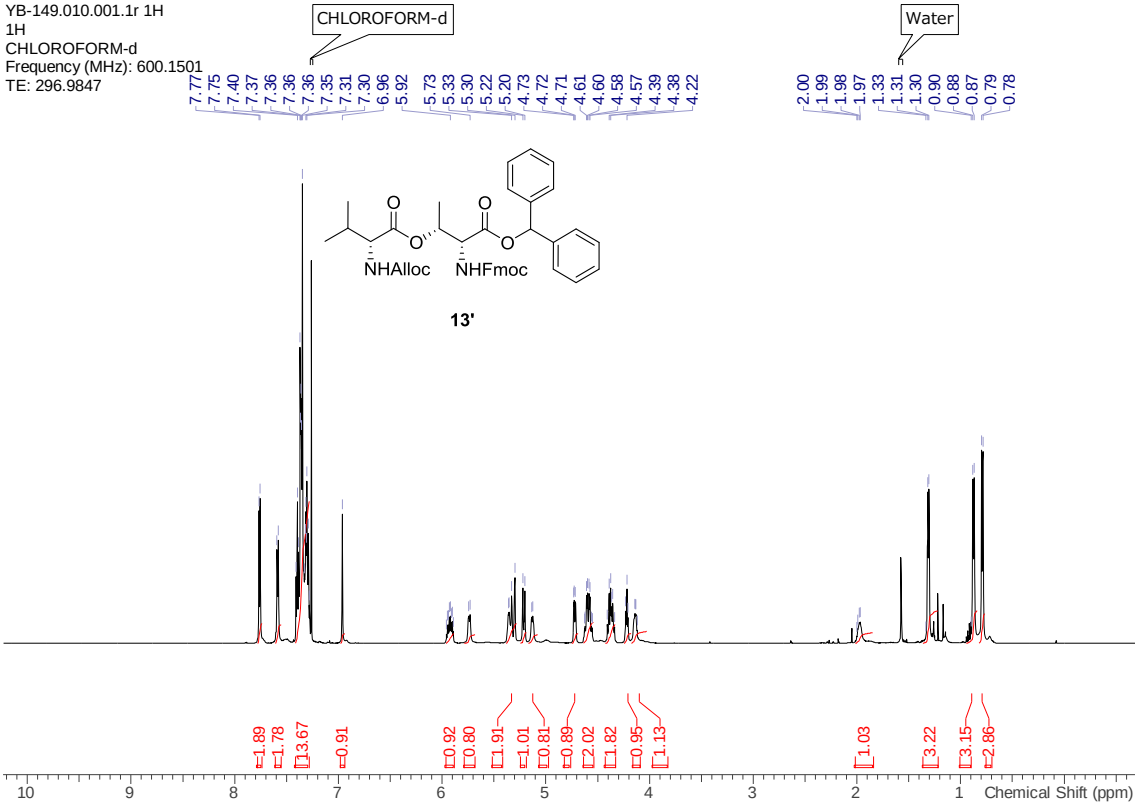


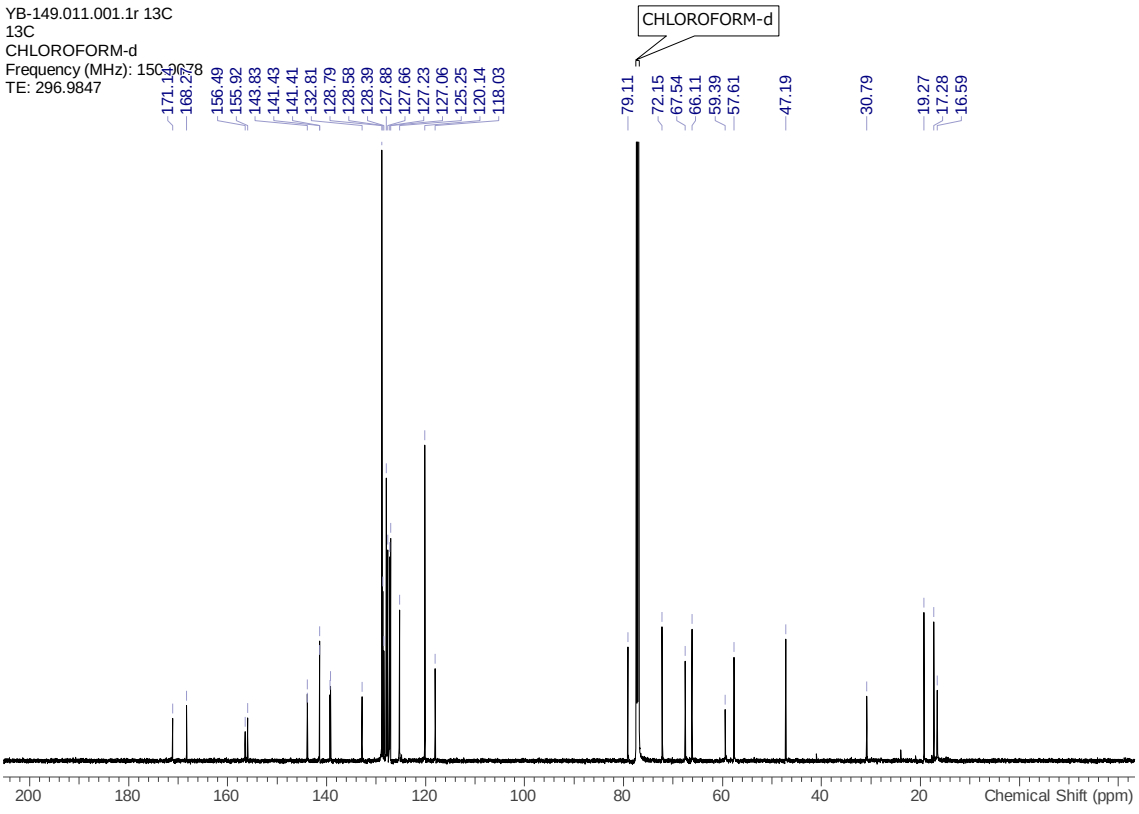

HSQC-DEPT ($CDCl_3$, 250, 63 MHz, 297 K)



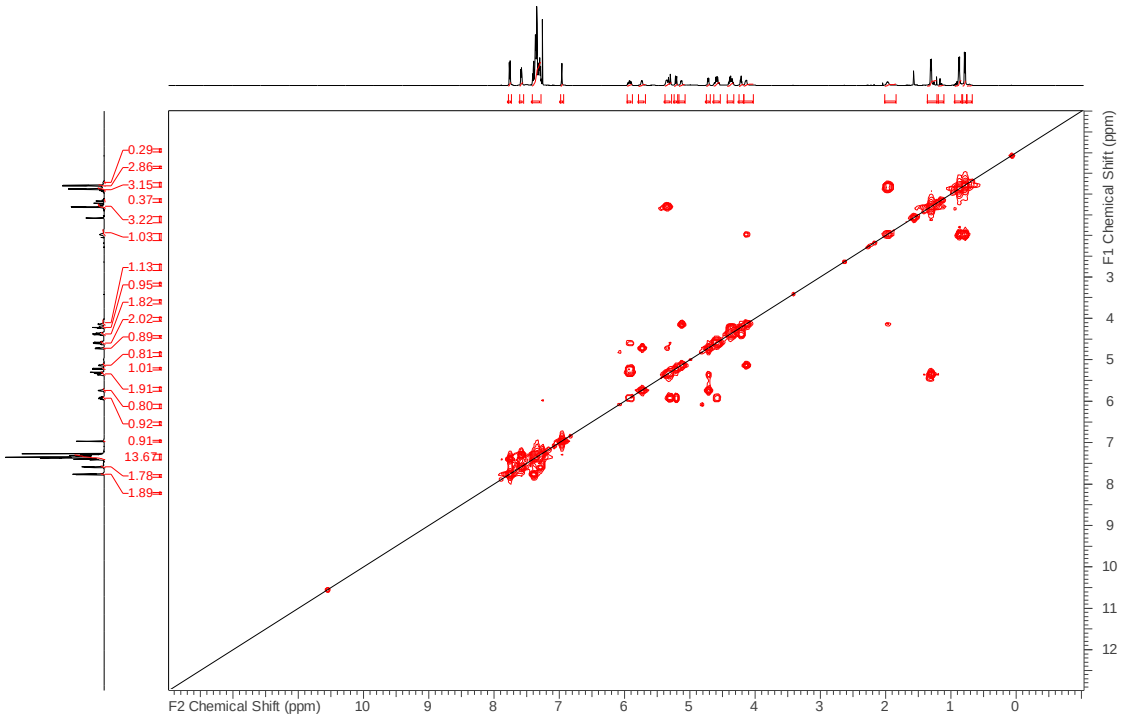
HMBC ($CDCl_3$, 250, 63 MHz, 297 K)



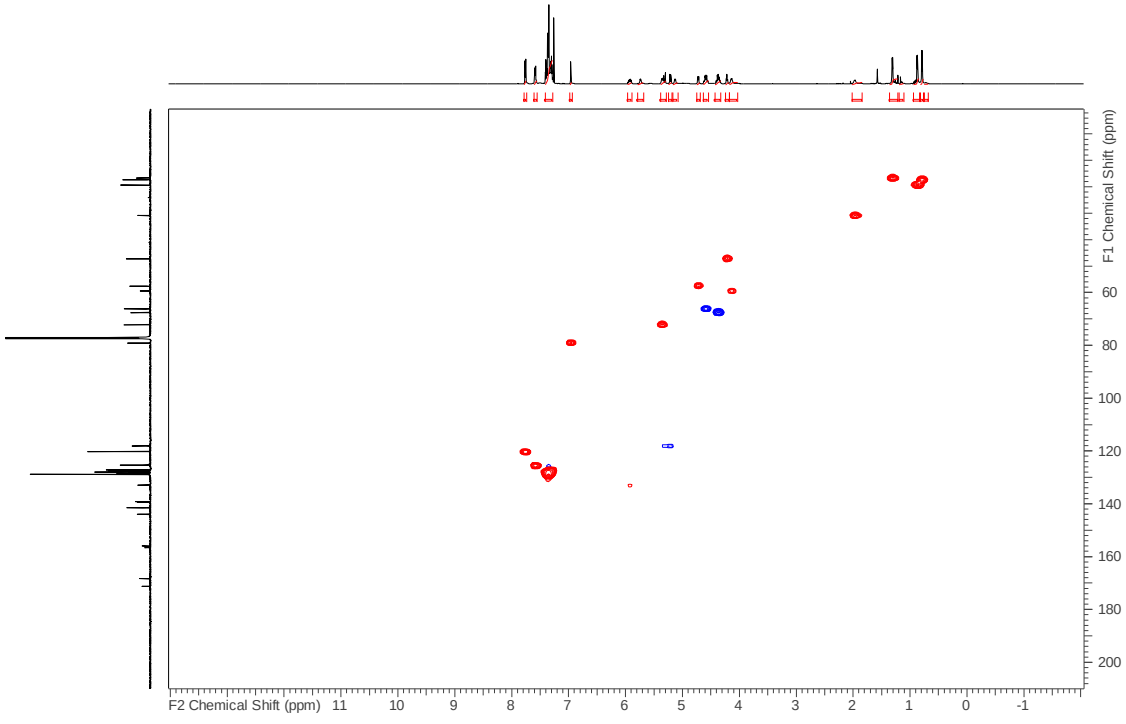

YB-149.010.001.1r 1H
1H
CHLOROFORM-d
Frequency (MHz): 600.1501
TE: 296.9847
CHLOROFORM-d
Water
7.77 7.75 7.40 7.37 7.36 7.36 7.36 7.35 7.31 7.30 6.96 5.92 5.73 5.33 5.30 5.22 5.20 4.73 4.72 4.71 4.61 4.60 4.58 4.57 4.39 4.38 4.22
2.00 1.99 1.98 1.97 1.33 1.31 1.30 0.90 0.88 0.87 0.79 0.78
NHAlloc
NHFmoc
13'
1.89 1.78 3.67 0.91 0.92 0.80 1.91 1.01 0.81 0.89 2.02 1.82 0.95 1.13 1.03 3.22 3.15 2.86
10 9 8 7 6 5 4 3 2 1
Chemical Shift (ppm)

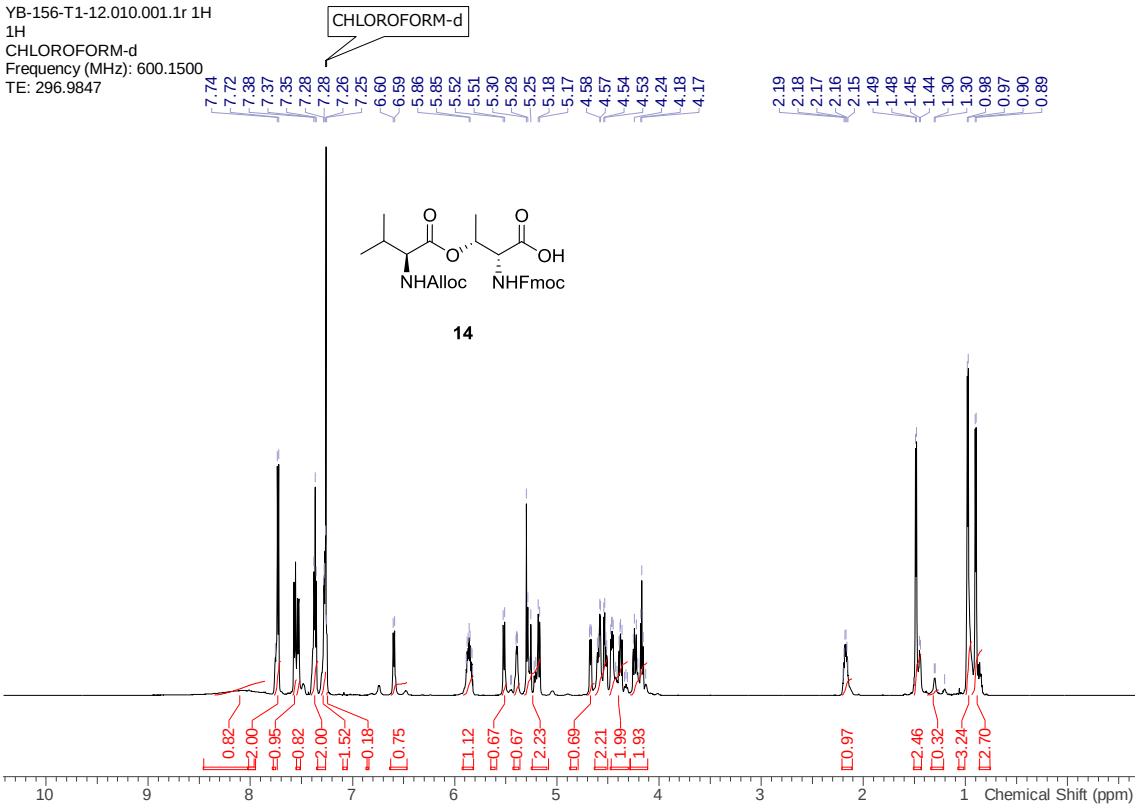

YB-149.011.001.1r 13C
13C
CHLOROFORM-d
Frequency (MHz): 150.0078
TE: 296.9847
CHLOROFORM-d
171.14 168.22 156.49 155.92 143.83 141.43 141.41 132.81 128.79 128.58 128.39 127.88 127.66 127.23 127.06 125.25 120.14 118.03
79.11 72.15 67.54 66.11 59.39 57.61 47.19 30.79 19.27 17.28 16.59
200 180 160 140 120 100 80 60 40 20
Chemical Shift (ppm)

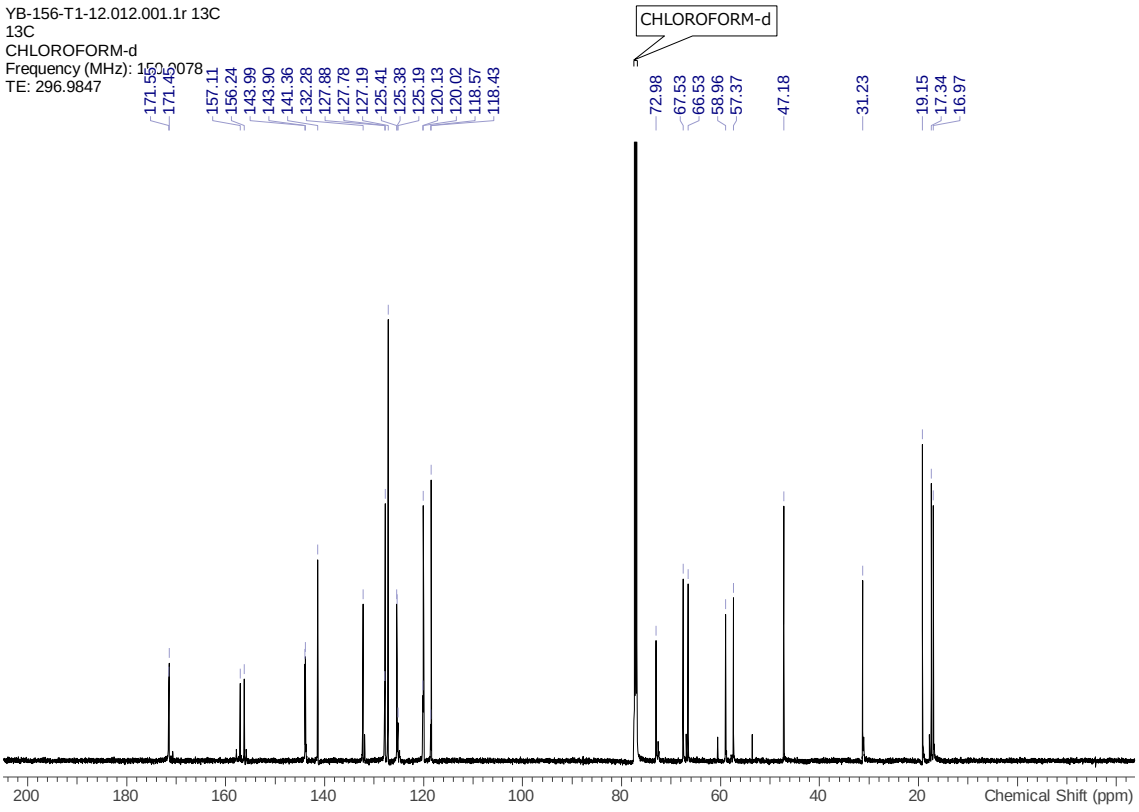
COSY ($CDCl_3$, 600 MHz, 297 K)



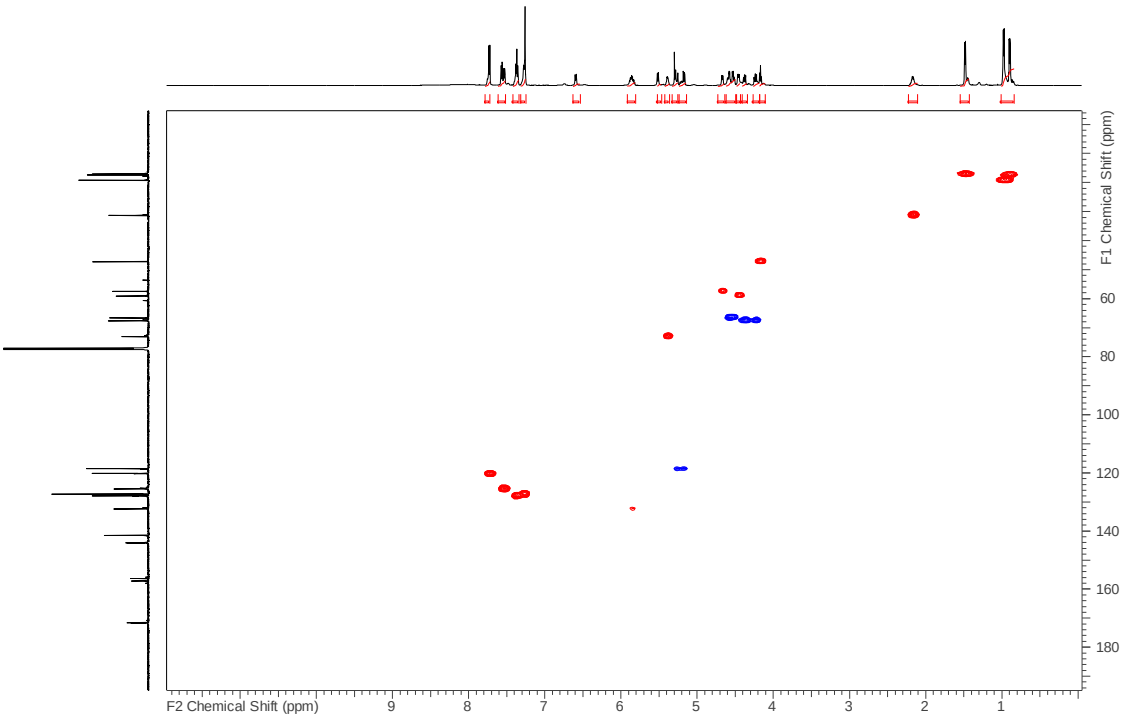

HSQC-DEPT ($CDCl_3$, 250 , 63 MHz, 297 K)

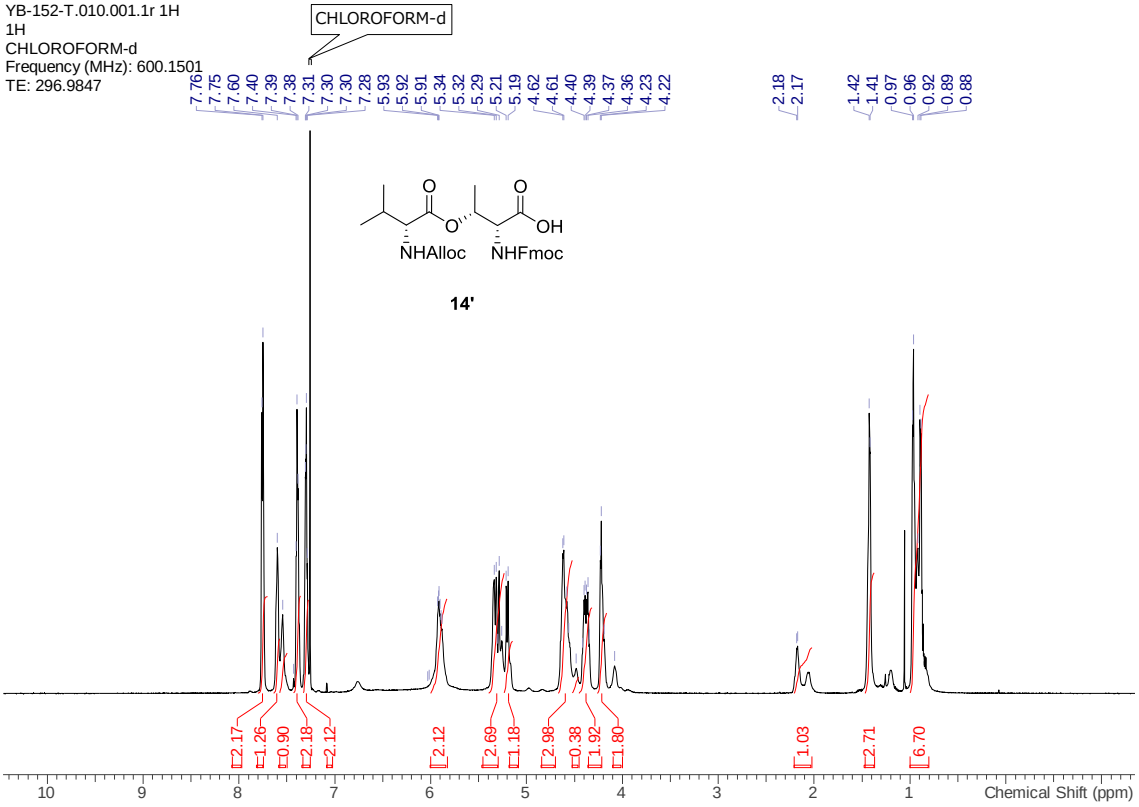


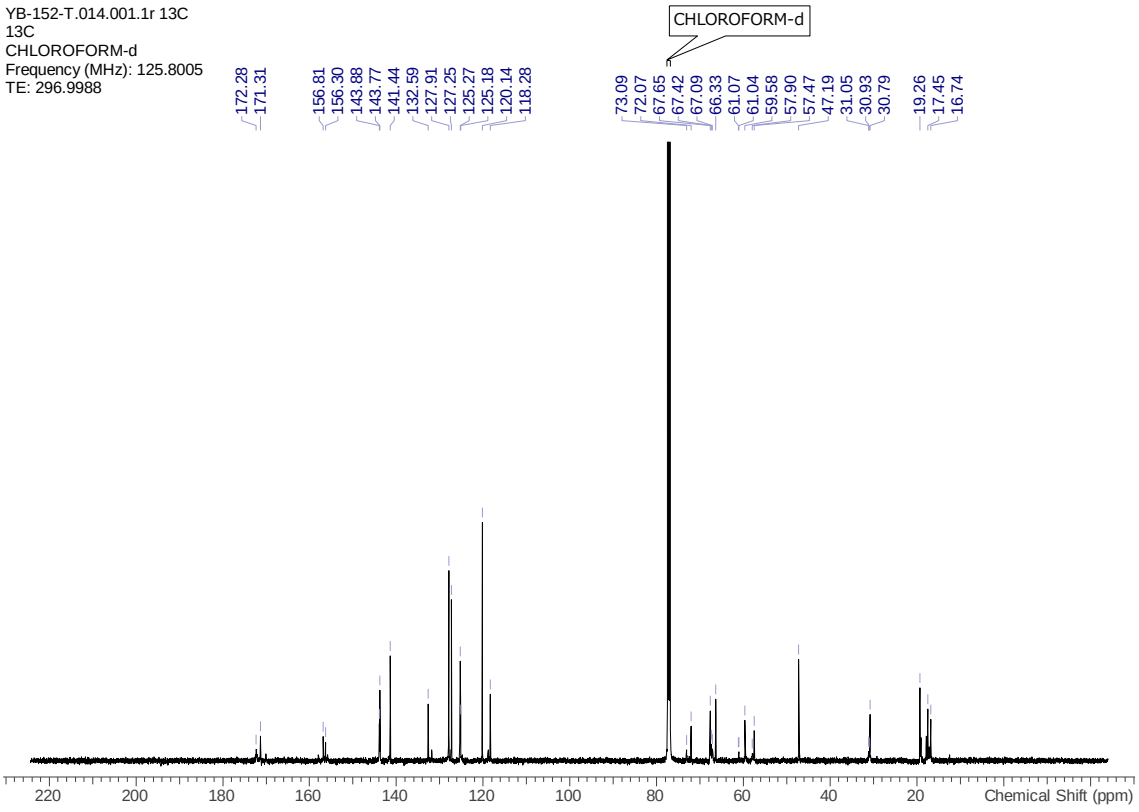

YB-156-T1-12.010.001.1r 1H
1H
CHLOROFORM-d
Frequency (MHz): 600.1500
TE: 296.9847
CHLOROFORM-d
7.74 7.72 7.38 7.37 7.35 7.28 7.28 7.26 7.25 6.60 6.59 5.86 5.85 5.52 5.51 5.30 5.28 5.25 5.18 5.17 4.58 4.57 4.54 4.53 4.24 4.18 4.17
2.19 2.18 2.17 2.16 2.15 1.49 1.48 1.45 1.44 1.30 1.30 0.98 0.97 0.90 0.89
O
O
O
OH
NHAlloc
NHFmoc
14
0.82 2.00 0.95 0.82 2.00 1.52 0.18 0.75 1.12 0.67 0.67 2.23 0.69 2.21 1.99 1.93 0.97 2.46 0.32 3.24 2.70
10 9 8 7 6 5 4 3 2 1 Chemical Shift (ppm)


YB-156-T1-12.012.001.1r 13C
13C
CHLOROFORM-d
Frequency (MHz): 150.9078
TE: 296.9847
CHLOROFORM-d
171.55 171.45 157.11 156.24 143.99 143.90 141.36 132.28 127.88 127.78 127.19 125.41 125.38 125.19 120.13 120.02 118.57 118.43
72.98 67.53 66.53 58.96 57.37 47.18 31.23 19.15 17.34 16.97
200 180 160 140 120 100 80 60 40 20 Chemical Shift (ppm)

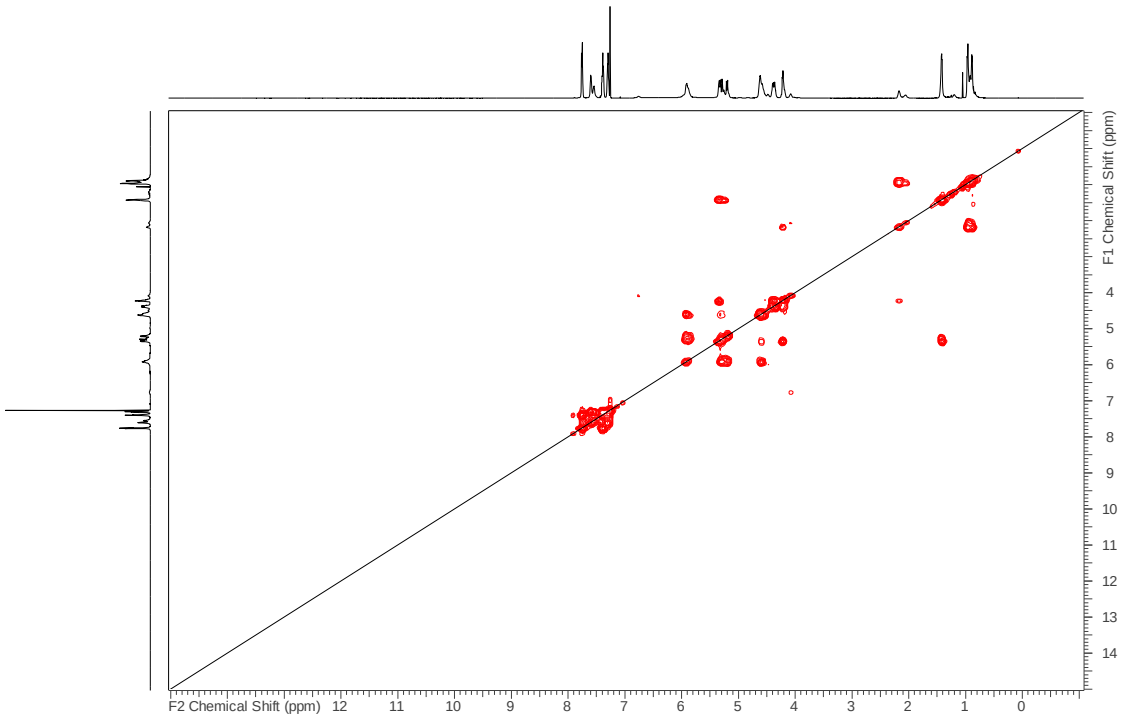
HSQC-DEPT ($CDCl_3$, 600, 151 MHz, 297 K)



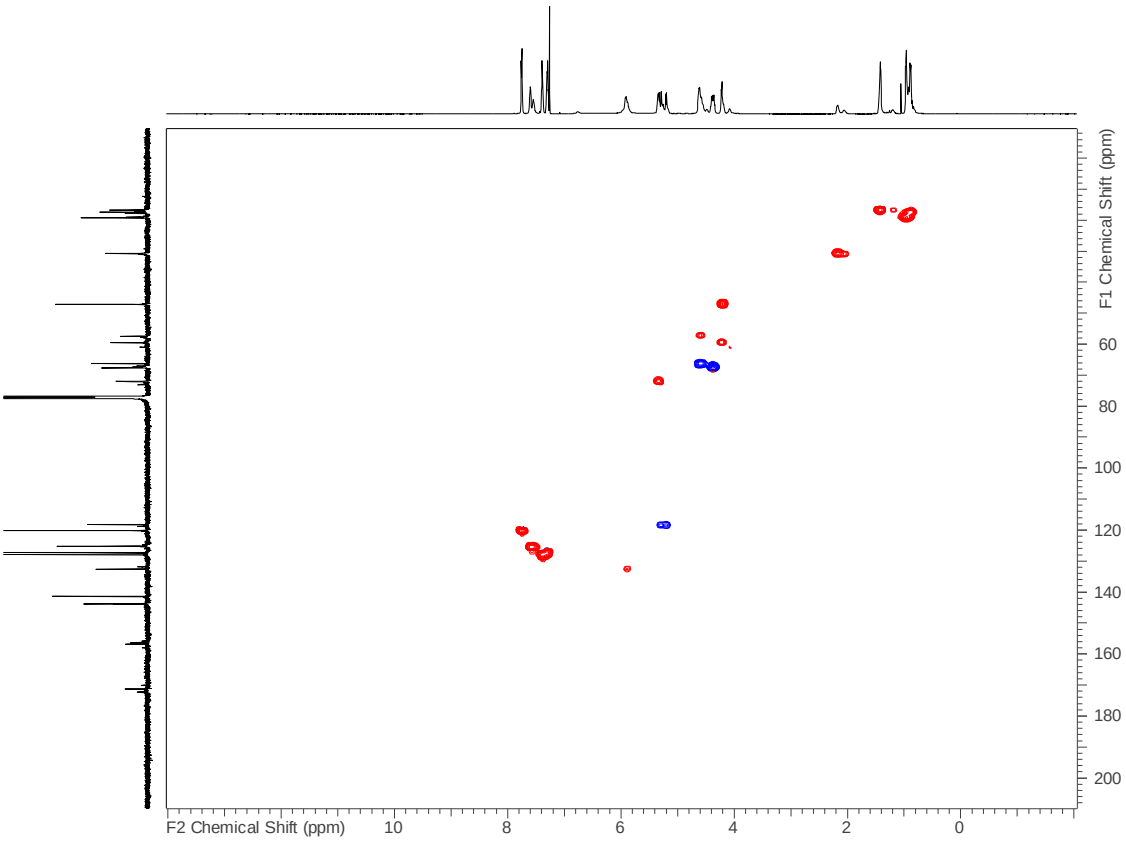

YB-152-T.010.001.1r 1H
1H
CHLOROFORM-d
Frequency (MHz): 600.1501
TE: 296.9847
CHLOROFORM-d
7.76 7.75 7.60 7.40 7.39 7.38 7.31 7.30 7.30 7.28 5.93 5.92 5.91 5.34 5.32 5.29 5.21 5.19 4.62 4.61 4.40 4.39 4.37 4.36 4.23 4.22
2.18 2.17
1.42 1.41 0.97 0.96 0.92 0.89 0.88
O
O
O
OH
NHAlloc
NHFmoc
14'
2.17 1.26 0.90 2.18 2.12 2.12 2.69 1.18 2.98 0.38 1.92 1.80 1.03 2.71 6.70
10 9 8 7 6 5 4 3 2 1
Chemical Shift (ppm)


YB-152-T.014.001.1r 13C
13C
CHLOROFORM-d
Frequency (MHz): 125.8005
TE: 296.9988
CHLOROFORM-d
172.28 171.31
156.81 156.30 143.88 143.77 141.44 132.59 127.91 127.25 125.27 125.18 120.14 118.28
73.09 72.07 67.65 67.42 67.09 66.33 61.07 61.04 59.58 57.90 57.47 47.19 31.05 30.93 30.79
19.26 17.45 16.74
220 200 180 160 140 120 100 80 60 40 20
Chemical Shift (ppm)

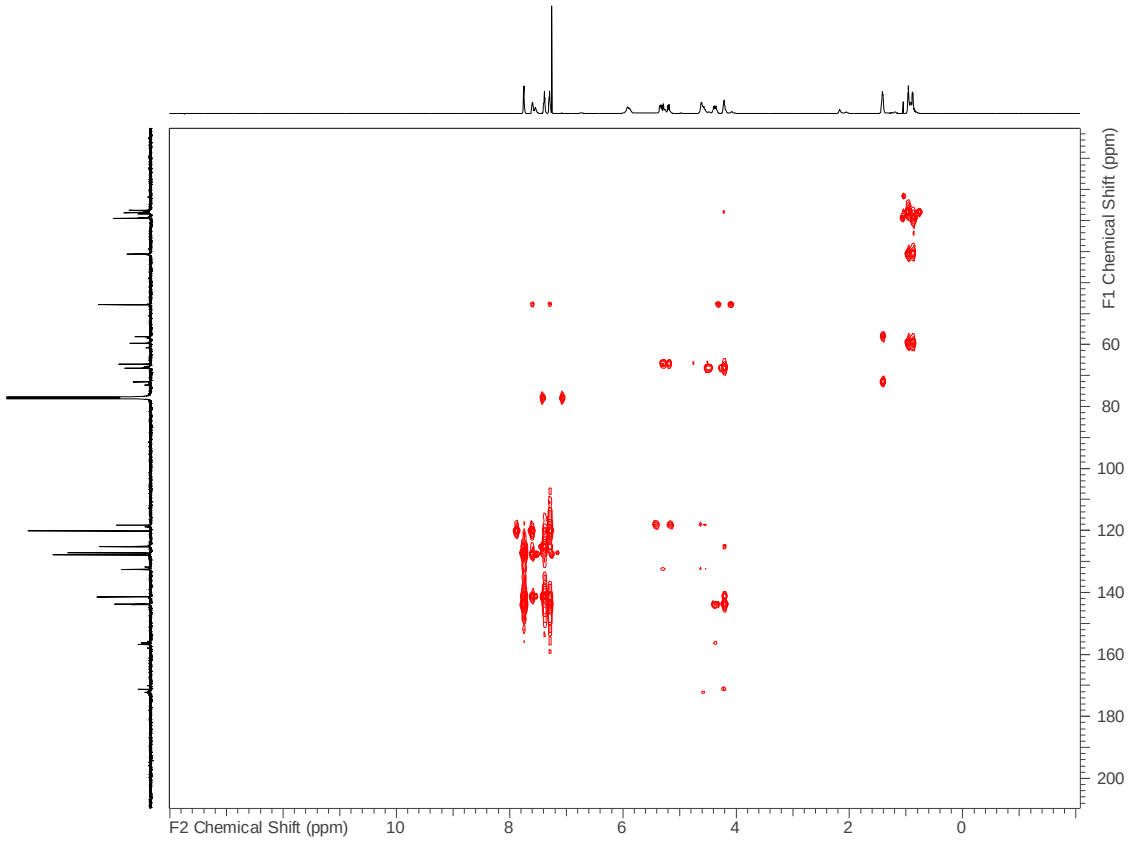
COSY ($CDCl_3$, 500 MHz, 297 K)

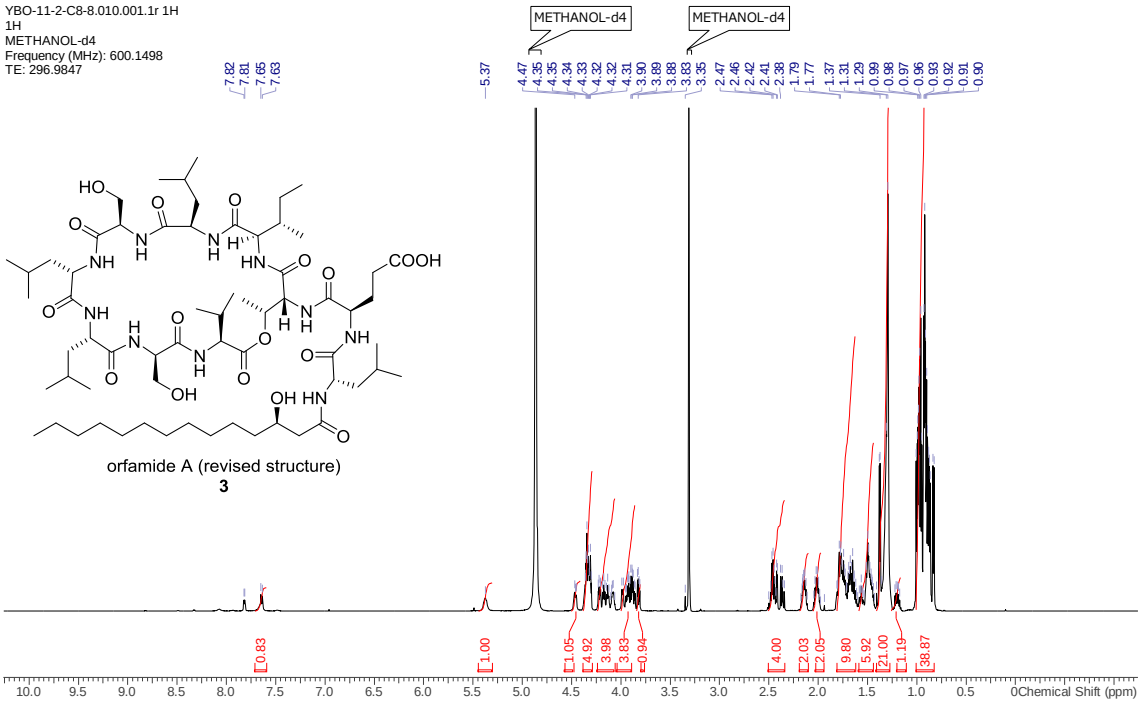



HSQC-DEPT ($CDCl_3$, 600, 151 MHz, 297 K)



HMBC ($CDCl_3$, 600, 151 MHz, 297 K)

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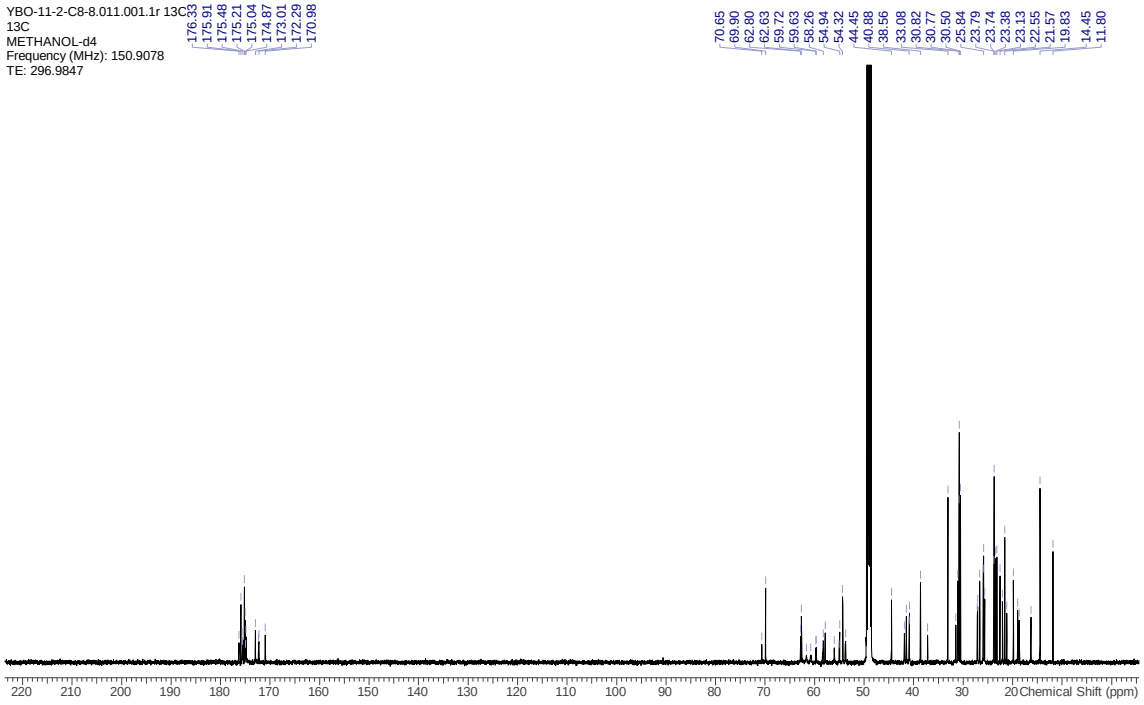
1H
METHANOL-d4
Frequency (MHz): 600.1498
TE: 296.9847
METHANOL-d4
METHANOL-d4
orfamide A (revised structure)
3
Chemical Shift (ppm)


YBO-11-2-C8-8.011.001.1r 13C
13C
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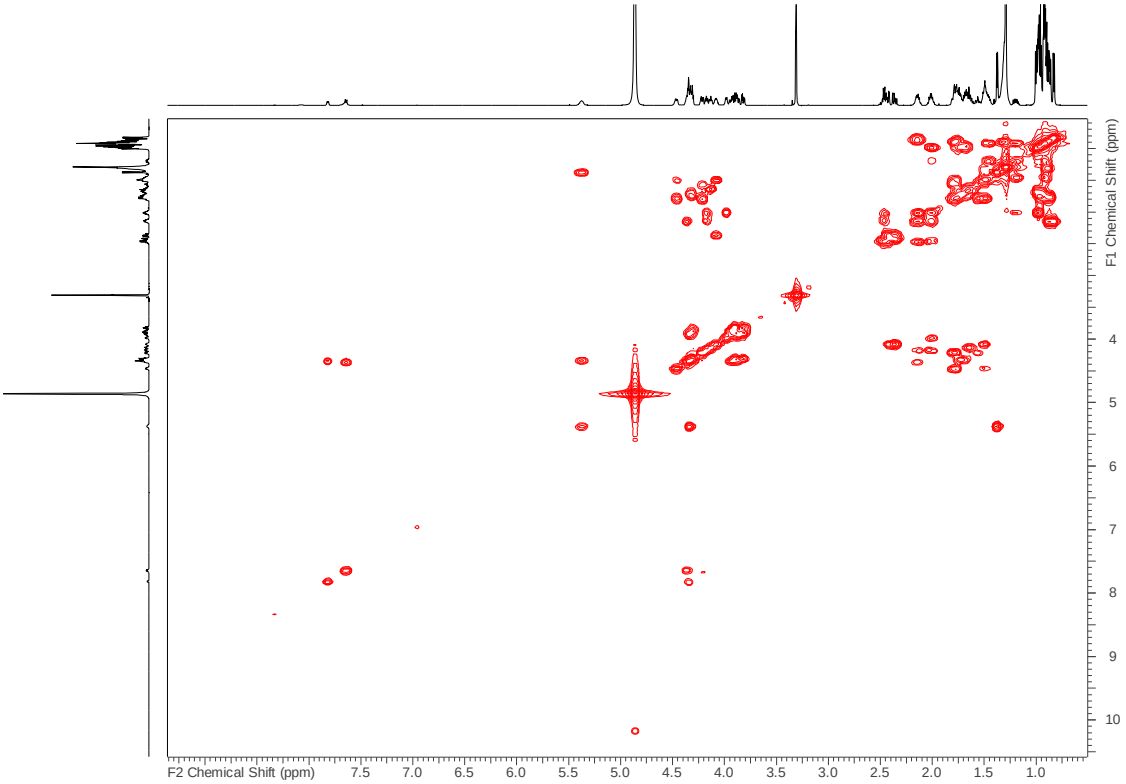
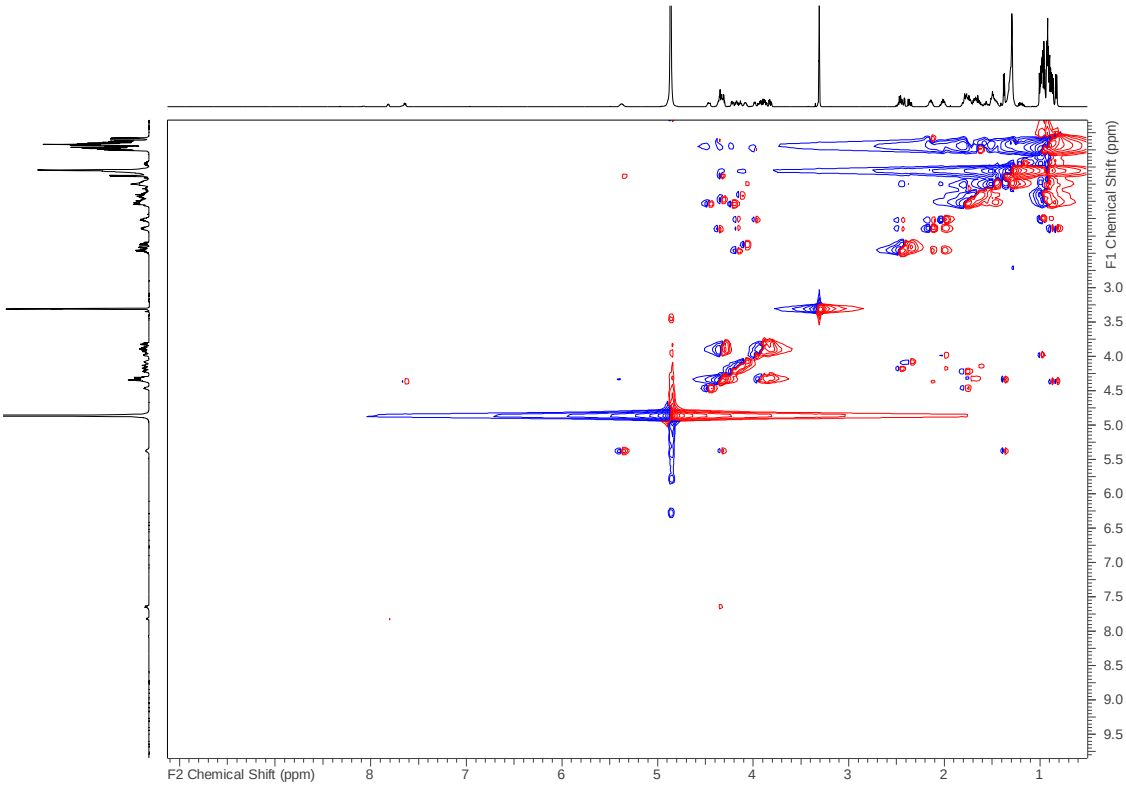
Frequency (MHz): 150.9078
TE: 296.9847
Chemical Shift (ppm)

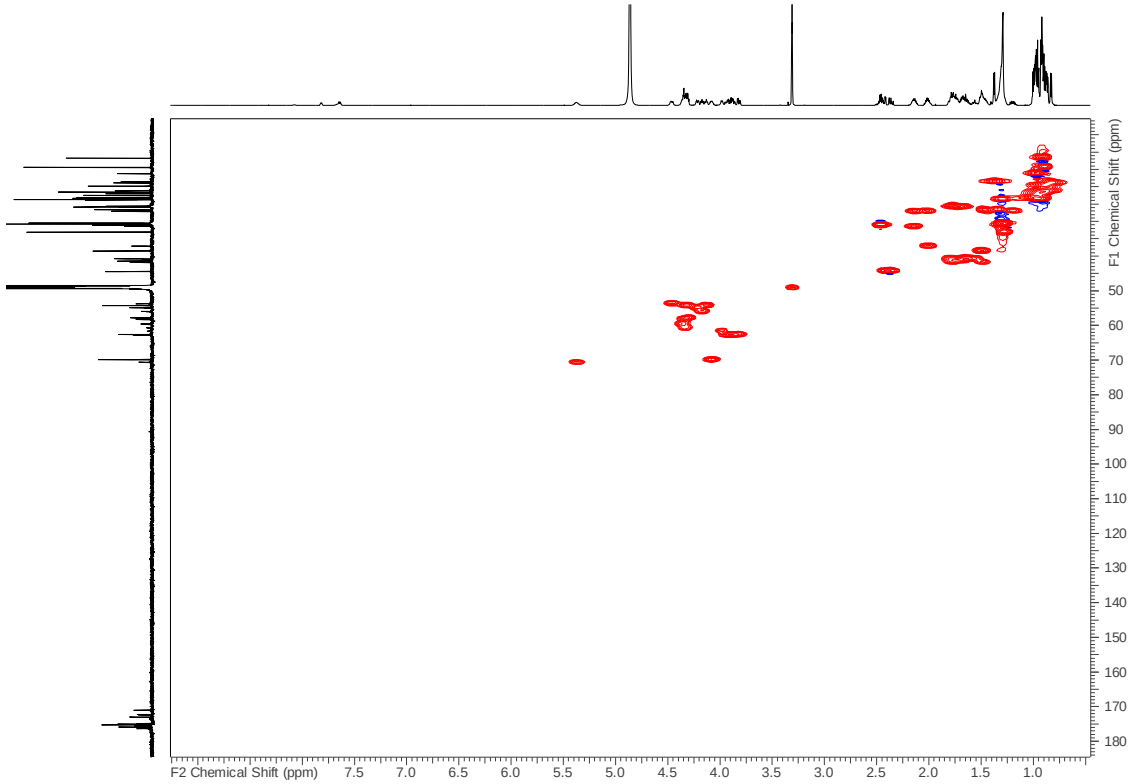
COSY (MeOH-$d_4$, 600 MHz, 297 K)



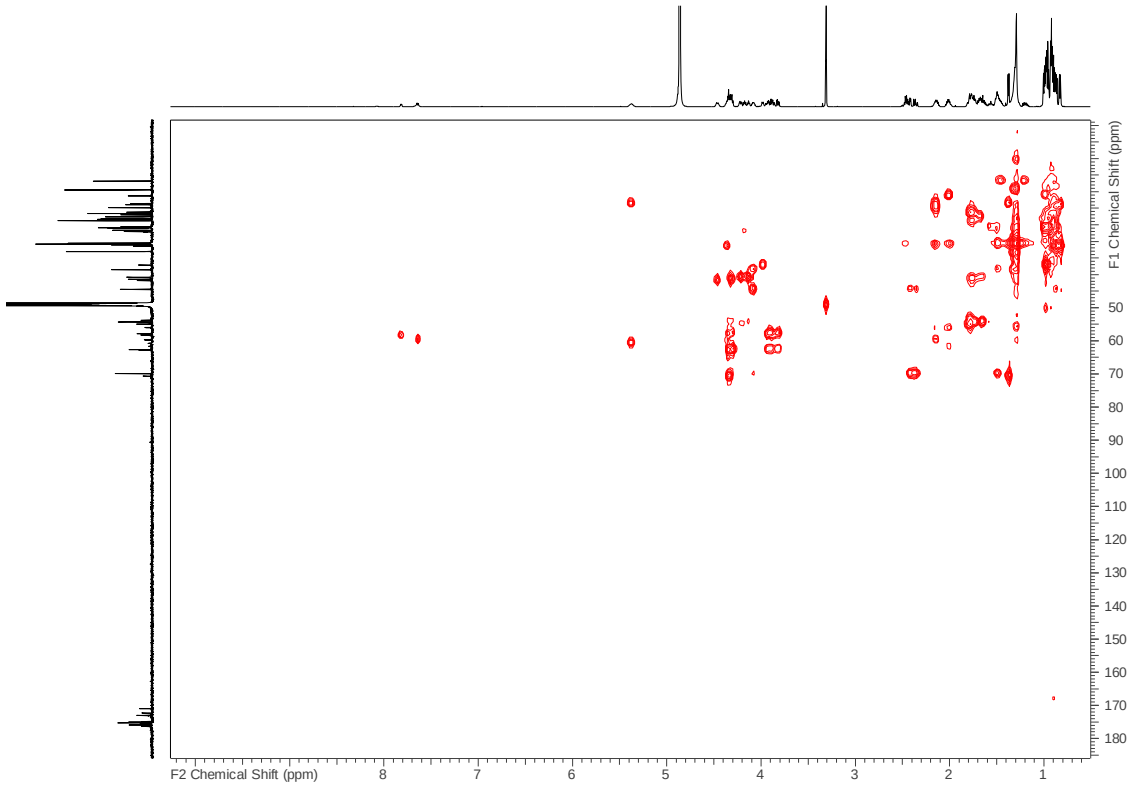

TOCSY (MeOH-$d_4$, 600 MHz, 297 K)



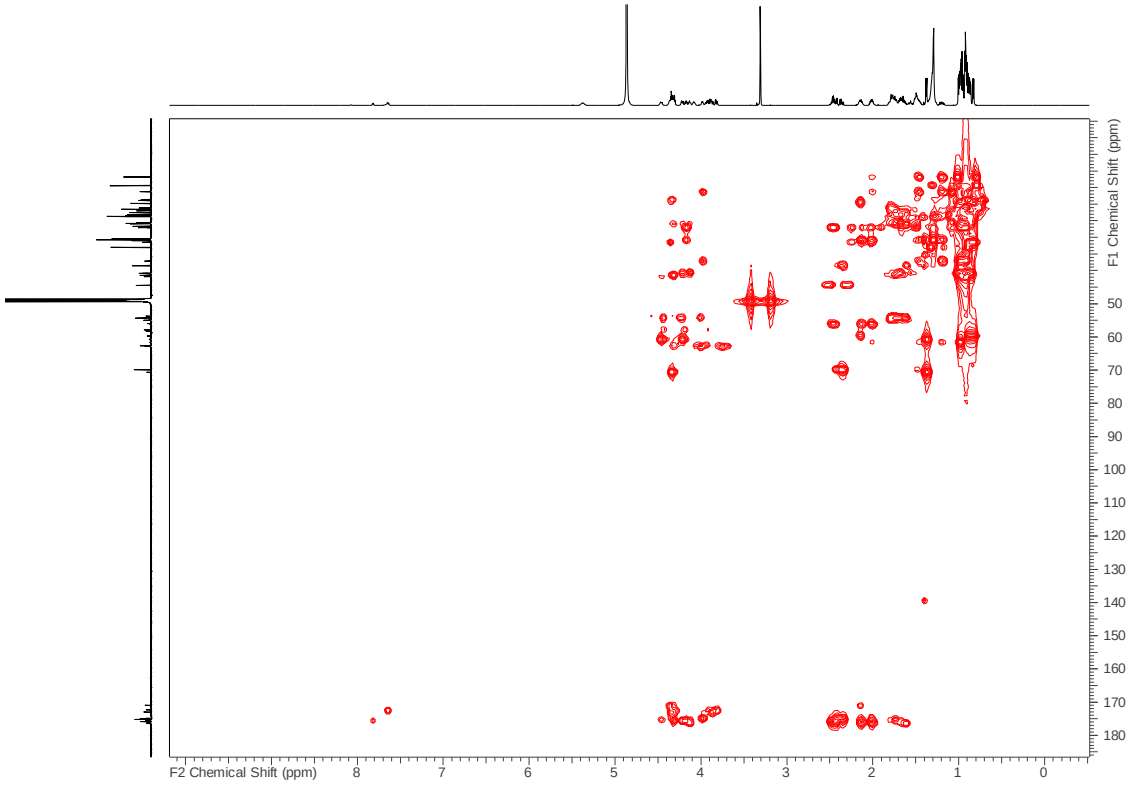
HSQC (MeOH-$d_4$, 600, 151 MHz, 297 K)

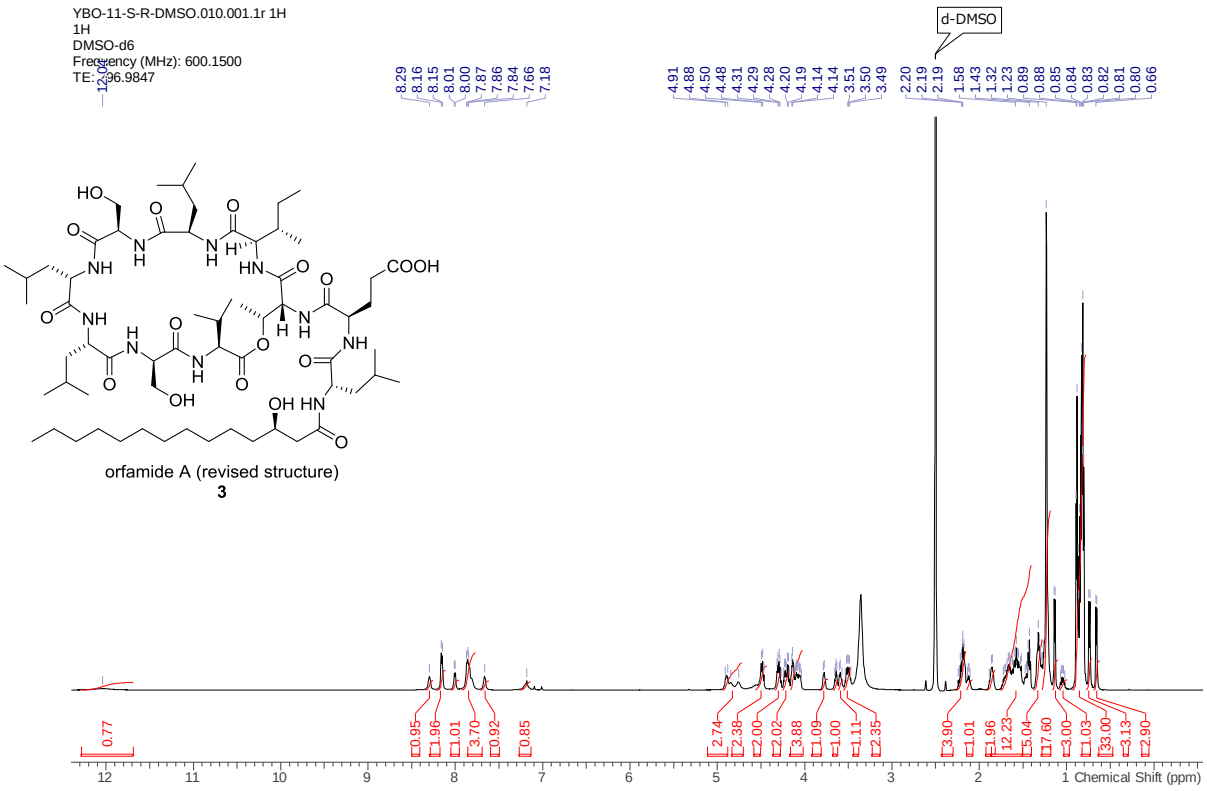



H2BC (MeOH-$d_4$, 600, 151 MHz, 297 K)



HMBC (MeOH-$d_4$, 600, 151 MHz, 297 K)

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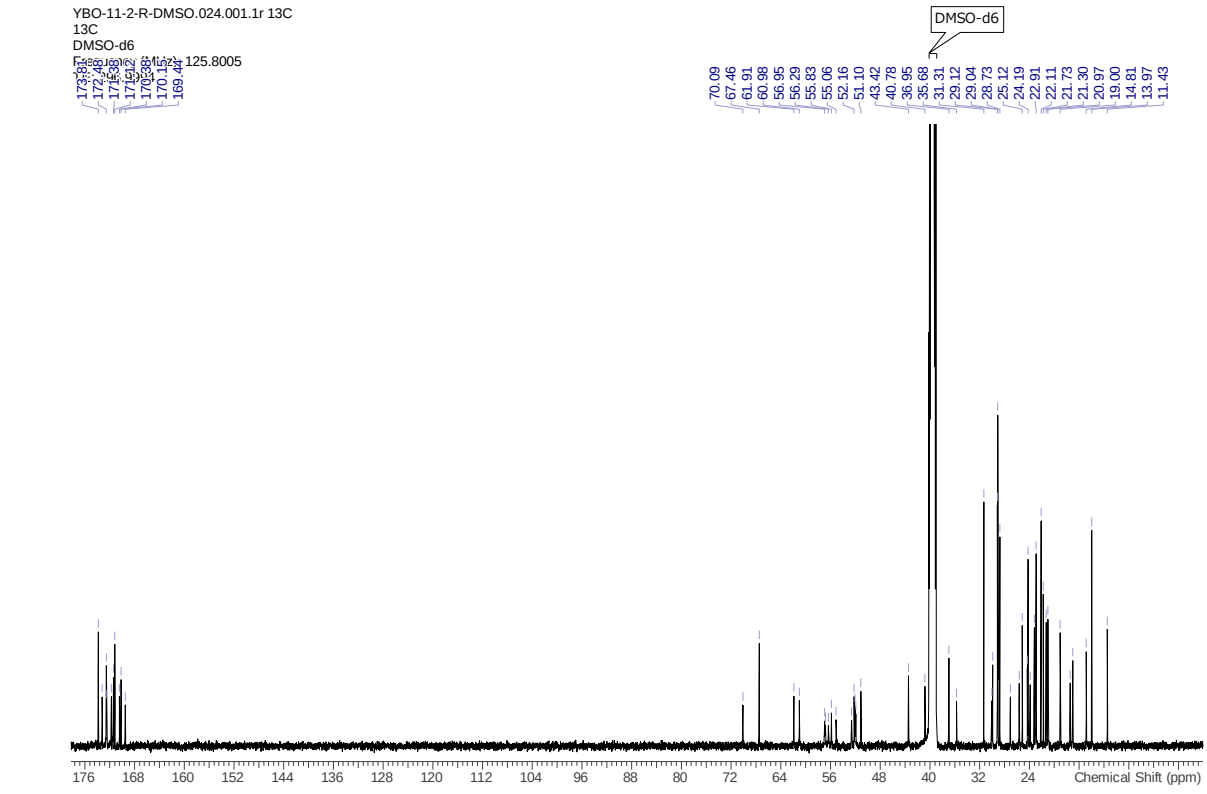
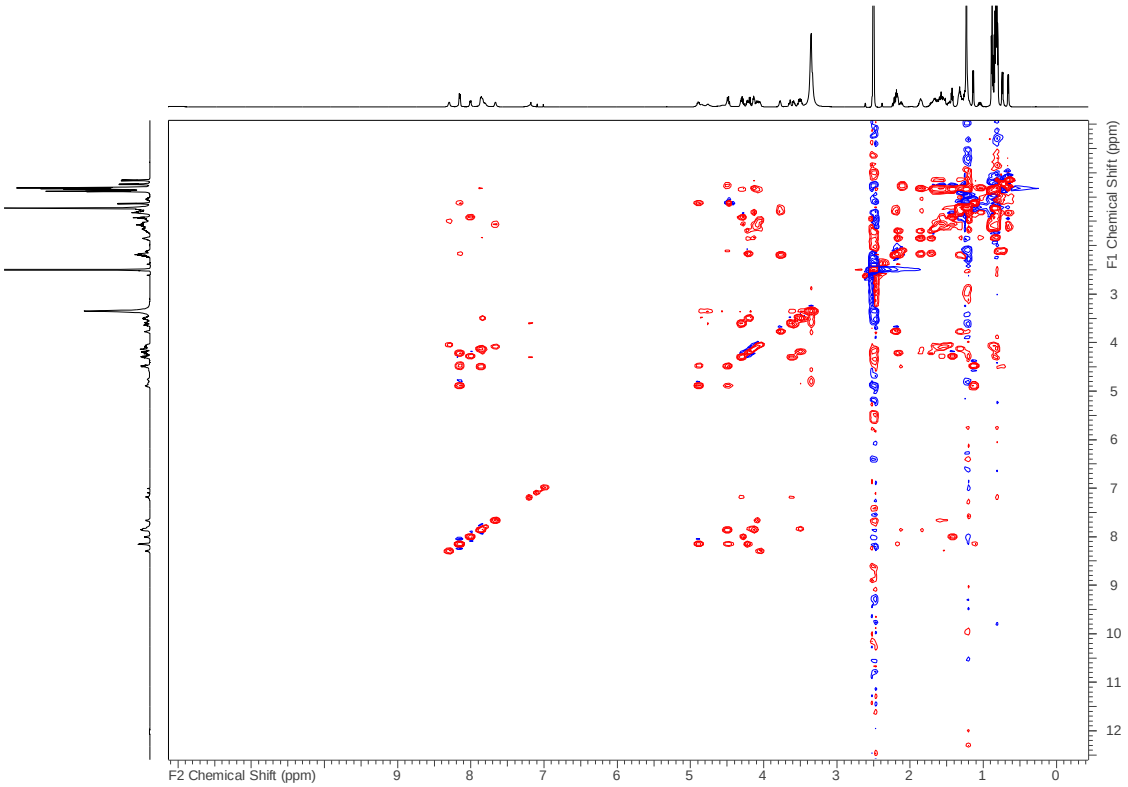
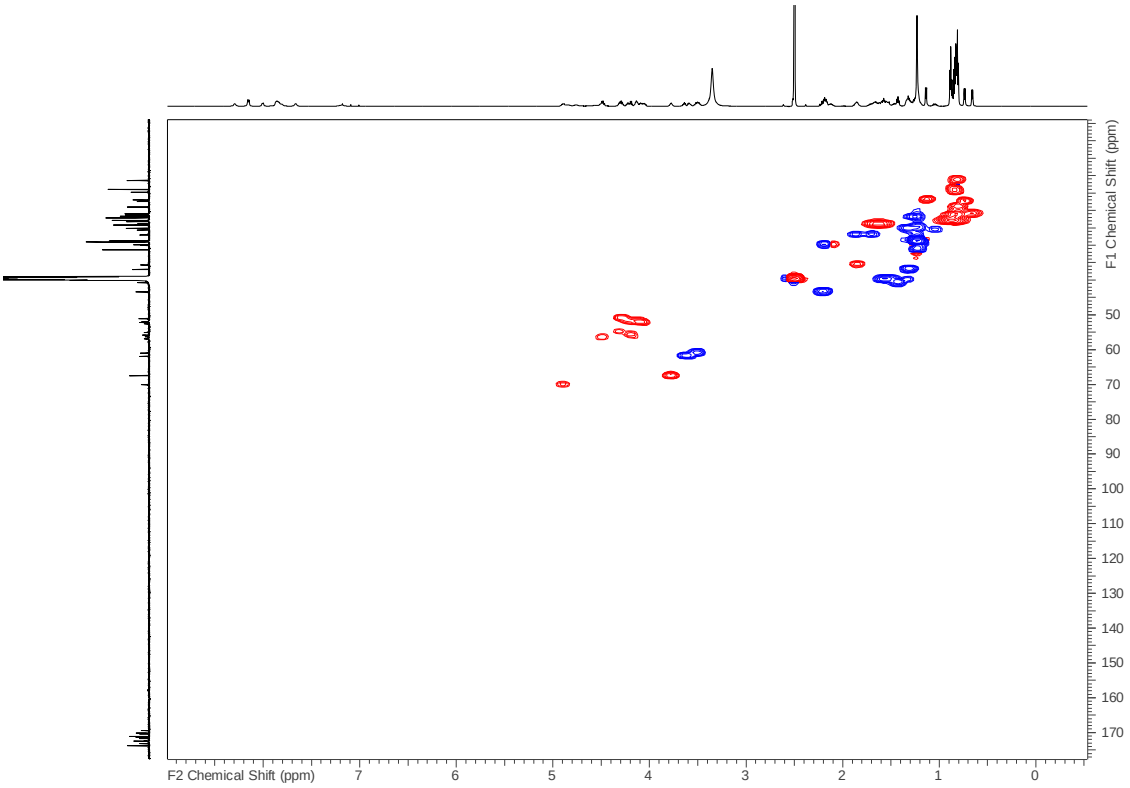
1H
DMSO-d6
Frequency (MHz): 600.1500
TE: 296.9847
d-DMSO
orfamide A (revised structure)
3
Chemical Shift (ppm)


YBO-11-2-R-DMSO.024.001.1r 13C
13C
DMSO-d6
DMSO-d6
Chemical Shift (ppm)

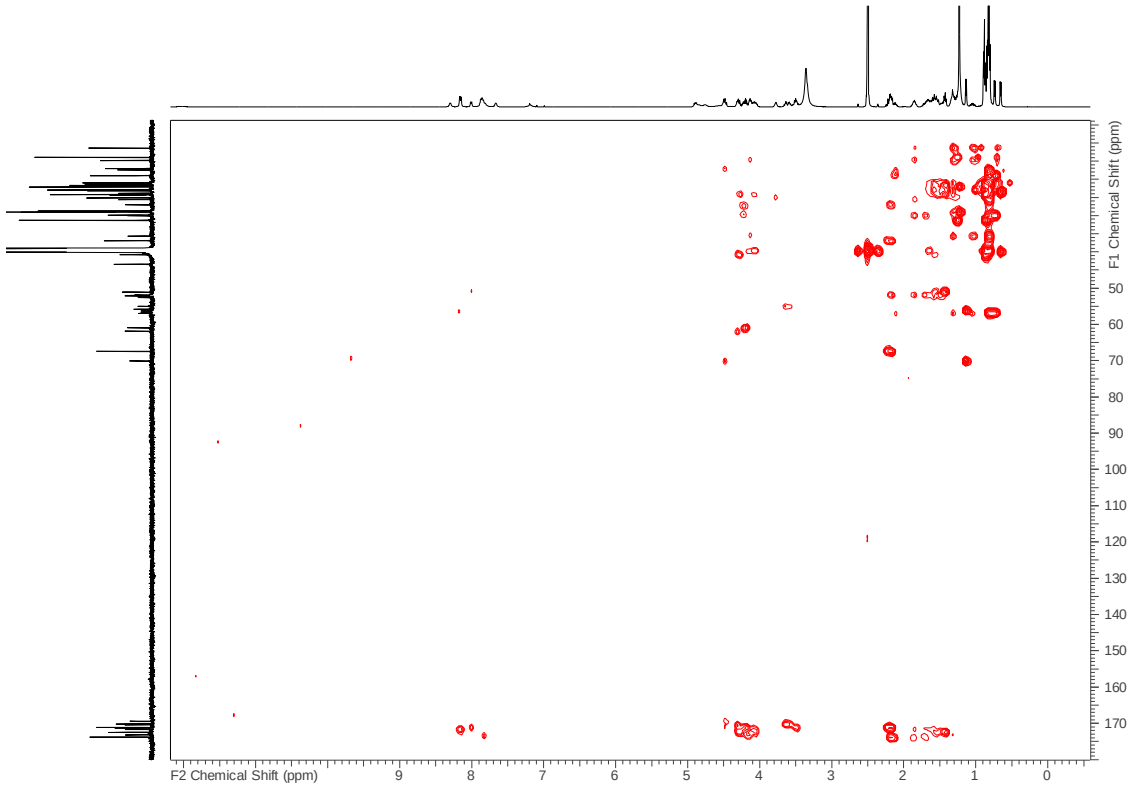
TOCSY (DMSO-$d_6$, 500 MHz, 297 K)

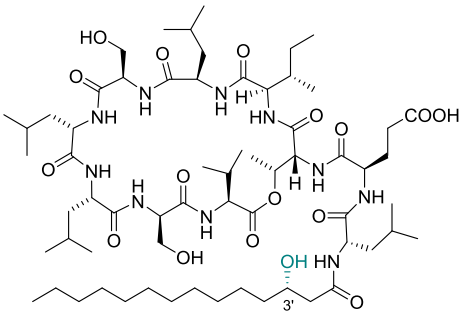



HSQC-DEPT (DMSO-$d_6$, 600, 151 MHz, 297 K)



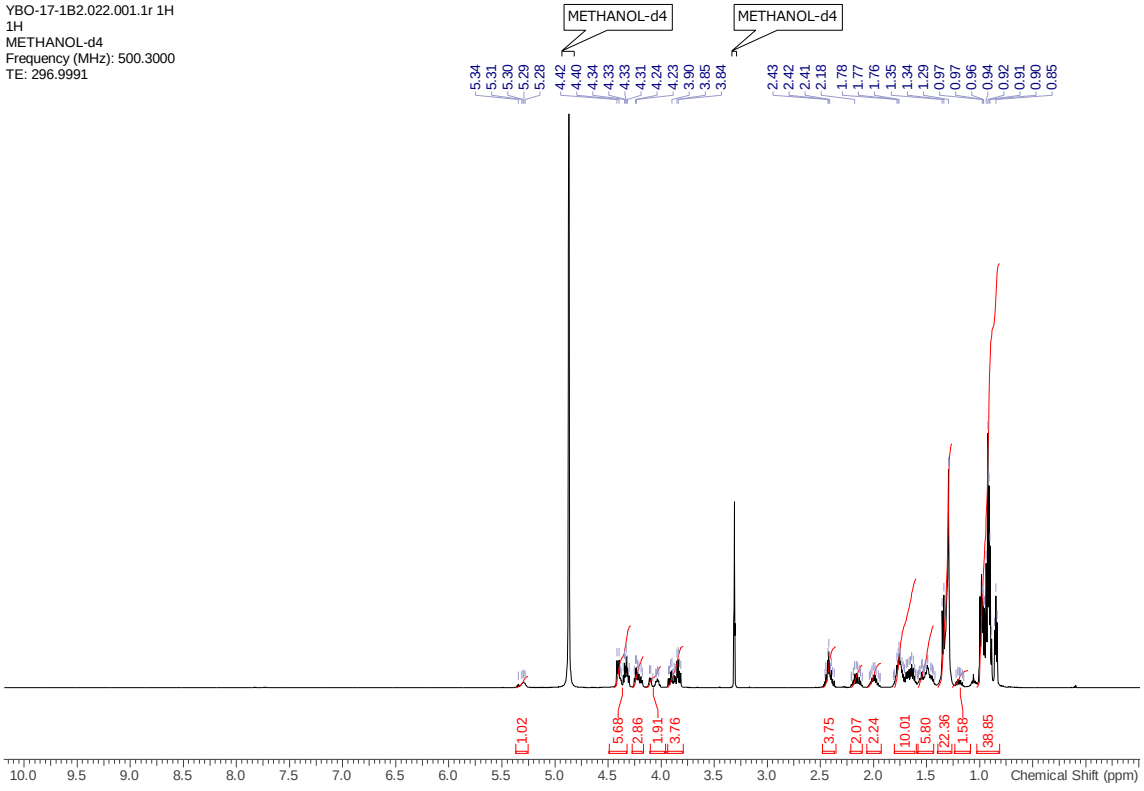
HMBC (DMSO-$d_6$, 500, 126 MHz, 297 K)

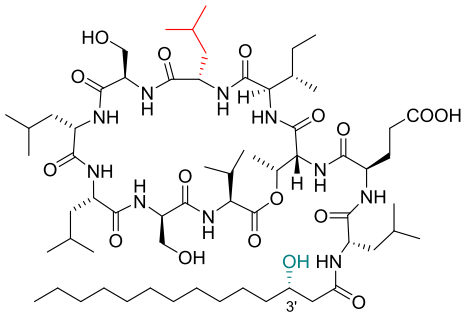





3'-*epi*-orfamide A

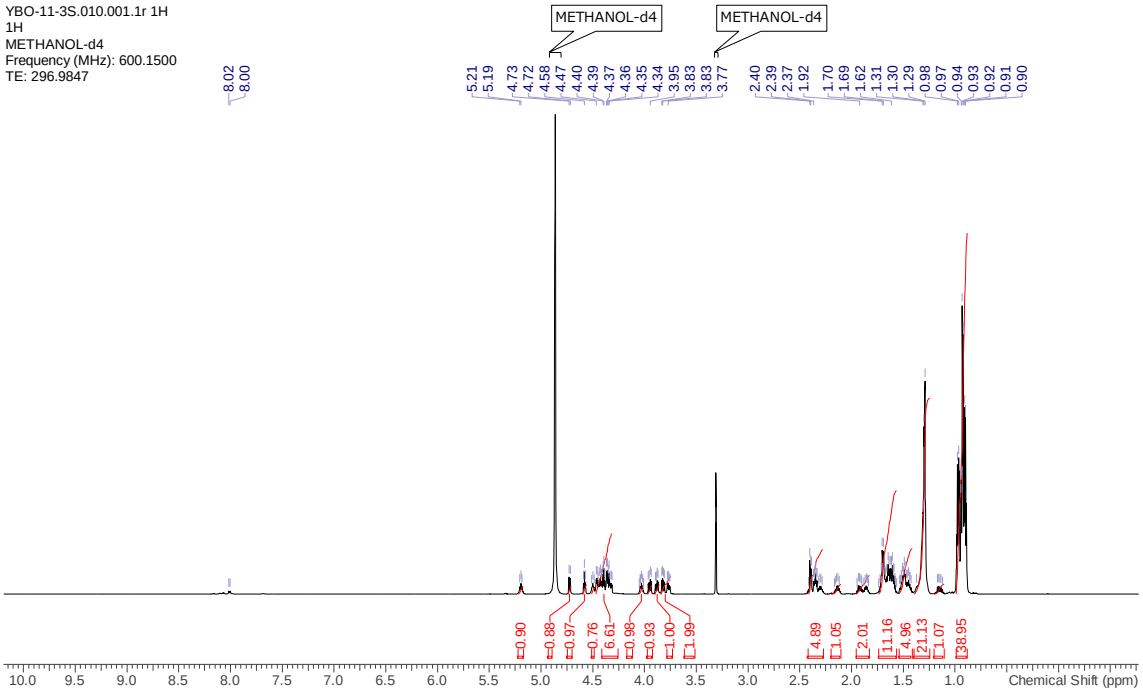
**8**

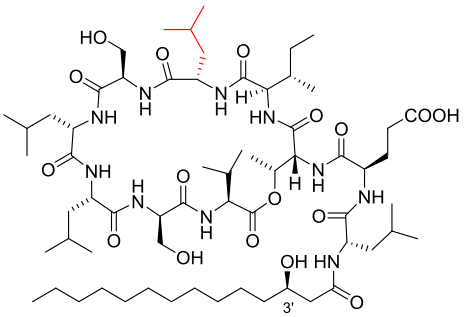





Proposed structcture of orfamide A

**2**





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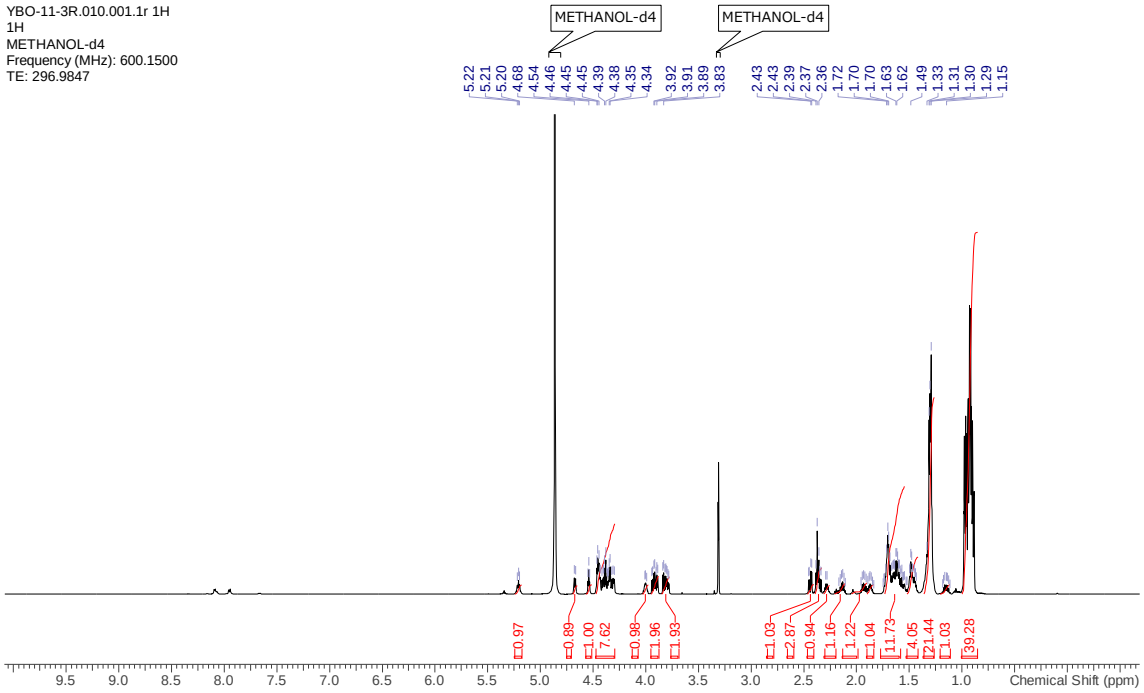
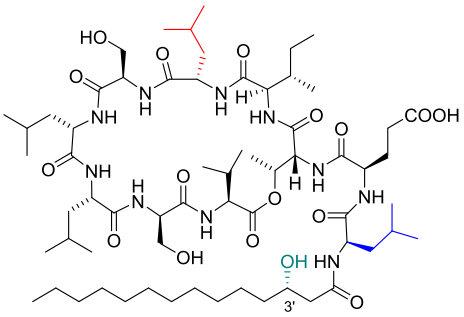
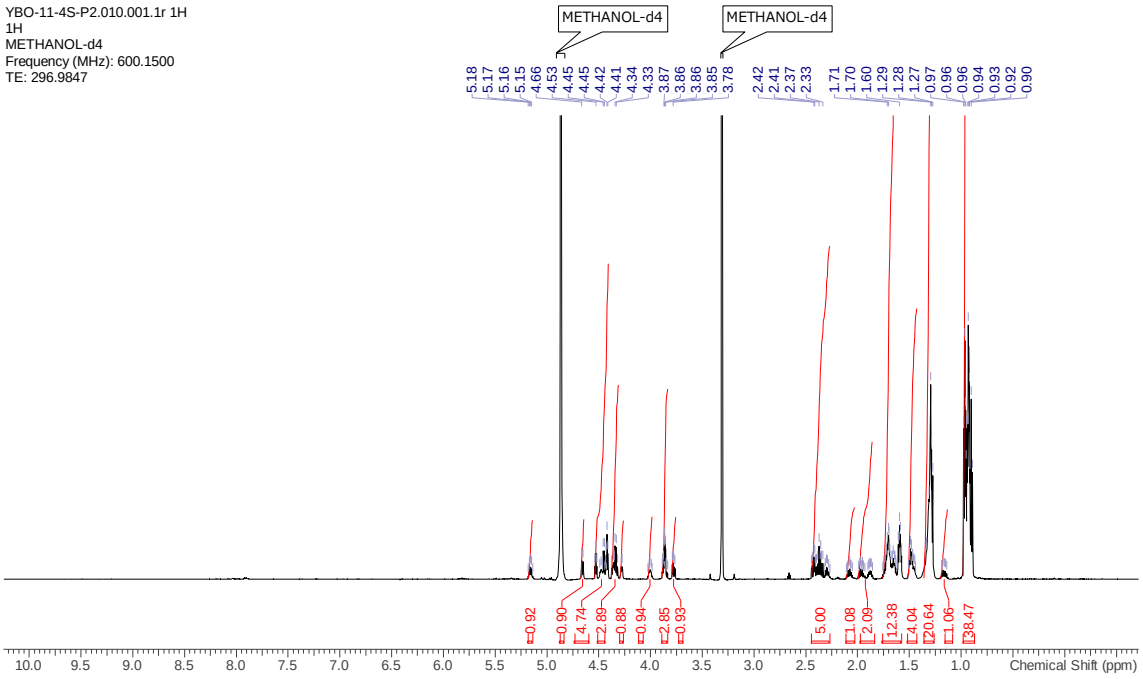
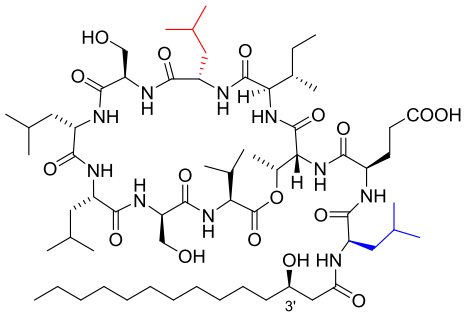

YBO-11-3R.010.001.1r 1H
1H
METHANOL-d4
Frequency (MHz): 600.1500
TE: 296.9847
METHANOL-d4
METHANOL-d4
5.22
5.21
5.20
4.68
4.54
4.46
4.45
4.45
4.39
4.38
4.35
4.34
3.92
3.91
3.89
3.83
2.43
2.43
2.39
2.37
2.36
1.72
1.70
1.70
1.63
1.62
1.49
1.33
1.31
1.30
1.29
1.15
0.97
0.89
1.00
7.62
0.98
1.96
1.93
1.03
2.87
0.94
1.16
1.22
1.04
1.73
4.05
21.44
1.03
39.28
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

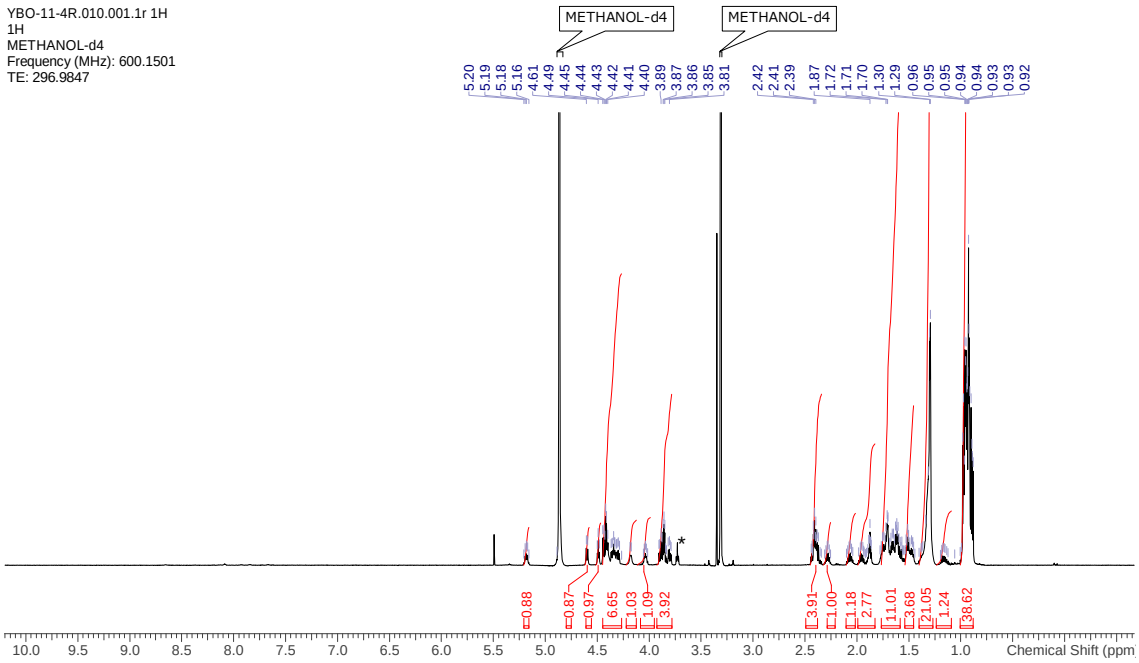


**6**

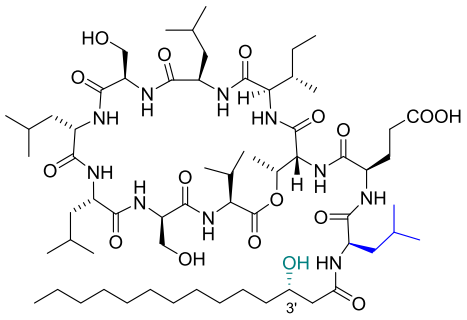

YBO-11-4S-P2.010.001.1r 1H
1H
METHANOL-d4
Frequency (MHz): 600.1500
TE: 296.9847
METHANOL-d4
METHANOL-d4
5.18
5.17
5.16
5.15
4.66
4.53
4.45
4.45
4.42
4.41
4.34
4.33
3.87
3.86
3.86
3.85
3.78
2.42
2.41
2.37
2.33
1.71
1.70
1.60
1.29
1.28
1.27
0.97
0.96
0.96
0.94
0.93
0.92
0.90
=0.92
=0.90
4.74
2.89
=0.88
=0.94
2.85
=0.93
5.00
1.08
2.09
12.38
4.04
20.64
1.06
38.47
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)



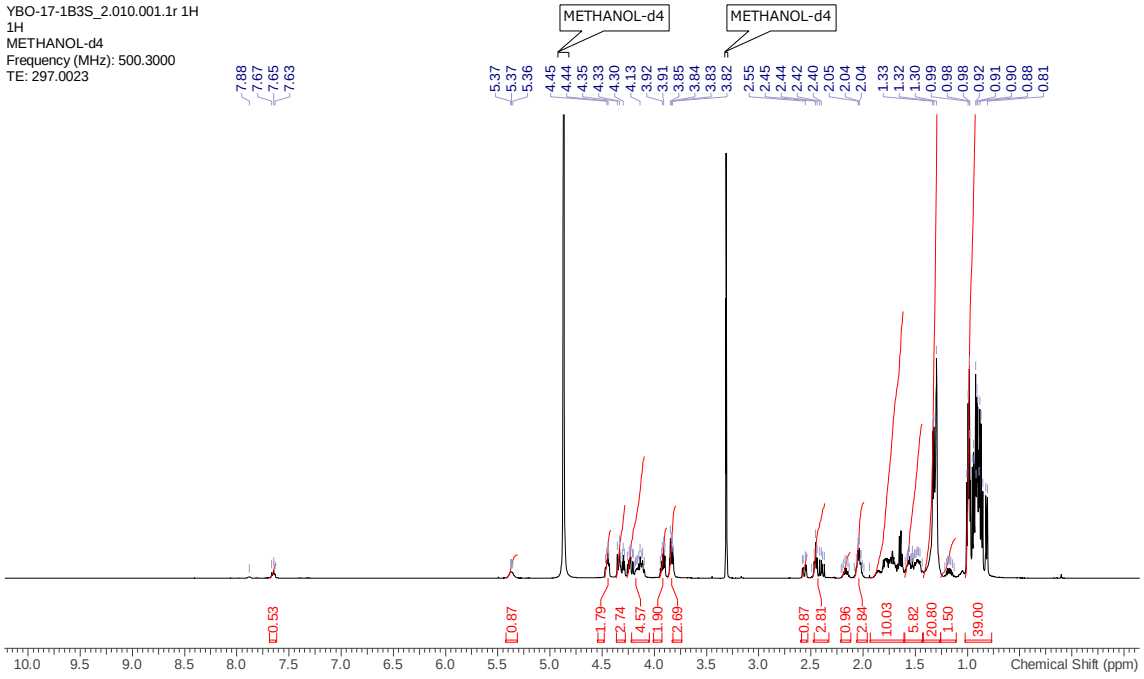
**7**


YBO-11-4R.010.001.1r 1H
1H
METHANOL-d4
Frequency (MHz): 600.1501
TE: 296.9847
METHANOL-d4
METHANOL-d4
5.20
5.19
5.18
5.16
4.61
4.49
4.45
4.44
4.43
4.42
4.41
4.40
3.89
3.87
3.86
3.85
3.81
2.42
2.41
2.39
1.87
1.72
1.71
1.70
1.30
1.29
0.96
0.95
0.95
0.94
0.94
0.93
0.93
0.92
0.88
0.87
0.97
6.65
1.03
1.09
3.92
3.91
1.00
1.18
2.77
11.01
3.68
21.05
1.24
38.62
*
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm

*Impurity



**9**


YBO-17-1B3S_2.010.001.1r 1H
1H
METHANOL-d4
Frequency (MHz): 500.3000
TE: 297.0023
METHANOL-d4
METHANOL-d4
7.88
7.67
7.65
7.63
5.37
5.37
5.36
4.45
4.44
4.35
4.33
4.30
4.13
3.92
3.91
3.85
3.84
3.83
3.82
2.55
2.45
2.44
2.42
2.40
2.05
2.04
2.04
1.33
1.32
1.30
0.99
0.98
0.98
0.92
0.91
0.90
0.88
0.81
0.53
0.87
1.79
2.74
4.57
1.90
2.69
0.87
2.81
0.96
2.84
10.03
5.82
20.80
1.50
39.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
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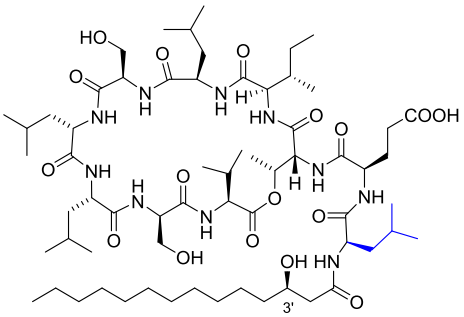
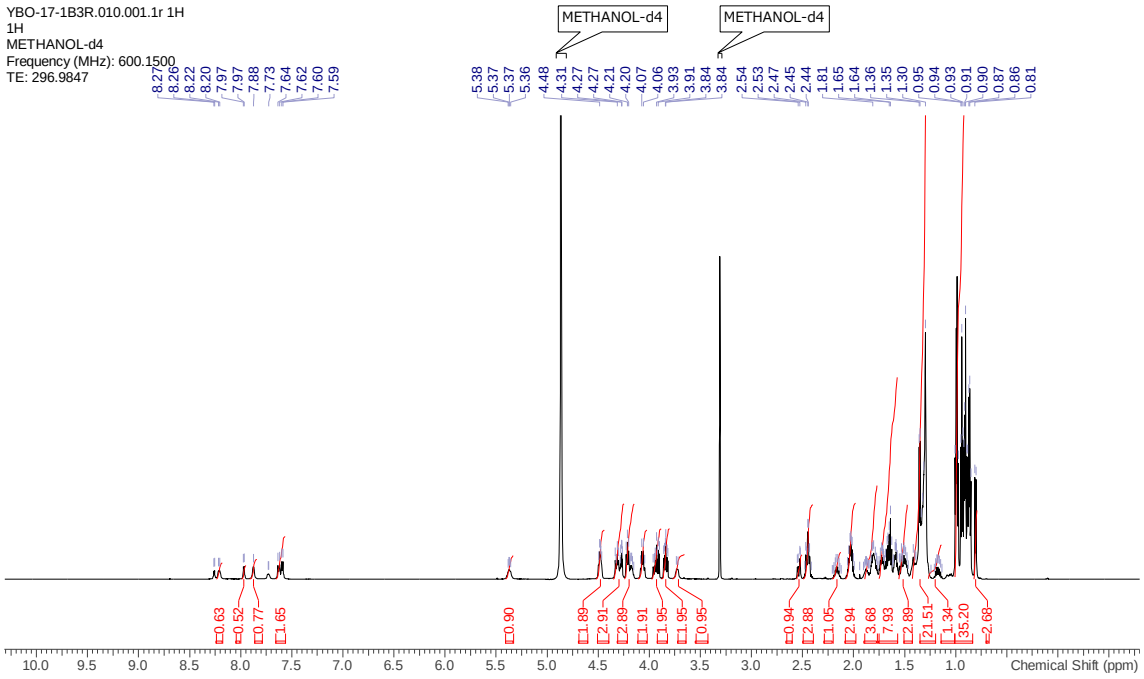
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)



**10**


YBO-17-1B3R.010.001.1r 1H
1H
METHANOL-d4
Frequency (MHz): 600.1500
TE: 296.9847
METHANOL-d4
METHANOL-d4
Chemical Shift (ppm)

## 5 Bioinformatics and genome analysis

The genome of *Pseudomonas protegens* Pf-5 (GenBank Accession Number CP000076) was analyzed with the online version of antiSMASH version 6.0.0alpha1 (Figure S11).[7] The NRPS genes *orfA*, *orfB*, and *orfC*, which code for the biosynthetic machinery of the orfamides, were located in region 4 of the genome (2.347.920-2.437.214 nt). Analysis of the A and C domains of *orfA*, *orfB*, and *orfC* revealed the module specificities (Table S8), which were in good agreement with the previously reported analysis (Table S9). Exceptions were found in the annotation of the $C_{Starter}$ domain of module 1 and in the C/E domain of module 7.

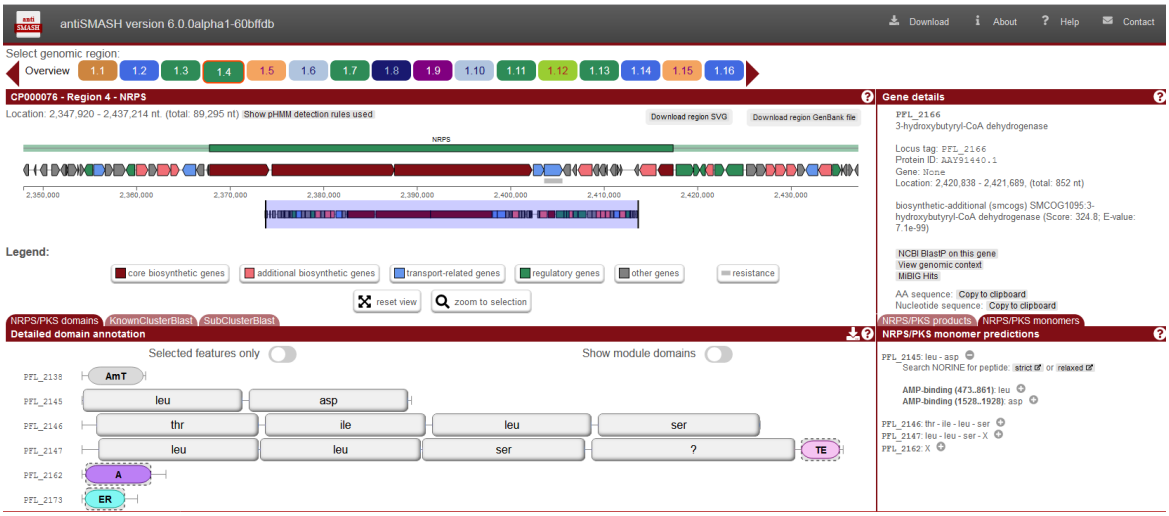



**Figure S13.** antiSMASH output of region 4.

**Table S8:** Results of the antiSMASH analysis of *orfA*, *orfB*, and *orfC*. antiSMASH uses NRPSPredictor2[8] to predict the A domain specificity. $C_{Starter}$ = lipoinitiation condensation domain; C/E = dual condensation/epimerization domain; $^{L}C_{L}$ = C domain that catalyzes the peptide bond formation between two L-amino acid.

| Module | A-Domain | C-Domain | Stachelhaus Code Match | Gene |
|---|---|---|---|---|
| 1 | Leu | $C_{starter}$ | 100% | *orfA* |
| 2 | Asp | C/E | 80% | *orfA* |
| 3 | Thr | C/E | 100% | *orfB* |
| 4 | Ile | C/E | 100% | *orfB* |
| 5 | Leu | C/E | 100% | *orfB* |
| 6 | Ser | C/E | 100% | *orfB* |
| 7 | Leu | C/E | 100% | *orfC* |
| 8 | Leu | $^{L}C_{L}$ | 100% | *orfC* |
| 9 | Ser | $^{L}C_{L}$ | 100% | *orfC* |
| 10 | ? | C/E | - | *orfC* |

**Table S9:** Results of the bioinformatics analysis according to Gross *et al*.[6]

| Module | C-Domain | Gene |
|---|---|---|
| 1 | - | *orfA* |
| 2 | C/E | *orfA* |
| 3 | C/E | *orfB* |
| 4 | C/E | *orfB* |
| 5 | C/E | *orfB* |
| 6 | C/E | *orfB* |
| 7 | - | *orfC* |
| 8 | $^{L}C_{L}$ | *orfC* |
| 9 | $^{L}C_{L}$ | *orfC* |
| 10 | C/E | *orfC* |

## 6 Calcium signal assay

The experiments were performed according to Aiyar et al. with some modifications.[9,10] Cells from the transgenic line AEQ34, expressing apo-aequorin were grown in TAP medium for 48 hours at 23 °C within a light-dark (LD) 12:12 cycle, and then incubated with coelenterazine overnight. The cell density was adjusted to 4-5 x $10^6$ cells / mL before the measurement was started. All compounds were dissolved in methanol and further diluted in TAP medium. They were used at the indicated end concentrations. As control, methanol proportional to 10 µM of compound was applied. Each line represents the mean of three independent biological replicates, and each biological replicate includes three technical replicates.

## 7 Anti Trypanosoma Assay

The bloodstream form of *Trypanosoma brucei brucei* (cell line 449)[11,12] were cultured at 37 °C in HMI-9 medium supplemented with heat-inactivated 10 % FCS, 50 units/ml penicillin, 50 µg/ml streptomycin and 0.2 µg/ml phleomycin (Sigma). The plate reader-based $IC_{50}$ determination of growth inhibition was performed as described previously.[13,14] Briefly, a 40 mM orfamide A stock solution was prepared in DMSO. The compound was pre-diluted in HMI-9 medium to a solution of 1.332 mM, which was further diluted in HMI-9 medium in ten 1:2 consecutive steps. A 10 µl aliquot of each dilution was mixed with 90 µl HMI-9 medium containing 2500 cells/ml in 96-well plate. Final compound concentrations tested ranged from 133.2 to 0.26 µM. The highest DMSO concentration that did not harm the cells (0.3 vol%) was used as negative control. Chlorhexidine, a known trypanocidal compound was used as positive control.[15,16] After 24 or 48 h incubation, 30 µl of ATPlite solution (PerkinElmer) was added to each well and the plate was subjected to luminescence measurement using a microplate reader (CLARIOstar Plus, BMG Labtech). *$EC_{50}$*-values were calculated by plotting the luminescence intensities against logarithmic compound concentration using GraphPad Prism software (GraphPad Software, La Jolla, CA). For the short-term toxicity analysis, logarithmically growing cells were diluted to a cell density of about 1.5 x $10^5$ cells/ml in 5 ml HMI-9 containing 6, 18 or 30 µM orfamide A, or only DMSO. The cells were incubated at 37°C. Viable cells were counted using a Neubauer chamber every hour for six hours, and then once more after 24 h. The mean and standard error of the mean (SEM) of two independent experiments were calculated using GraphPad Prism software. For the morphology analysis by fluorescence microscopy, about 1.2 x $10^6$ cells were incubated in the presence of 30 µM orfamide A or only DMSO at 21 °C, in order to retard cell lysis. Cell fixation, permeabilization, and DAPI staining were performed as described previously.[17]

**A**

| | IC50-value ± SEM (µM) | |
|---|---|---|
| | 24 h | 48 h |
| **Orfamide A** | 6.1 ± 0.4 | 11.1 ± 0.4 |
| **Chlorhexidine** | 0.5 ± 0.008 | 0.4 ± 0.09 |

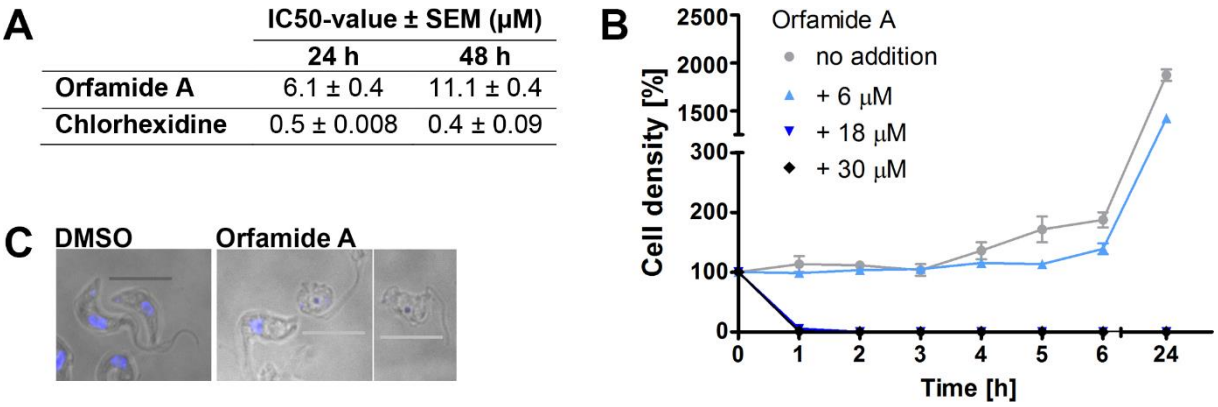



**Figure S13.** Trypanocidal activity of orfamide A against the bloodstream form of *T. brucei*. A) The cells were cultured for 24 or 48 h in the presence of various concentrations of orfamide A and subjected to ATPlite measurements. Chlorhexidine served as positive control. The data represent mean values ± SEM of two independent experiments, each performed in triplicate. B) The parasites were incubated for 24 h in the presence of different concentrations of orfamide A, or DMSO only (no addition). Living cells were counted every hour for six hours, and after 24 h. The data are mean ± SEM of two independent experiments. C) The cells were kept in the presence of 30 µM orfamide A or DMSO only at 21 °C for 1 h, fixed, stained with DAPI (blue) and subjected to immunofluorescence microscopy. The images represent merged DIC and DAPI images. Size of bar: 10 µM.

## 8 References


(1) G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw, K. I. Goldberg, *Organometallics* **2010**, *29*, 2176–2179.

(2) M. De Vleeschouwer, D. Sinnaeve, J. Van Den Begin, T. Coenye, J. C. Martins, A. Madder, *Chem. Eur. J.* **2014**, *20* , 7766–7775.

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