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Supporting Information

Total Chemical Synthesis of the Antibacterial, Head-to-Tail Cyclized Protein AS-48 by KAHA Ligation

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1. General Methods

1.1. Reagents and solvents

Fmoc-amino acids with suitable side-chain protecting groups, HCTU (*O*-(1*H*-6-Chlorobenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluroniumhexafluorophosphate) and HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) were purchased from Peptides International (Louisville, KY, USA) and ChemImpex (Wood Dale, IL, USA). Solvents for flash chromatography (EtOAc, MeOH) were of technical grade and distilled prior to use. HPLC grade CH_3CN from Sigma-Aldrich was used for analytical and preparative HPLC purification. DMF (> 99.8%) from Sigma-Aldrich was directly used without further purification for solid phase peptide synthesis. Other commercially available reagents and solvents were purchased from Sigma- Alrich (Buchs, Switzerland), Acros Organics (Geel, Belgium) and TCI Europe (Zwijndrecht, Belgium). Boc-(*S*)-5-oxaproline[1] was prepared as previously reported by our group.

1.2. Characterization

^1H and ^{13}C NMR spectra were recorded on Bruker DRX400, Bruker AVIII400 and Bruker AVIII600 spectrometers. Chemical shifts for ^1H NMR (400 and 600 MHz) and ^{13}C NMR (101 and 150 MHz) are expressed in parts per million and are referred to residual undeuterated solvent signals. Coupling constants are reported in Hertz (Hz) and the corresponding splitting patterns are indicated as follows: s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublet; td, triplet of doublet; t, triplet; m, multiplet; appt d, apparent triplet of a doublet; d apt, doublet of an apparent triplet; d appt d, doublet of an apparent triplet of a doublet. High- resolution mass spectra were recorded by the Mass Service of the Laboratory of Organic Chemistry at ETH Zurich either with a Bruker maXis instrument (ESI-MS measurements) equipped with an ESI source and a Qq-TOF detector or with a Bruker solariX instrument (MALDI-FTICR-MS) using 4-hydroxy-α-cyanocinnamic acid as matrix.

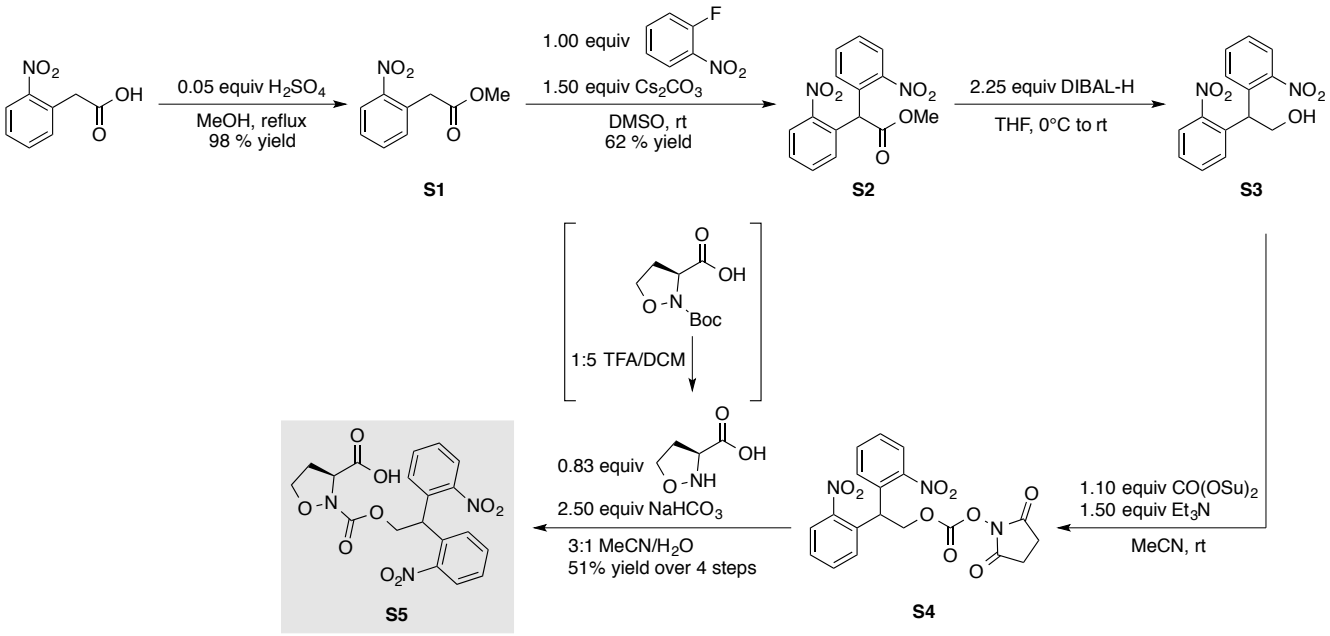
1.3. Reactions and purification

All reactions were performed using standard techniques under an atmosphere of N_2. Reactions and fractions from flash chromatography were monitored by thin layer chromatography using precoated glas plates (Merck, silica 60 F254) and visualized by staining with basic $KMnO_4$ solution. Flash chromatography was performed on Silicycle SiO_2 Type F60 (230-400 mesh) using a forced flow of air at 0.5-1.0 bar. Unless otherwise stated, peptides and protein segments were analyzed and purified by reversed phase high performance liquid chromatography (RP-HPLC) on Jasco analytical and preparative instruments equipped with dual pumps, mixer and in-line degasser, a variable wavelength UV detector (simultaneous monitoring of the eluent at 220 nm, 254 nm and 301 nm) and a Rheodyne injector fitted with a 20 or 1000 µl injection loop. If required, the columns were heated using an Alltech column heater or a water bath (preparative HPLC). The mobile phase for RP-HPLC were Milipore-H_2O containing 0.1 % (v/v) TFA and HPLC grade CH_3CN containing 0.1 % (v/v) TFA. Analytical HPLC was performed on Shiseido Capcell Pak C18 MGII (5 µm, 4.6 mm I.D. x 250 mm) or Shiseido Capcell Pak C18 (UG 80, 5 µm, 4.6 mm I.D. x 250 mm) columns at a flow rate of 1 ml/min. Preparative HPLC was performed on Shiseido Capcell Pak MGII (5 µm, 20 mm I.D. x 250 mm) or Vydac 248MS C18 (10 µm, 22 mm I.D. x 250 mm) columns at a flow rate of 10 ml/min.

1.4. Solid phase peptide synthesis

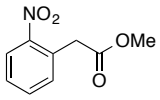
Peptides were synthesized on a Multisyntech Syro I parallel synthesizer using Fmoc SPPS chemistry. The following Fmoc amino acids with side-chain protection groups were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(1-Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH.

2. Preparation of Photoprotected (*S*)-5-Oxaproline S5



Scheme S1: Preparation of protected photoprotected (S)-5-Oxaproline **S5**.

2.1. Methyl 2-(2-nitrophenyl)acetate S1



S1

2-Nitrophenylacetic acid (10.0 g, 55.2 mmol, 1.00 equiv) was dissolved in MeOH (110 mL). H_2SO_4 (147 µL, 2.76 mmol, 0.05 equiv) was added and the mixture was heated to reflux for 3 h. MeOH was removed under reduced pressure. The brown residue was dissolved in EtOAc (150 mL) and washed with sat. aq. $NaHCO_3$ (2 x 50 mL) and brine (30 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated to obtain the product **S1** (10.6 g, 54.2 mmol, 98 %) as yellow oil which solidified upon storage at –20 °C. The analytical data matches the reported values.[2]

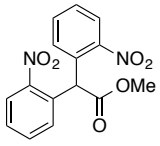
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (dd, *J* = 8.2, 1.3 Hz, 1H, CH), 7.60 (td, *J* = 7.5, 1.4 Hz, 1H, CH), 7.51 – 7.44 (m, 1H, CH), 7.36 (dd, *J* = 7.6, 1.4 Hz, 1H, CH), 4.03 (s, 2H, CH_2), 3.71 (s, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5 (CO), 148.9 (C), 133.7 (CH), 133.5 (C), 129.9 (CH), 128.8 (CH), 125.4 (CH), 52.4 (CH_3), 39.7 (CH_2).

IR (thin film): 1738, 1524, 1436, 1414, 1347, 1311, 1288, 1218, 1170, 1000, 864, 789, 711 cm^{-1}.

HR-MS (ESI): calculated for $C_9H_9NNaO_4$ $[M+Na]^+$: 218.0424, found: 218.0428.

2.2. Methyl 2,2-bis(2-nitrophenyl)acetate S2



S2

Methyl 2-(2-nitrophenyl)acetate (**S1**) (10.6 g, 54.1 mmol, 1.00 equiv.) was dissolved in DMSO (20 mL). 2-Fluoronitrobenzene (5.71 mL, 54,1 mmol, 1.00 equiv) was added in one portion by syringe. The flask was placed in a water bath at rt. Cs_2CO_3 (30.5 g, 79.7 mmol, 1.50 equiv) was added in one portion resulting in a blue suspension. The reaction mixture was stirred for 2 h at rt. The reaction mixture was cooled to 0 °C and H_2O (200 mL) was slowly added. The mixture was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with citric acid (10 %w/w, 20 mL), H_2O (50 mL) and brine (20 mL). The combined aqueous layers were re-extracted with EtOAc (50 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated to obtain the crude product (16.8 g) as yellow oil, which crystallized upon storage at –20 °C. The crude material was purified by recrystallization from a mixture of petroleum ether 60/90 (60 mL) and EtOAc (25 ml). The precipitate was collected by filtration and washed with hexanes/EtOAc 9:1 (100 mL) to obtain the pure product **S2** (10.6 g, 33.5 mmol, 62 %) as pale yellow solid. The analytical data matches the reported values.[3]

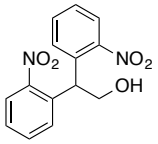
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (dd, *J* = 8.1, 1.5 Hz, 2H, CH), 7.60 (td, *J* = 7.6, 1.5 Hz, 2H, CH), 7.52 (td, *J* = 7.8, 1.5 Hz, 2H, CH), 7.19 (dd, *J* = 7.8, 1.5 Hz, 2H, CH), 6.35 (s, 1H, CH), 3.78 (s, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 171.2 (CO), 149.0 (C), 133.7 (CH), 132.3 (C), 131.1 (CH), 129.1 (CH), 125.8 (CH), 53.1 (CH_3), 50.3 (CH).

IR (thin film): 1741, 1607, 1577, 1525, 1436, 1348, 1306, 1272, 1203, 1172, 1005, 854, 787, 744, 712 cm-1. HR-

HR-MS (ESI): calculated for $C_{15}H_{12}N_2NaO_6$ $[M+Na]^+$: 339.0588, found: 339.0588.

2.3. 2,2-bis(2-nitrophenyl)ethan-1-ol S3



S3

Methyl 2,2-bis(2-nitrophenyl)acetate (**S2**) (5.34 g, 16.9 mmol, 1.00 equiv) was dissolved in anhydrous THF (50 mL) at rt in an oven dried flask. The solution was cooled to 0 ℃ and diisobutylaluminium hydride (1 M in toluene, 38.0 mL, 38.0 mmol, 2.25 equiv) was added dropwise within 1 h at 0 ℃. The mixture was allowed to warm to rt and was stirred for 1 h. The mixture was cooled to -20 ℃ and H_2O (1.50 mL) was added dropwise. Aqueous NaOH (15 %w/w, 1.50 mL) was added dropwise. H_2O (3.80 mL) was added dropwise, the mixture was allowed to warm up to rt and was stirred for 30 min. $MgSO_4$ (3.00 g) was added and the suspension was filtered through a plug of celite (washed with 2 x 30 mL EtOAc). The filtrate was washed with brine (20 mL), dried over $MgSO_4$, filtered and concentrated to obtain the crude material (4.95 g) as brown solid, which used for the next step without further purification. An analytically pure sample was obtained by flash chromatography (SiO_2, hexanes/EtOAc 9:1 to 7:3).[4]

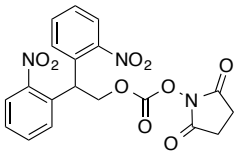
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (dd, *J* = 8.1, 1.4 Hz, 2H, CH), 7.61 – 7.55 (m, 2H, CH), 7.51 (dd, *J* = 7.9, 1.6 Hz, 2H, CH), 7.42 (ddd, *J* = 8.1, 7.2, 1.6 Hz, 2H, CH), 5.51 (t, *J* = 6.2 Hz, 1H, CH), 4.36 (dd, *J* = 6.5, 2.8 Hz, 2H, CH_2), 2.03 (bs, 1H, OH).

^{13}C NMR (101 MHz, $CDCl_3$) δ 149.8 (C), 135.0 (CH), 133.1 (C), 130.2 (CH), 128.2 (CH), 125.5 (CH), 64.4 (CH_2), 43.6 (CH).

IR (thin film): 1523, 1350, 1051, 855, 786, 741, 712 cm^{-1}.

HR-MS (ESI): calculated for $C_{14}H_{12}N_2NaO_5$ $[M+Na]^+$: 311.0638, found: 311.0631.

2.4. 2,2-Bis(2-nitrophenyl)ethyl (2,5-dioxopyrrolidin-1-yl) carbonate S4



S4

2,2-bis(2-nitrophenyl)ethan-1-ol (**S3**) (4.86 g, 16.9 mmol, 1.00 equiv) was dissolved in MeCN (85 mL) at rt. N,N-Disuccinimidyl carbonate (4.75 g, 18.6 mmol, 1.10 equiv) was added in one portion. Et_3N (3.51 ml, 25.3 mmol, 1.50 equiv) was added dropwise within 5 min. The resulting clear orange solution was stirred for 2 h at rt. The solvent was removed under reduced pressure. The residue was dissolved in EtOAc (250 mL) and washed with aq. citric acid (10 %w/w, 50 mL) and H_2O (50 mL). The combined aqueous layers were extracted with EtOAc (20 mL), dried over $MgSO_4$, filtered and concentrated to obtain the crude material (8.04 g) as yellow foam, which was used for the next reaction without further purification. An analytically pure sample can be obtained by flash chromatography (SiO_2, hexanes/EtOAc 7:3 to 3:7).

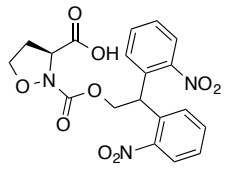
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (dd, *J* = 8.2, 1.4 Hz, 2H, CH), 7.63 (td, *J* = 7.7, 1.5 Hz, 2H, CH), 7.50 – 7.41 (m, 4H, CH), 5.85 (t, *J* = 5.8 Hz, 1H, CH), 5.10 (d, *J* = 5.8 Hz, 2H, CH_2), 2.80 (s, 4H, CH_2).

^{13}C NMR (101 MHz, $CDCl_3$) δ 168.4 (CO), 151.4 (CO), 149.4 (CO), 133.6 (CH), 133.2 (C), 130.1 (CH), 128.9 (CH), 125.9 (CH), 70.5 (CH_2), 41.0 (CH), 25.5 (CH_2).

IR (thin film): 1814, 1790, 1741, 1525, 1352, 1258, 1213, 1091, 855, 745, 714 cm^{-1}.

HR-MS (MALDI): calculated for $C_{19}H_{15}N_3NaO_9$ $[M+Na]^+$: 452.0701, found: 452.0699.

2.5. (*S*)-((2,2-bis(2-nitrophenyl)ethoxy)carbonyl)-5-oxaproline ((*S*)-di-NPEOC-5-oxaproline) S5



S5

Boc-(*S*)-5-Oxaproline (1.00 g, 4.60 mmol, 1.00 equiv) was dissolved in CH_2Cl_2 (46 mL) at rt and TFA (11.5 mL) was added. The yellow solution was stirred for 1 h at rt. The solvents were removed under reduced pressure. The residue dissolved in toluene and the solvent was removed under reduced pressure to remove residual TFA (3 x repeated). The residue was dissolved in 3:1 MeCN/H_2O (46 mL) and 2,2-bis(2-nitrophenyl)ethylsuccinimidylcarbonat (**S4**) (2.37 g, 5.53 mmol, 1.20 equiv) was added. $NaHCO_3$ (1.16 g, 13.8 mmol, 3.00 equiv) was added slowly. The mixture was stirred at rt for 3.5 h. Ethylenediamine* (300 µL) was added and the mixture was stirred for 10 min. The mixture was diluted with EtOAc (150 mL) and 1 M HCl (250 mL) was added. The phases were separated and the aqueous layer was extracted with MTBE (50 mL). The combined organic layers were washed with H_2O (50 mL) and brine (25 mL), dried over $MgSO_4$ and concentrated to obtain the crude material (2.32 g) as a light green foam, which was purified by flash chromatography (SiO_2, hexanes/EtOAc 8:2 to 3:7 to EtOAc/MeOH 4:1) to afford pure product **S5** (1.73 g, 4.01 mmol, 51 % yield over 4 steps) as light brown foam. *Ethylendiamine was used to convert excess **S4** to a water-soluble product that can be removed by acidic aqueous workup.

1H NMR (400 MHz, $CDCl_3$) δ 7.99 – 7.92 (m, 2H, CH), 7.61 – 7.55 (m, 2H, CH), 7.47 – 7.40 (m, 4H, CH), 5.77 (t, *J* = 6.3 Hz, 1H, CH), 5.37 (bs, 1H, COO*H*), 4.90 (dd, *J* = 6.4, 1.3 Hz, 2H, CH_2), 4.65 (q, *J* = 9.5, 5.2 Hz, 1H, CH), 4.10 (td, *J* = 7.9, 4.3 Hz, 1H, CH_2), 3.79 – 3.67 (m, 1H, CH_2), 2.64 (dddd, *J* = 12.6, 9.5, 7.1, 4.3 Hz, 1H, CH_2), 2.51 (dtd, *J* = 12.8, 7.9, 5.2 Hz, 1H, CH_2)

13C NMR (101 MHz, $CDCl_3$) δ 173.9 (CO), 156.1 (CO), 150.0 (C), 149.5 (C), 134.0* (C), 133.9* (C), 133.3* (CH), 133.2* (CH), 130.2* (CH), 130.1* (CH), 128.7* (CH), 128.6* (CH), 125.71* (CH), 125.69* (CH), 69.1 (CH_2), 66.9 (CH_2), 59.4 (CH), 40.7 (CH), 33.0 (CH_2). *signals of rotamers.

IR (thin film): 1726, 1525, 1350, 1305, 1215, 1078, 855, 787, 744, 711 cm^{-1}.

HR-MS (ESI): calculated for $C_{19}H_{17}N_3NaO_9$ $[M+Na]^+$: 454.0857, found: 454.0855.

3. Synthesis of AS-48

Sequence natural protein:

MAKEFGIPAA VAGTVLNVVE AGGWVTTIVS ILTAVGSGGL SLLAAAGRES IKAYLKKEIK KKGKRAVIAW

Segment 1:

(di-NPEOC-Opr–IKAYLKKEI KKKGKRAVIA WMAKEFGIPA AVAGTVLNVV EAGGW–V(Ketoacid)

Segment 2:

Opr–TIVSILTAVG SGGLSLLAAA GR–E(Protected Ketoacid)

3.1. SPPS of Segment 1 (1)

Segment 1 (**1**) was assembled by Fmoc SPPS on 0.3 mmol scale. Protected valine α-ketoacid was prepared and loaded on the resin (Peptides International Rink amide polystyrene resin, crosslinked with 1 % divinylbenzene, loading 0.32 mmol/g) as previously described.[5,6] The last residue – photoprotected (*S*)-5-oxaproline **S5** – was coupled using 1.75 equiv **S5**, 1.75 equiv HATU and 4 equiv DIPEA in DMF for 2 h.

Coupling: 4.00 equiv of Fmoc protected amino acid, 4.00 equiv of HCTU (for residues 1-29) or HATU (for residues 30-46), 8.00 equiv of DIPEA, solvent: DMF, 2 x 45 min, followed by capping with 20 % Ac_2O in DMF.

Deprotection: 5 min wash with 0.8 M LiCl in DMF, 2 x 10 min with 20 % piperidine in DMF.

Cleavage and Purification: The peptide resin (0.30 mmol) was treated for 2.5 h with 45 mL of a mixture of: 81 : 5 : 5 : 5 : 2.5 : 1.5 TFA/thioanisole/phenol/H_2O/dimethylsulfide/ethane-1,2-dithiol. The mixture was concentrated at reduced pressure and added to a cooled (−20 °C) solution of Et_2O (80 mL). The mixture was centrifuged and decanted. The residue was suspended in Et_2O (80 mL), sonicated (2 min), centrifuged and decanted (repeated 2 x). The crude peptide was dried at reduced pressure and purified by preparative HPLC (gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 25% to 65 % in 45 min) to obtain 51.0 mg (3 % based on initial resin loading) of purified **1**.

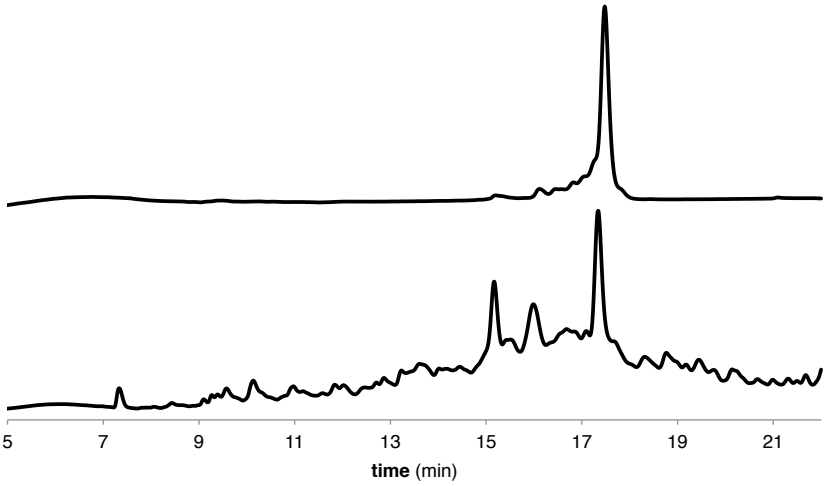


Figure S1: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 30 % to 60 % in 15 min) of crude (lower trace) and purified (upper trace) Segment 1 (**1**).

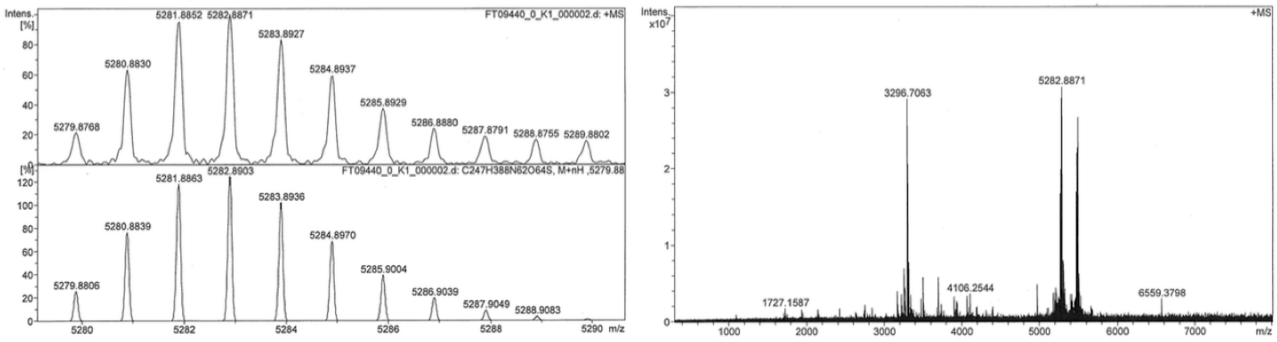


Figure S2: MALDI mass spectrum (left: zoomed-in [top: observed, bottom: calculated]; right: zoomed out) of purified Segment 1 (**1**). This peptide contains photolabile protecting groups leading to side-reactions during laser irraditation by MALDI MS and thus affecting the quality of the spectrum.

3.2. SPPS of Segment 2 (2)

Segment 2 (**2**) was assembled by Fmoc SPPS on 0.50 mmol scale. Photoprotected glutamic acid α-ketoacid was prepared and loaded on the resin (Peptides International Rink amide polystyrene resin, crosslinked with 1 % divinylbenzene, loading 0.32 mmol/g) as previously described.[Error! Bookmark not defined.,Error! Bookmark not defined.] The last residue – Boc-(*S*)-5-oxaproline – was coupled using 2.00 equiv Boc-(*S*)-5-oxaproline, 2.00 equiv HATU and 5 equiv DIPEA in DMF for 2 h.

Coupling: 4.00 equiv of Fmoc protected amino acid, 4.00 equiv of HCTU, 8.00 equiv of DIPEA, solvent: DMF, 2 x 45 min, followed by capping with 20 % Ac_2O in DMF.

Deprotection: 5 min wash with 0.8 M LiCl in DMF, 2 x 10 min with 20 % piperidine in DMF.

Cleavage and Purification: The peptide resin (0.50 mmol) was treated for 1.5 h with 35 mL of a mixture of: 95 : 2.5 : 2.5 TFA/triisopropylsilane/H_2O. The mixture was concentrated at reduced pressure and added to a cooled (–20 °C) solution of diethyl ether (70 mL). The mixture was centrifuged and decanted. The residue was suspended in diethyl ether (70 mL), sonicated (2 min), centrifuged and decanted (repeated 2 x). The crude peptide was dried at reduced pressure and purified by preparative HPLC (gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 40 % to 75 % in 45 min) to obtain 63.1 mg (5 % based on initial resin loading) of purified **2**.

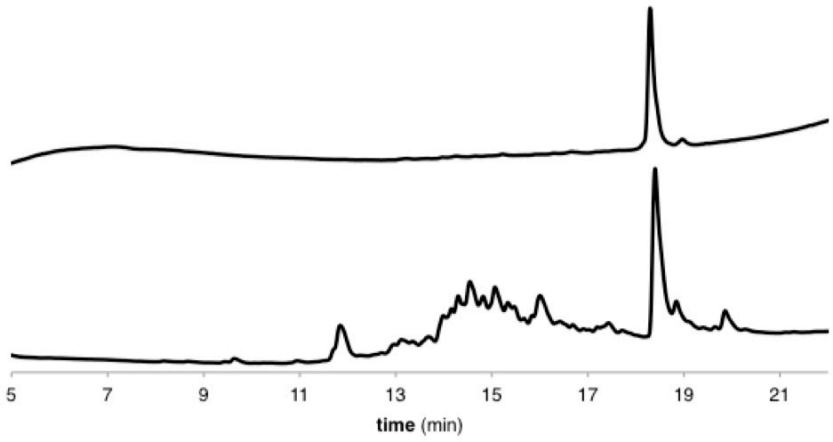


Figure S3: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 5 % to 95 % in 20 min) of crude (lower trace) and purified (upper trace) Segment 2 (**2**).

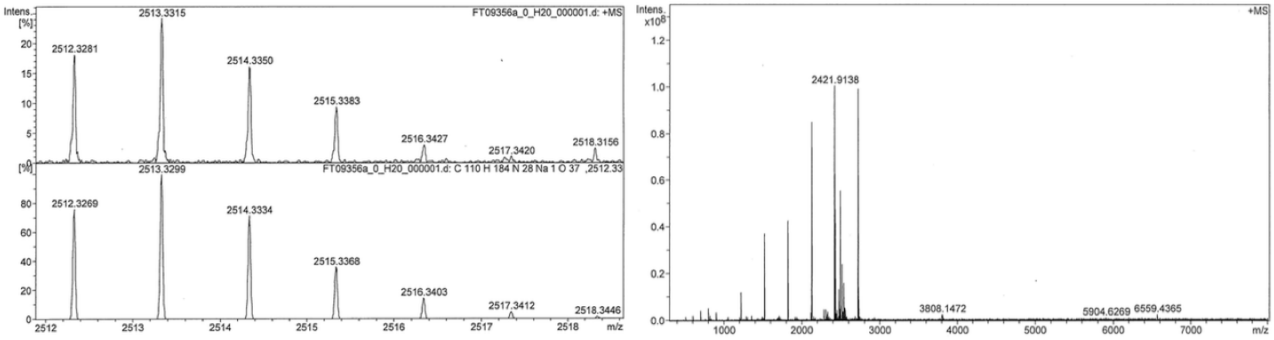


Figure S4: MALDI mass spectrum (left: zoomed-in [top: observed, bottom: calculated]; right: zoomed out [internal calibration standards at m/z 1521.9715, 1821.9523, 2121.9332, 2421.9140, 2721.8948]) of purified Segment 2 (**2**). This peptide contains photolabile protecting groups leading to side-reactions during laser irraditation by MALDI MS and thus affecting the quality of the spectrum.

3.3. Protein synthesis

Ligation and Photodeprotection

Segment 1 (**1**) (33.0 mg, 6.25 µmol, 1.00 equiv) and Segment 2 (**2**) (18.0 mg, 7.22 µmol, 1.16 equiv) were dissolved in 1:1 HFIP/AcOH (312 µL, 20 mM) containing 0.1 M H_2O (0.562 µL, 31.2 µmol). The mixture was shaken at 45 °C. Additional segment 2 (**2**) was added after 2.5 h (5.5 mg, 2.20 µmol, 0.35 equiv) and 4 h (2.3 mg, 738 nmol, 0.15 equiv). After a total of 5 h the ligation (product: **S6**) was complete. The mixture was diltuted with 7:3 DMSO/H_2O (5 mL) and irradiated with a handheld UV lamp at 365 nm for 3 h at rt. The crude mixture was purified by preparative HPLC (gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 40 % to 65 % in 35 min) to obtain pure AS-48 linear **3** (4.3 mg, 597 nmol, 10 %).

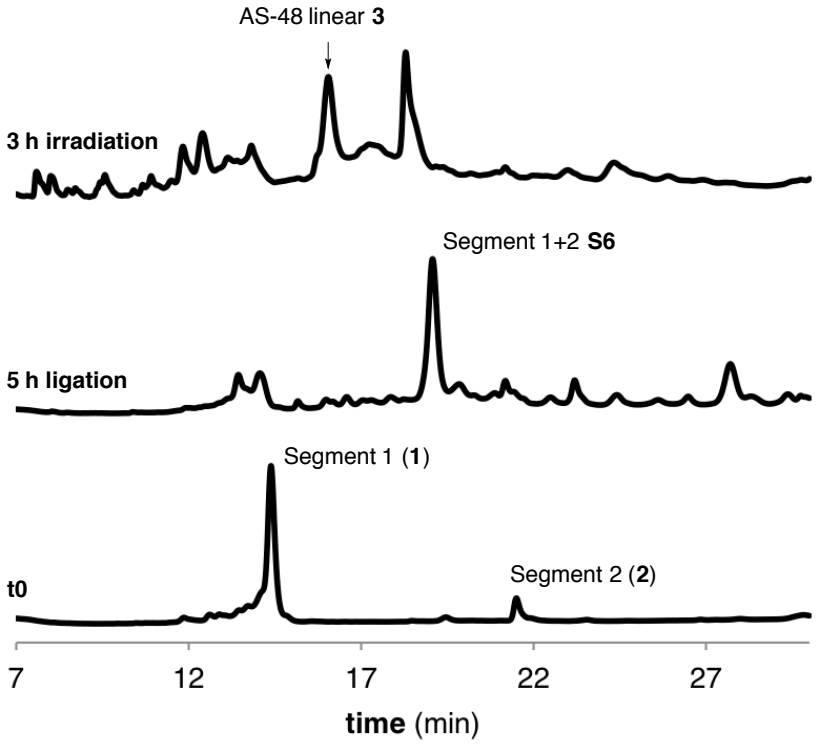


Figure S5: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 40 % to 70 % in 22 min) of ligation of Segment 1 (**1**) and Segment 2 (**2**) (bottom trace: t = 0 h, middle trace: t = 8 h) and one-pot deprotection by UV irradiation at 365 nm after 4 h (upper trace).

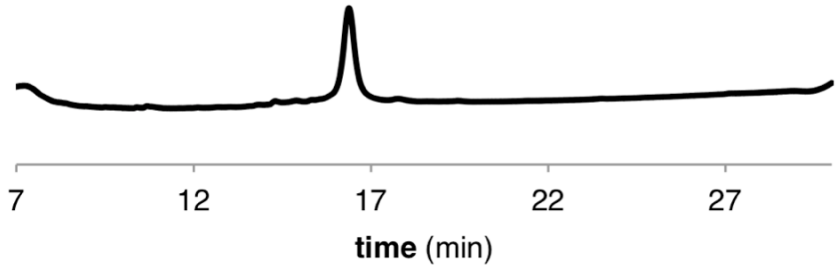


Figure S6: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 40 % to 70 % in 20 min) of purified AS-48 linear **3**.

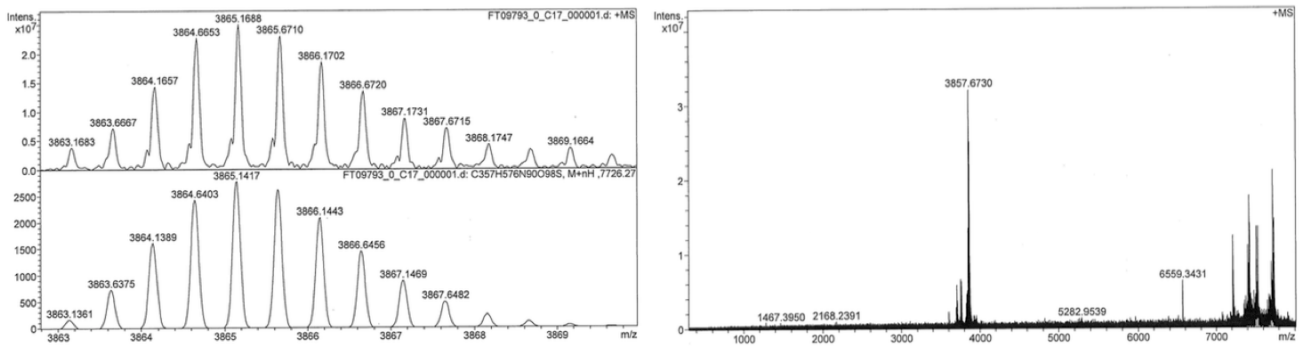


Figure S7: MALDI mass spectrum (left: zoomed-in [top: observed, bottom: calculated]; right: zoomed out) of double charged ion $[M+2H]^{2+}$ of S1+2 **S6**. This peptide contains photolabile protecting groups leading to side-reactions during laser irraditation by MALDI MS and thus affecting the quality of the spectrum.

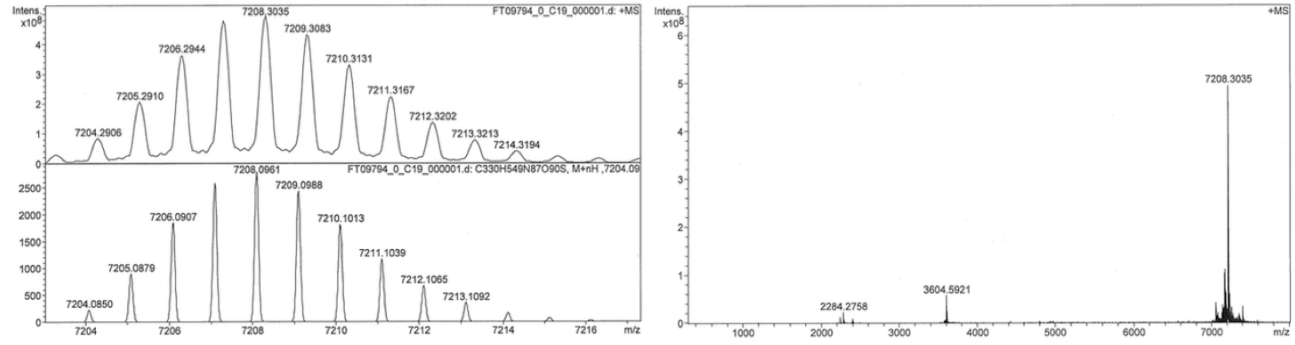


Figure S8: MALDI mass spectrum (left: zoomed-in [top: observed, bottom: calculated]; right: zoomed out) of purified AS-48 linear **3**.

Cyclization and Rearrangement

Purified AS-48 linear **3** (3.3 mg, 458 nmol) was dissolved in 7:3 DMSO/H_2O (916 µL, 0.5 mM) containing 0.1 M oxalic acid. The mixture was shaken at 60 °C for 20 h to (product: AS-48 cyclic$_{depsi/depsi}$ **4**). The mixture was diluted with pH 9 phosphate buffer (0.3 M, 2.5 mL) containing 6 M guanidinium hydrochloride and shaken at rt. The mixture was adjusted multiple times during the course of the reaction to pH 9 with 25% aqueous ammonia. After 12 h the mixture was acidified with TFA to pH 3 and purified by preparative HPLC (gradient: MeCN (0.1 % TFA) in H_2O (0.1% TFA) 40 % to 80 % in 30 min) to obtain pure synthetic AS-48 **5** (1.0 mg, 139 nmol, 30 %).

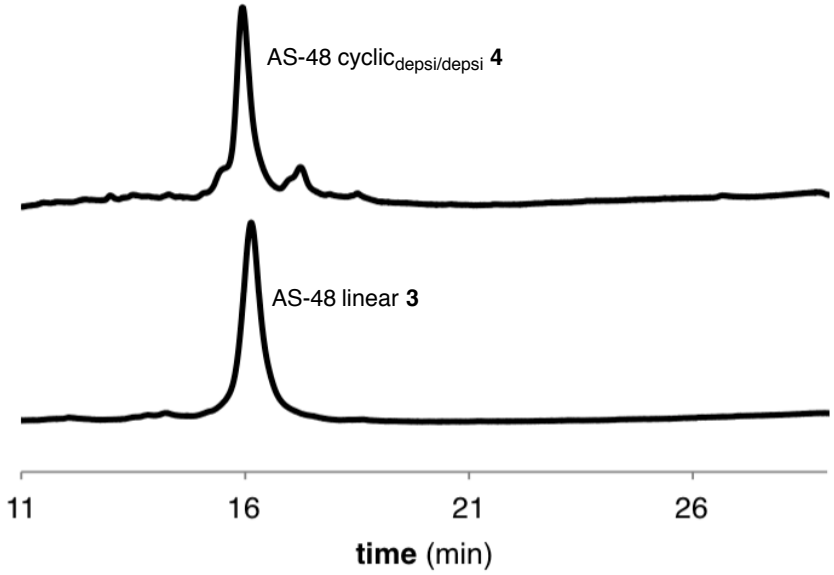


Figure S9: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1% TFA) 40 % to 70 % in 22 min) of the cyclization of AS-48 linear **3** to AS-48 cyclic$_{depsi/depsi}$ **4**. The retention time of product and starting material are essentially identical.

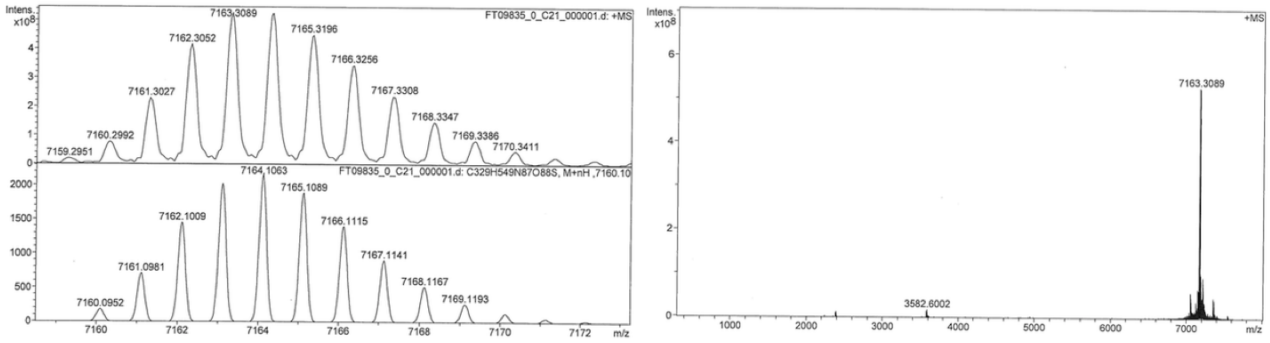


Figure S10: MALDI mass spectrum (left: zoomed-in [top: observed, bottom: calculated]; right: zoomed out) of AS-48 cyclic$_{depsi/depsi}$ **4**.

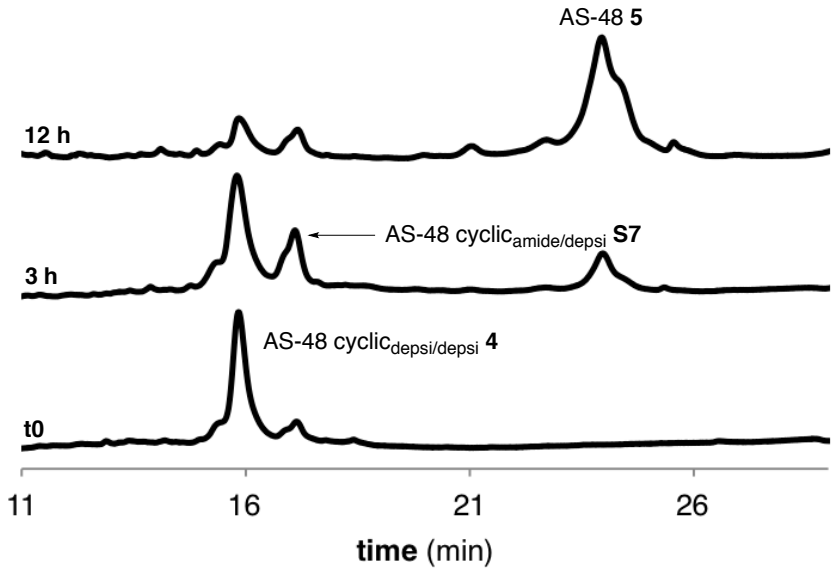


Figure S11: Analytical HPLC traces (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 40 % to 70 % in 30 min) of the rearrangement of AS-48 cyclic$_{depsi/depsi}$ **4** to synthetic AS-48 **5** at t = 0 h (bottom trace), t = 3 h (middle trace) and t = 12 h (upper trace).

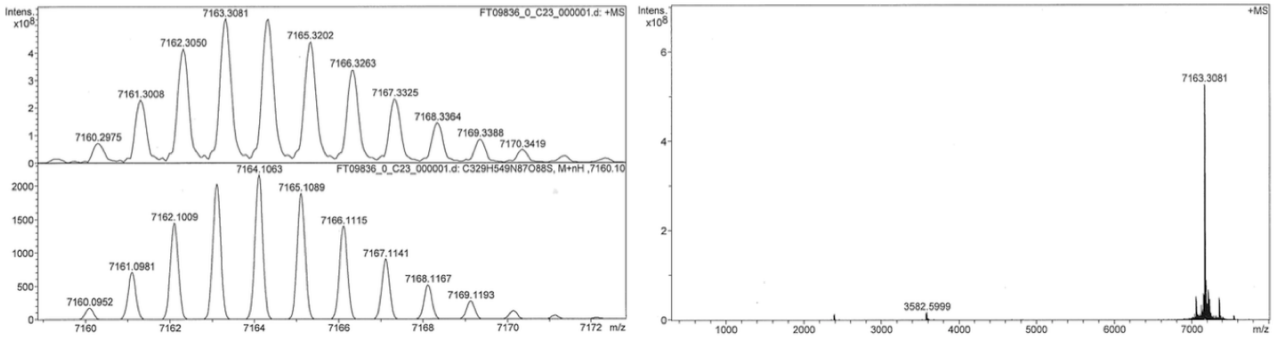


Figure S12: MALDI mass spectrum (left: zoomed-in [top: observed, bottom: calculated]; right: zoomed out) of synthetic AS-48 **5**.

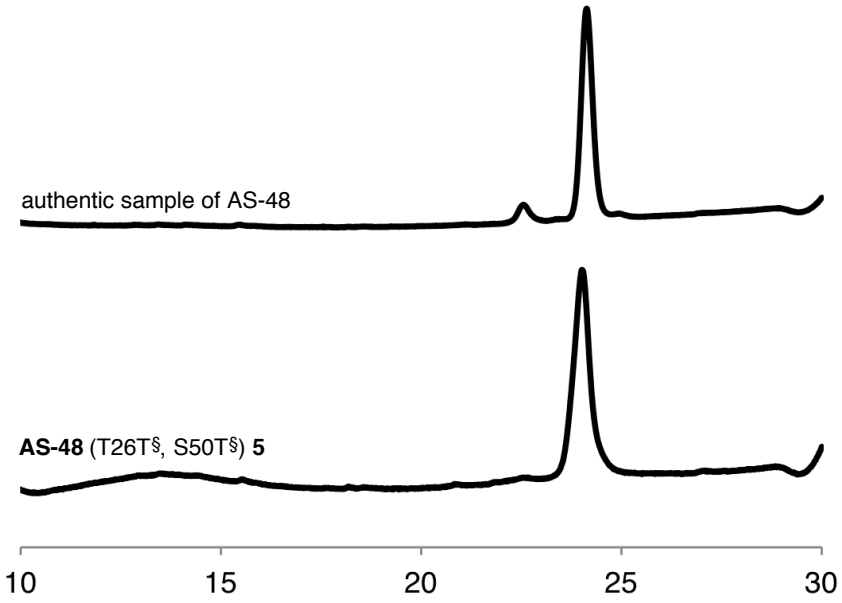


Figure S13: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 5 % to 95 % in 20 min) of synthetic AS-48 (T26T§, S50T§) **5** (bottom trace) and an authentic sample of AS-48 (upper trace).

3.4. Failed Ligations

Ligation in 9:1 DMSO/H_2O at 60-90 °C

Segment 1 (**1**) (3.3 mg, 625 nmol, 1.00 equiv), Segment 2 (**2**) (1.6 mg, 625 nmol, 1.00 equiv) and oxalic acid (0.4 mg, 4.2 µmol) were dissolved in 9:1 DMSO/H_2O (41.7 µL, 15 mM). The mixture was shaken at 60 °C for 18 h. No conversion was observed. The mixture was heated to 95 °C and shaken for 3 h. The desired product was not observed.

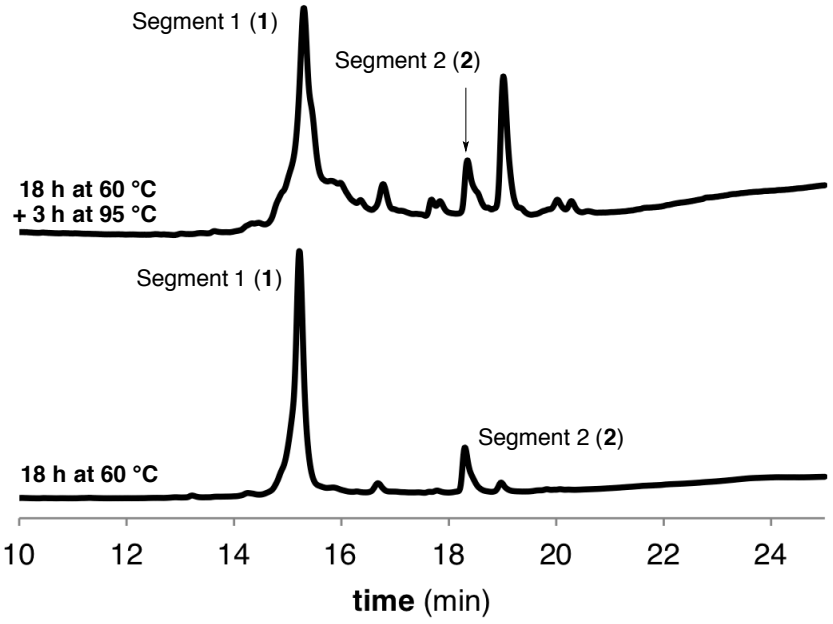


Figure S14: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 5 % to 95 % in 20 min) of ligation of Segment 1 (**1**) and Segment 2 (**2**) (bottom trace: after 18 h at 60 °C, upper trace: after 18 h at 60 °C and 3 h at 95 °C).

Ligation in 10:1 NMP/H_2O at 60 °C

Segment 1 (**1**) (3.3 mg, 625 nmol, 1.00 equiv), Segment 2 (**2**) (1.6 mg, 625 nmol, 1.00 equiv) and oxalic acid (0.3 mg, 2.8 µmol) were dissolved in 10:1 NMP/H_2O (27.5 µL, 23 mM). The mixture was shaken at 60 °C for 21 h. The desired product was not observed.

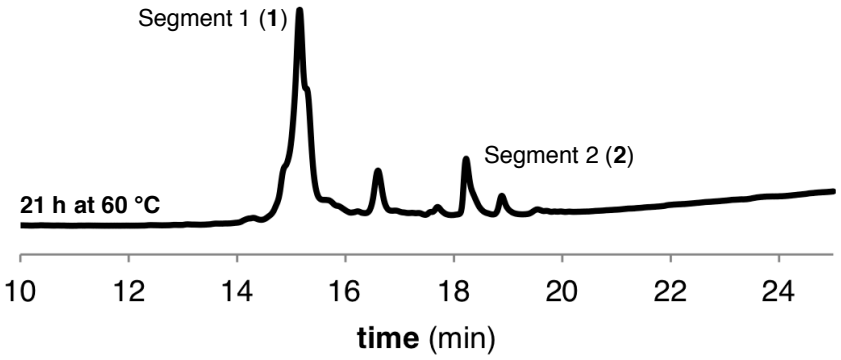


Figure S15: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 5 % to 95 % in 20 min) of ligation of Segment 1 (**1**) and Segment 2 (**2**) after 21 h at 60 °C.

Ligation in 9:1 DMSO/H_2O with 5 M GdnCl at 60 °C

Segment 1 (**1**) (3.3 mg, 625 nmol, 1.00 equiv), Segment 2 (**2**) (1.6 mg, 625 nmol, 1.00 equiv), oxalic acid (0.3 mg, 2.8 µmol) and guanidinium chloride (14.8 mg, 156 µmol) were dissolved in 9:1 DMSO/H_2O (31.3 µL, 20 mM). The mixture was shaken at 60 °C for 18 h. The desired product was not observed.

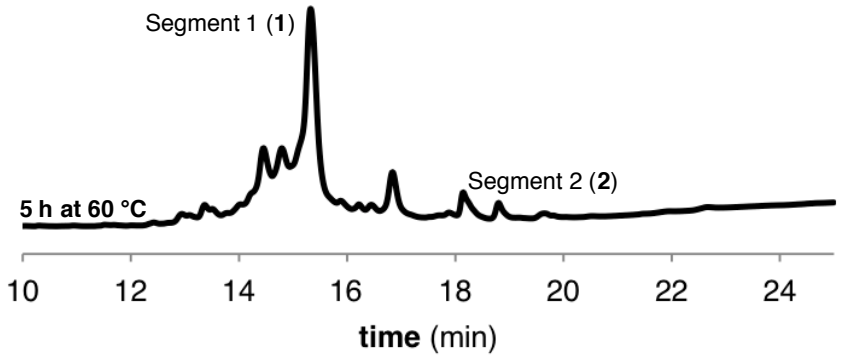


Figure S16: Analytical HPLC trace (220 nm, gradient: MeCN (0.1 % TFA) in H_2O (0.1 % TFA) 5 % to 95 % in 20 min) of ligation of Segment 1 (**1**) and Segment 2 (**2**) after 5 h at 60 °C.

4. CD spectra

CD spectra were recorded on an AVIV Circular Dichroism Spectrometer Model 430 at 25 °C in a quartz cell with a path length of 0.5 mm in 10 mM pH 3 phosphate buffer. The data is displayed as mean residue ellipticity. Concentrations were determined by measuring the absorption at 280 nm (Nanodrop 2000 Instrument) using a calculated extinction coefficient for AS-48 of ε = 12490 L $cm^{-1}mol^{-1}$. The following concentrations were measured: native AS-48 8 mM; synthetic AS-48 after folding (storage for 1 month in pH 3 buffer at 4 °C) 8 mM; linear AS-48 (**3**) 22 mM, synthetic AS-48 before folding 21 mM.

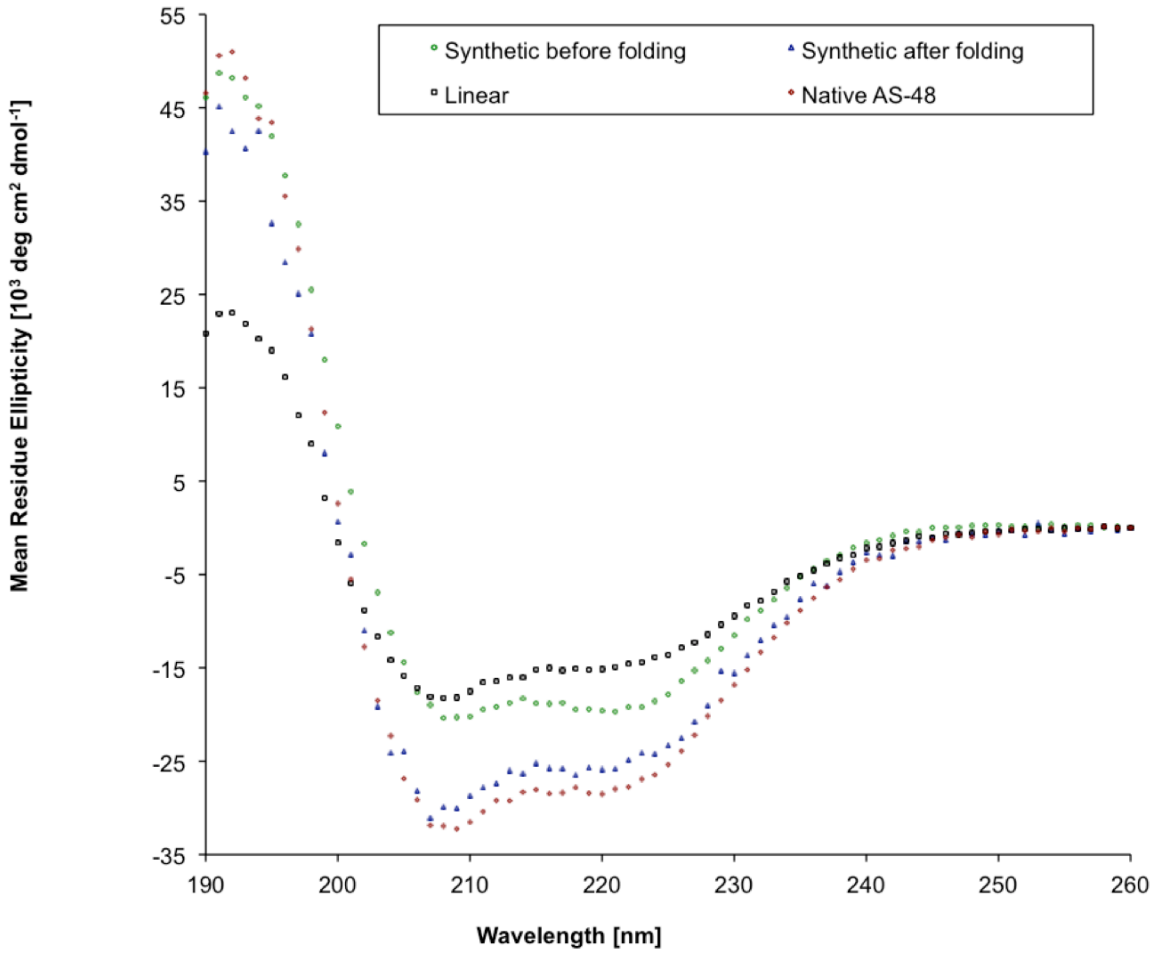


Figure S17: CD spectra of native AS-48 (red rhombs), synthetic AS-48 after folding (blue triangles), linear AS-48 **3** (black squares), synthetic AS-48 before folding (green circles).

5. Agar Well Bacterial Growth inhibition assay

Hard BHI (Brain Heart Infusion Broth, Sigma Aldrich) medium was prepared: BHI (7.4 g), Agar (1.5 g) and MilliQ-H_2O (200 mL) were mixed and autoclaved for 20 min, cooled to 80 °C, filled into Petri dishes (25 mL medium per dish) and stored at 4 °C. Soft BHI medium was prepared: BHI (7.4 g), Agar (1.5 g) and MilliQ-H_2O (200 mL) were mixed and autoclaved for 20 min, cooled to 80 °C, filled in 15 mL falcon tubes (10 mL medium per falcon tube) and stored at 4 °C. Liquid BHI medium was prepared: BHI (7.4 g) and MilliQ-H_2O (200 mL) were mixed, autoclaved for 20 min and stored at 4 °C. Inoculation: A glycerol stock of L. innocua (Strain: Cect [Colección Española de Cultivos Tipo] 4030; 50 mg) was added to liquid BHI medium (7 mL) and incubated at 37 °C for 15 h. Assay: Onto a petri dish containing hard BHI medium were added 5 µL of a solution of AS-48 (concentrations for synthetic folded AS-48: 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 µM; for authentic AS-48: 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.780, 0.039 µM) in phosphate buffer (pH 3, 10 mM). As a negative control, 5 µL of phosphate buffer without protein was added to the medium (see Figure **S18**). The spots on the agar plate were allowed to dry at rt. In a separate falcon tube soft BHI medium (10 mL) was molten (warmed to 90 °C, then cooled to 45 °C) and 150 µL of the bacteria culture (prepared as described above) was added and mixed. This solution was added to the previously prepared agar plates with spots of AS-48 and allowed to solidify for 10 min at rt. The agar plates were incubated at 37 °C for 16 h. For unfolded synthetic AS-48 the MIC was determined to be 17.1 µM-10 µM, for folded synthetic AS-48 **5** 0.313-0.156 µM and for the authentic sample of AS-48 0.156-0.078 µM.

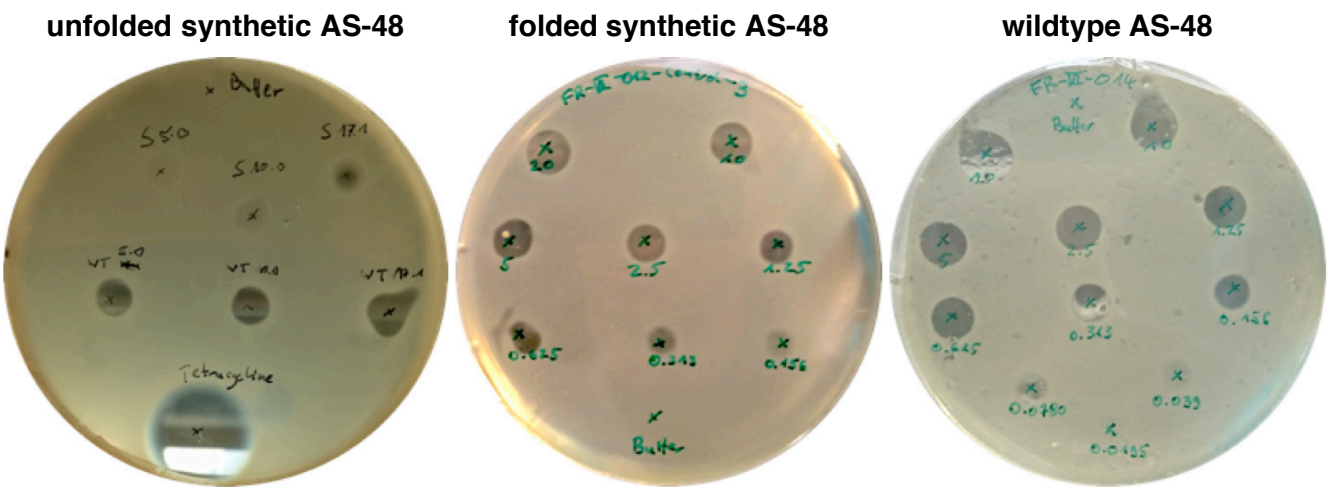


Figure S18: Agar well bacterial growth inhibition assay with unfolded synthetic AS-48 **5** (left picture; for spots marked with WT, wildytpe AS-48 was used as positive control, the bottom spot represents positive control with tetracycline), folded synthetic AS-48 **5** (middle picture) and an authentic sample of AS-48 (right picture) after 16 h of incubation at 37 °C with *L. innocua*.

6. Membrane Incorporation Assay

Preparation of MLV (multilamellar vesicles) solution

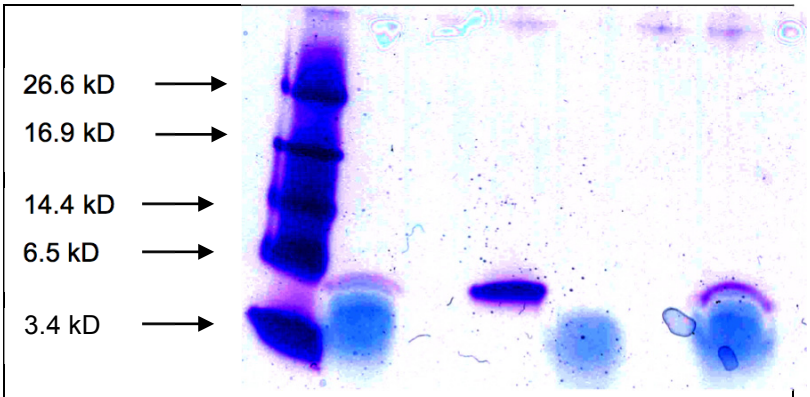
Phosphatidyl choline (600 µL, 10 mg/mL solution in $CHCl_3$) was added to a 50 mL round bottom flask. $CHCl_3$ (400 µL) was added and the solvent was evaporated in a rotary evaporator at reduced pressure with a water-bath temperature of 40 °C. The residue was dried at high vacuum for 1 h. The residue was incubated with milliQ-H_2O (1 mL) at rt for 2 h with occasional swirling. The solution was stored at 4 °C.

Incorporation into MLVs

For the assay the following solutions were used: SDS (0.6 %w/v in milliQ-H_2O), MLV (as described above, 6 mg/mL), synthetic AS-48 **5** (10 µM, in 10 mM pH 3 phosphate buffer), authentic AS-48 (10 µM, in 10 mM pH 3 phosphate buffer). Following Table **S1** the solutions (each 25 µL) were mixed and incorporated at rt for 1 h.

Gel filtration

If indicated in Table **S1**, the above-prepared mixture was applied onto a size-exclusion column (6 mm inner diameter, filled with 300 µL Sigma-Aldrich Octyl Sepharose CL-4B suspension in ethanol, equilibrated with 3 mL milliQ-H_2O) and eluted with milliQ-H2O (1 mL). The eluate was lyophilized, dissolved in milliQ-H_2O (30 µL) and analyzed by SDS-PAGE (125 V, 400 mA, BIO-RAD mini-PROTEAN Tris-Tricine precast gel).



Lane	1	2	3	4	5	6
SDS	+	+	-	+	+	+
MLV	+	-	-	+	-	+
CL-4B	+	+	-	+	+	+
Concentration [µM]	10	10	5	-	10	10
Type	Syn	Syn	WT	-	WT	WT

Table S1: SDS PAGE analysis of the membrane incorporation assay. "+" indicates that the corresponding solution was used in the mixture, "-" indicates that instead the same volume of milliQ-H2O was used. Syn indicates that synthetic AS-48 **5** was used, WT indicates an authentic sample of AS-48 was used. "Concentration" refers to the protein concentration. As shown in lane 4, the broad blue band arises from MLV.

7. References

(1) V. R. Pattabiraman, A. O. Ogunkoya, J. W. Bode, *Angew. Chem. Int. Ed,.* 2012, **51**, 5114–5118.

(2) P. Sudta, N. Kirk, A. Bezos, A. Gurlica, R. Mitchell, T. Weber, A. C. Willis, S. Prabpai, P. Kongsaeree, P. R. Parish, S. Suksamrarn, M. Kelso, J. *Aust. J. Chem.,* 2013, **66**, 864–873.

(3) R. A. Bunce, J. E. Schammerhorn, *Org. Prep. Proc. Int.,* 2005, **37**, 550–555.

(4) A. Hasan, K-P. Stengele, H. Giegrich, P. Cornwell, K. R. Isham, R. A. Sachleben, W. Pfleiderer, R. S. Foote, *Tetrahedron* ,1997, **53**, 4247–4264.

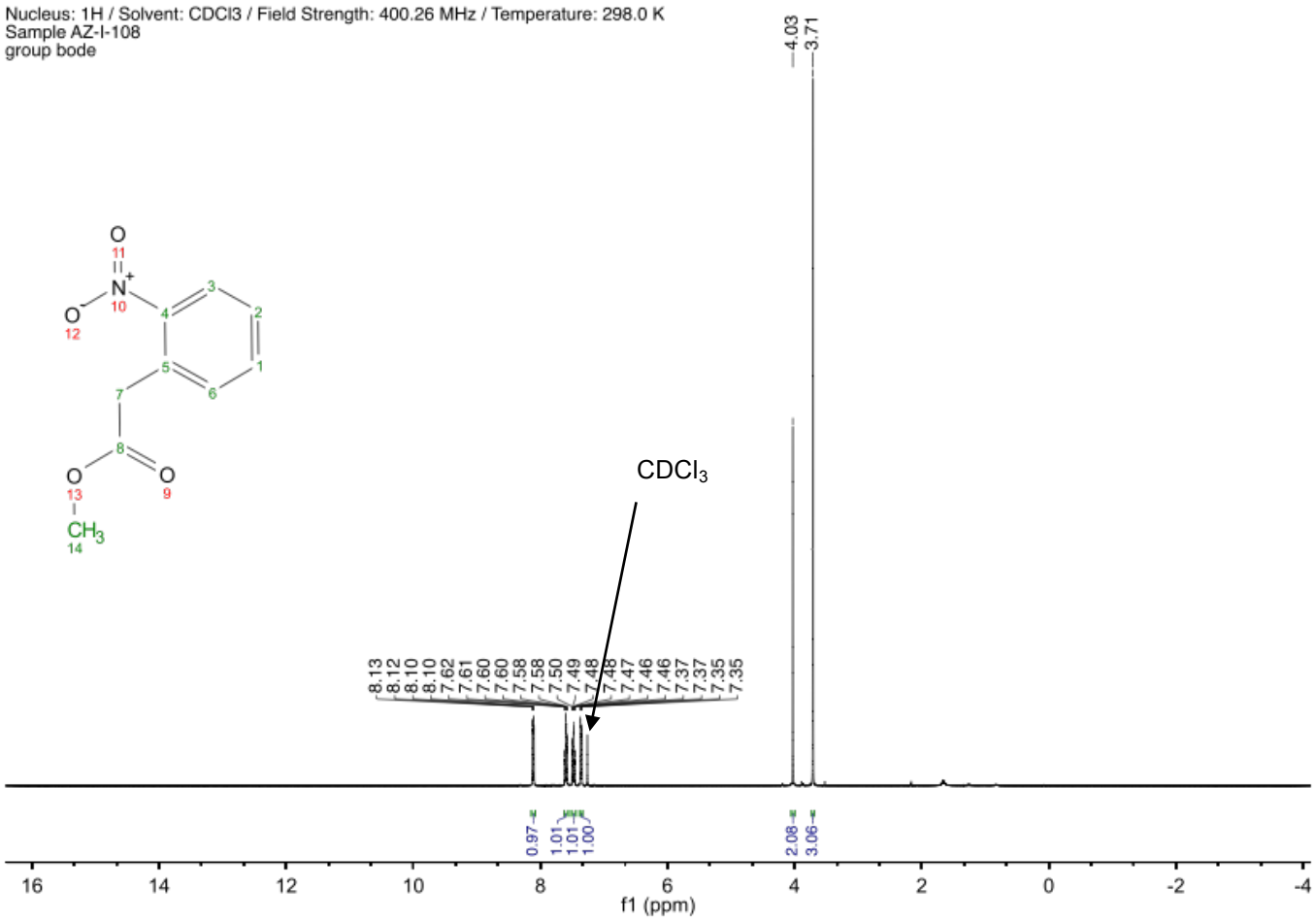
(5) F. Thuaud, F. Rohrbacher, A. Zwicky, J. W. Bode, *Org. Lett.,* 2016, **18**, 3670–3673.

(6) T. G. Wucherpfennig, F. Rohrbacher, V. R. Pattabiraman, J. W. Bode, *Angew. Chem. Int. Ed.,* 2014, **53**, 12244–12247.

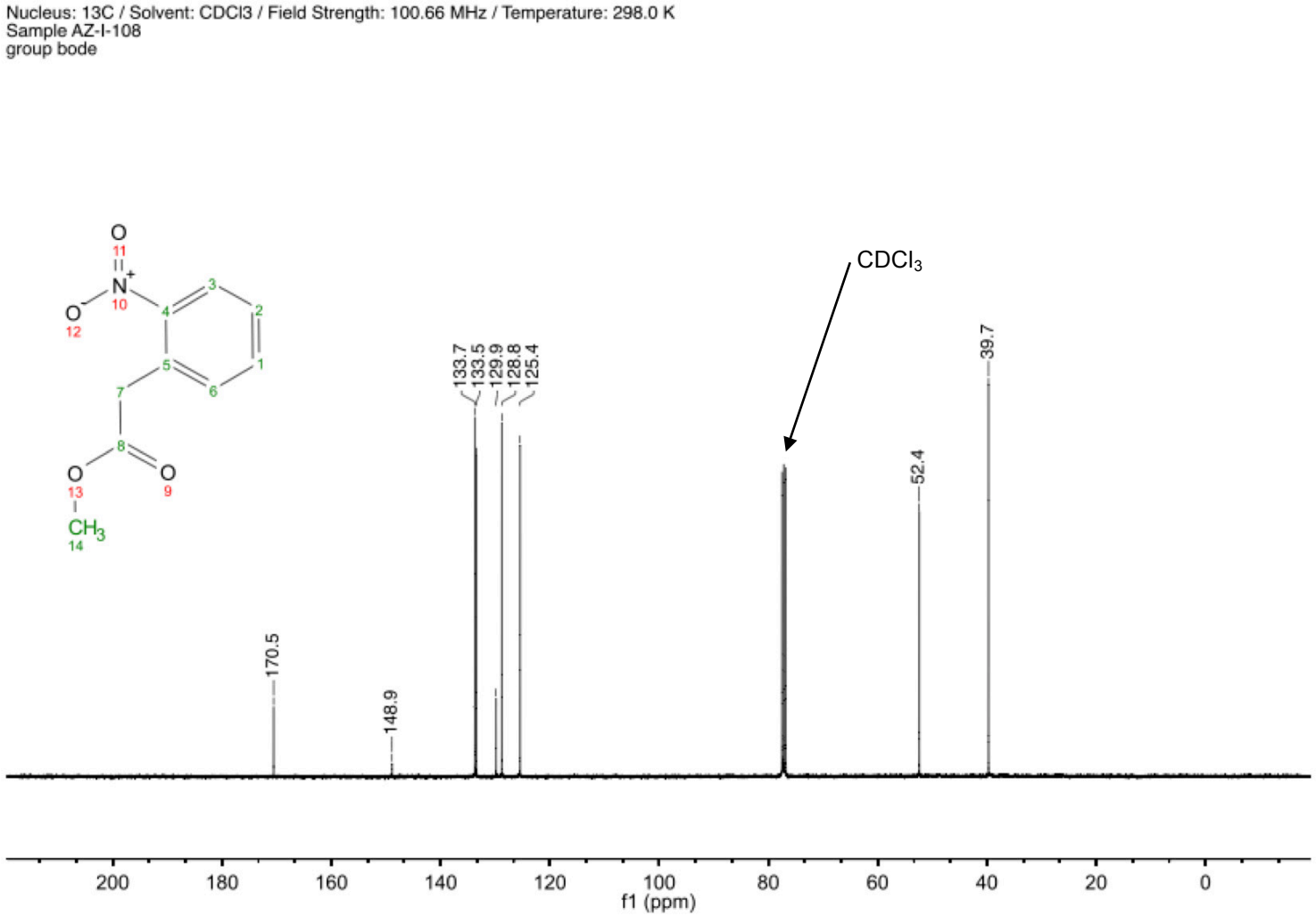
8. NMR Spectra

8.1. Methyl 2-(2-nitrophenyl)acetate S1

^{1}H NMR



^{13}C NMR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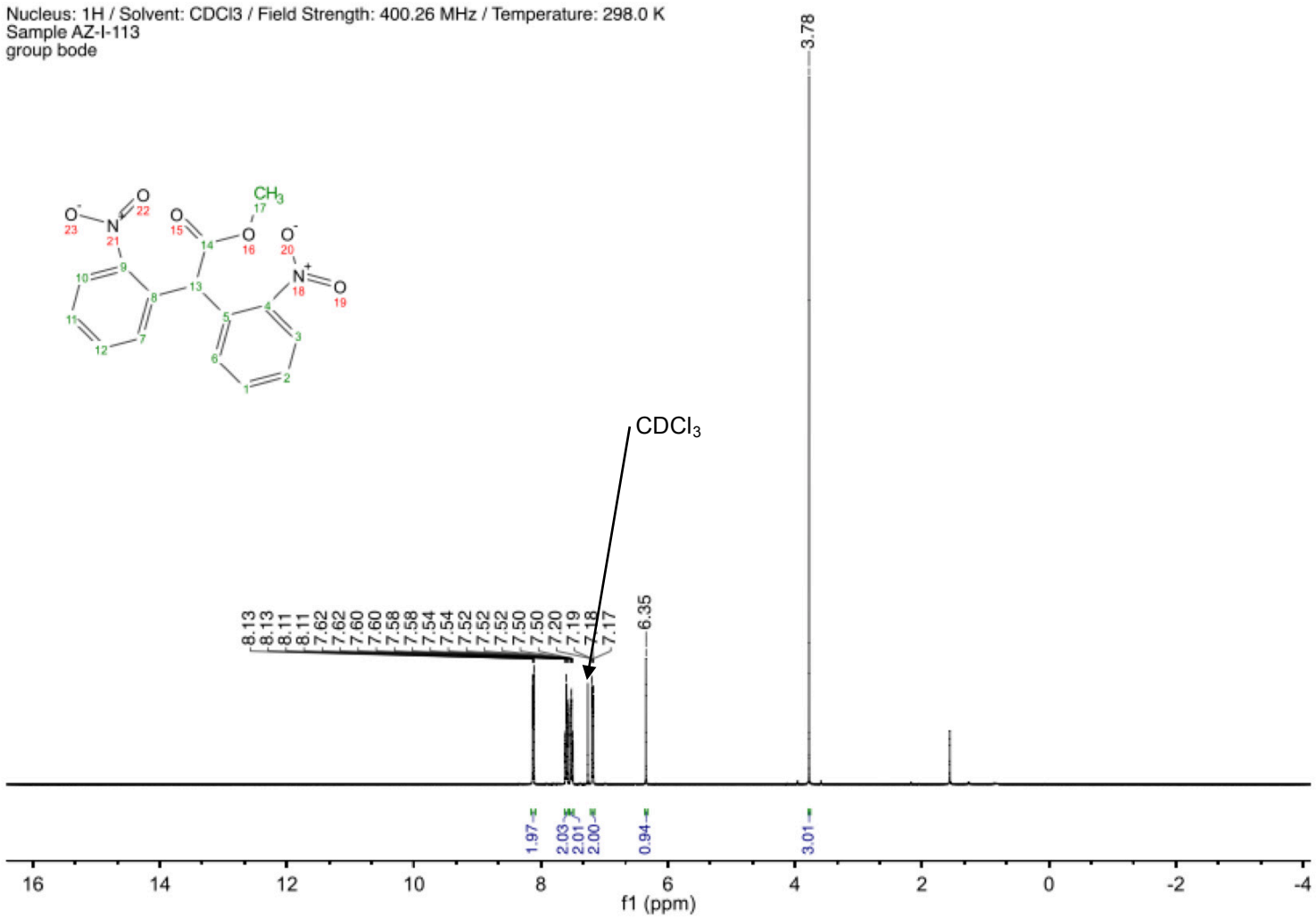
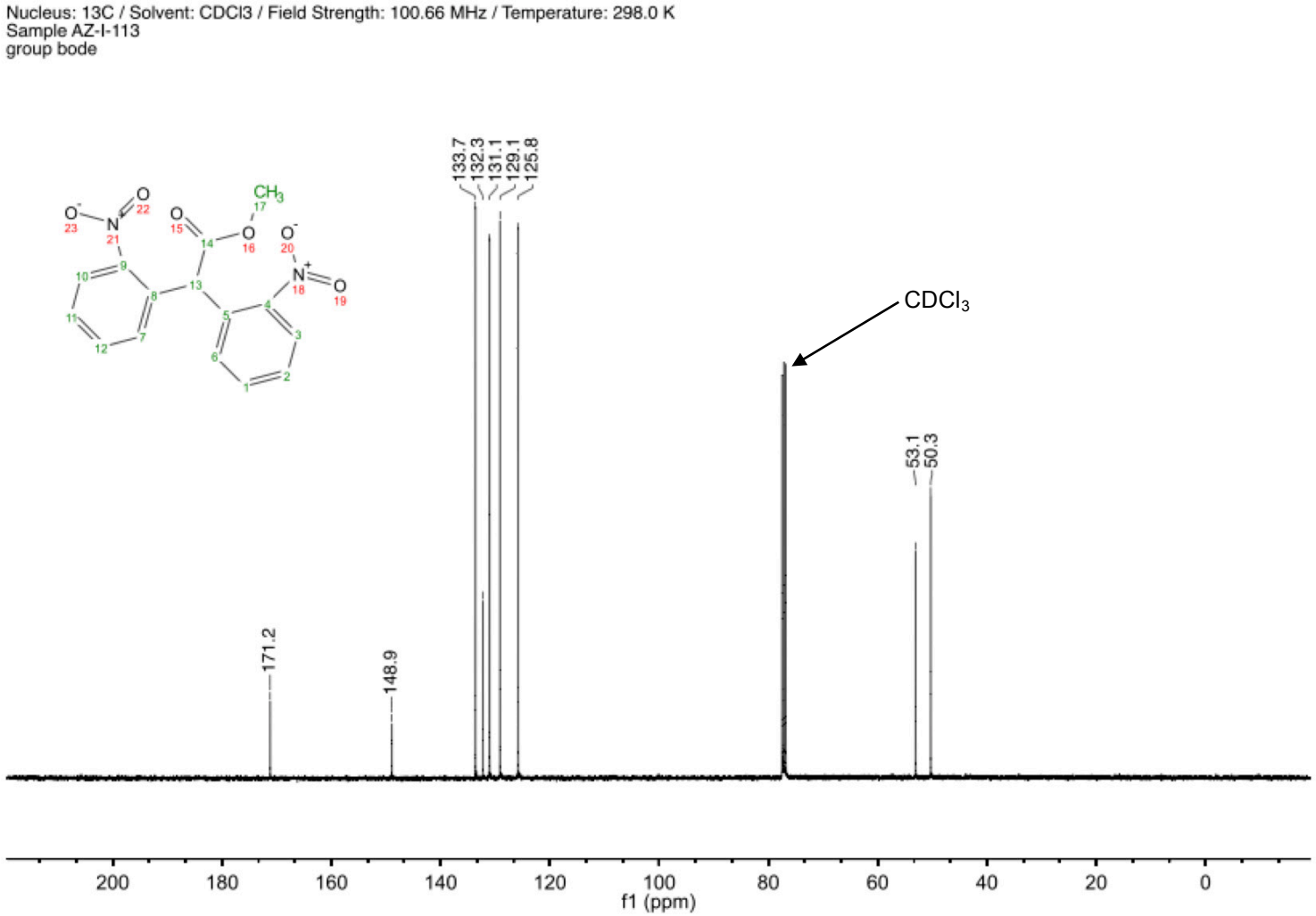
Sample AZ-I-108
group bode
CDCl3
170.5
148.9
133.7
133.5
129.9
128.8
125.4
52.4
39.7
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)

8.2. Methyl 2,2-bis(2-nitrophenyl)acetate S2

^{1}H-NMR



^{13}C NMR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132.3
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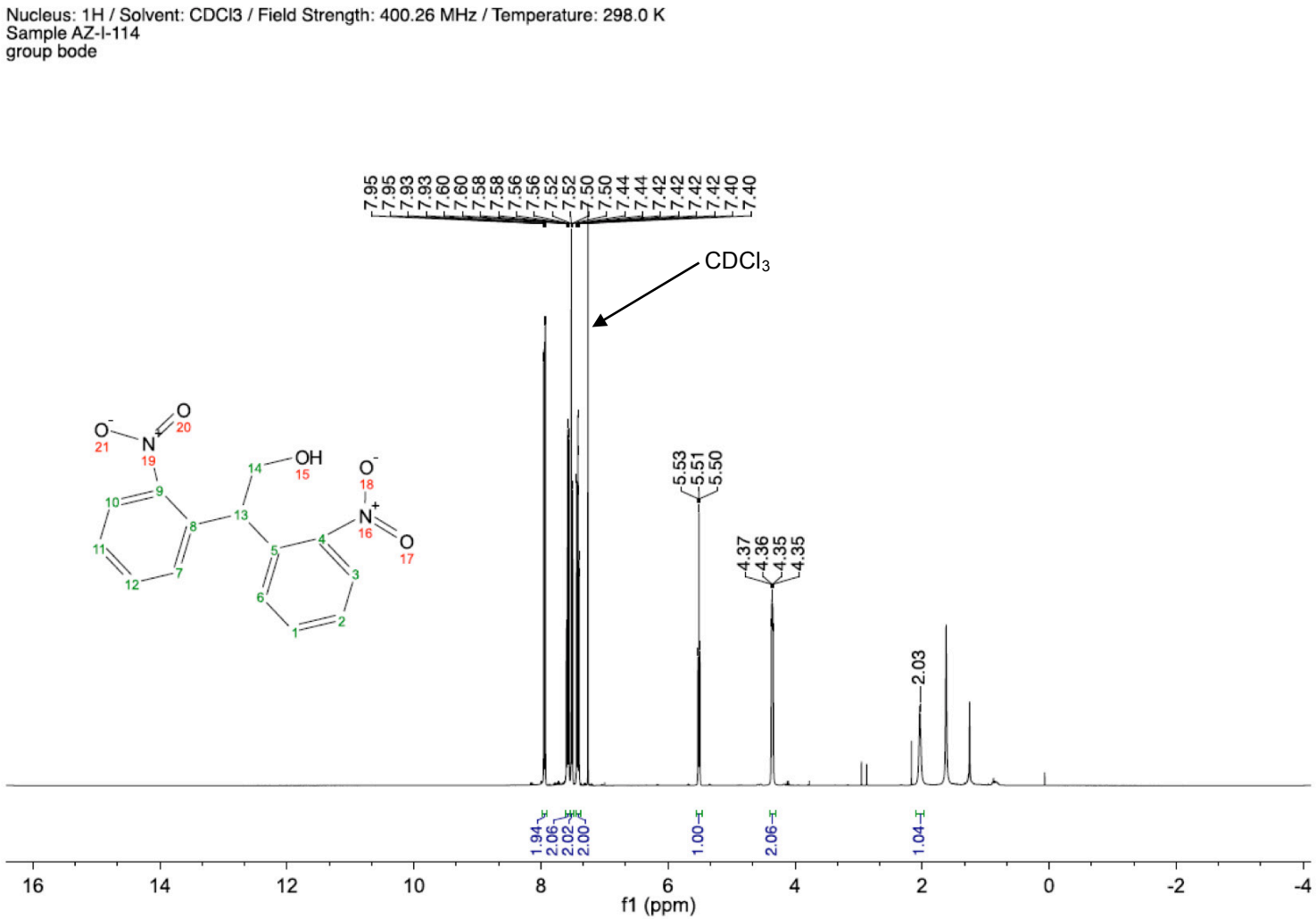
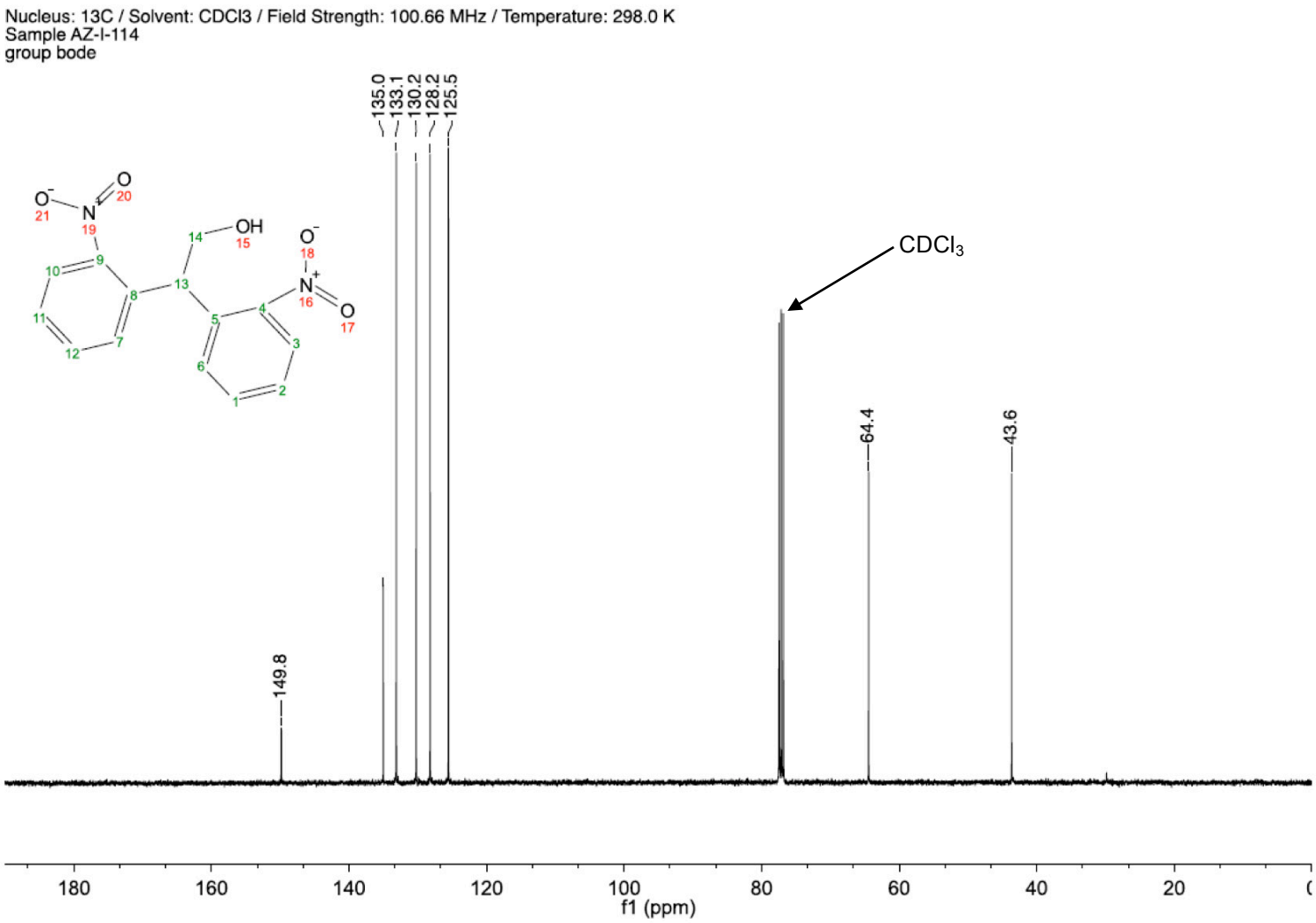
125.8
CDCl3
53.1
50.3
171.2
148.9
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)

8.3. 2,2-bis(2-nitrophenyl)ethan-1-ol S3

^{1}H-NMR



^{13}C NMR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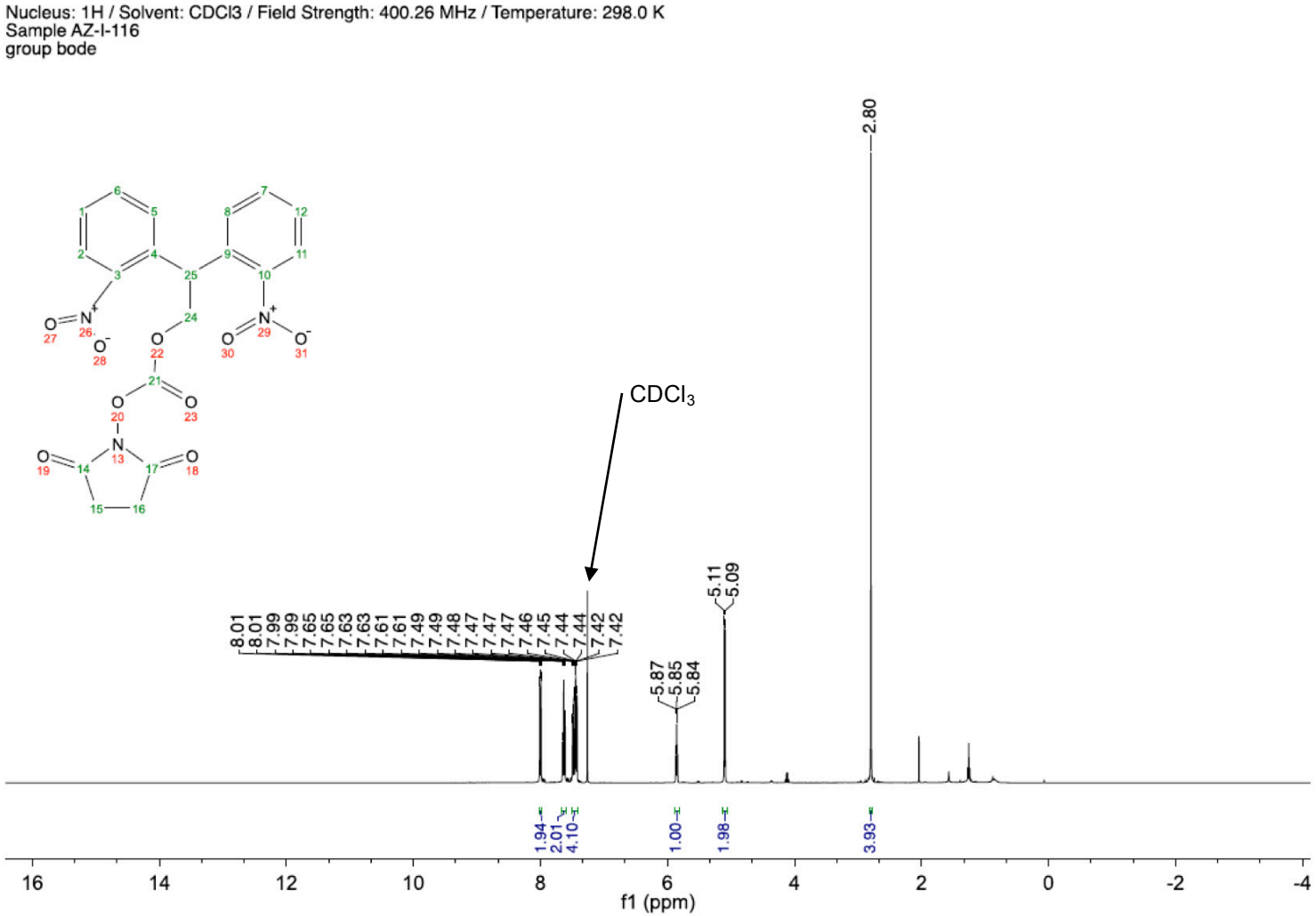
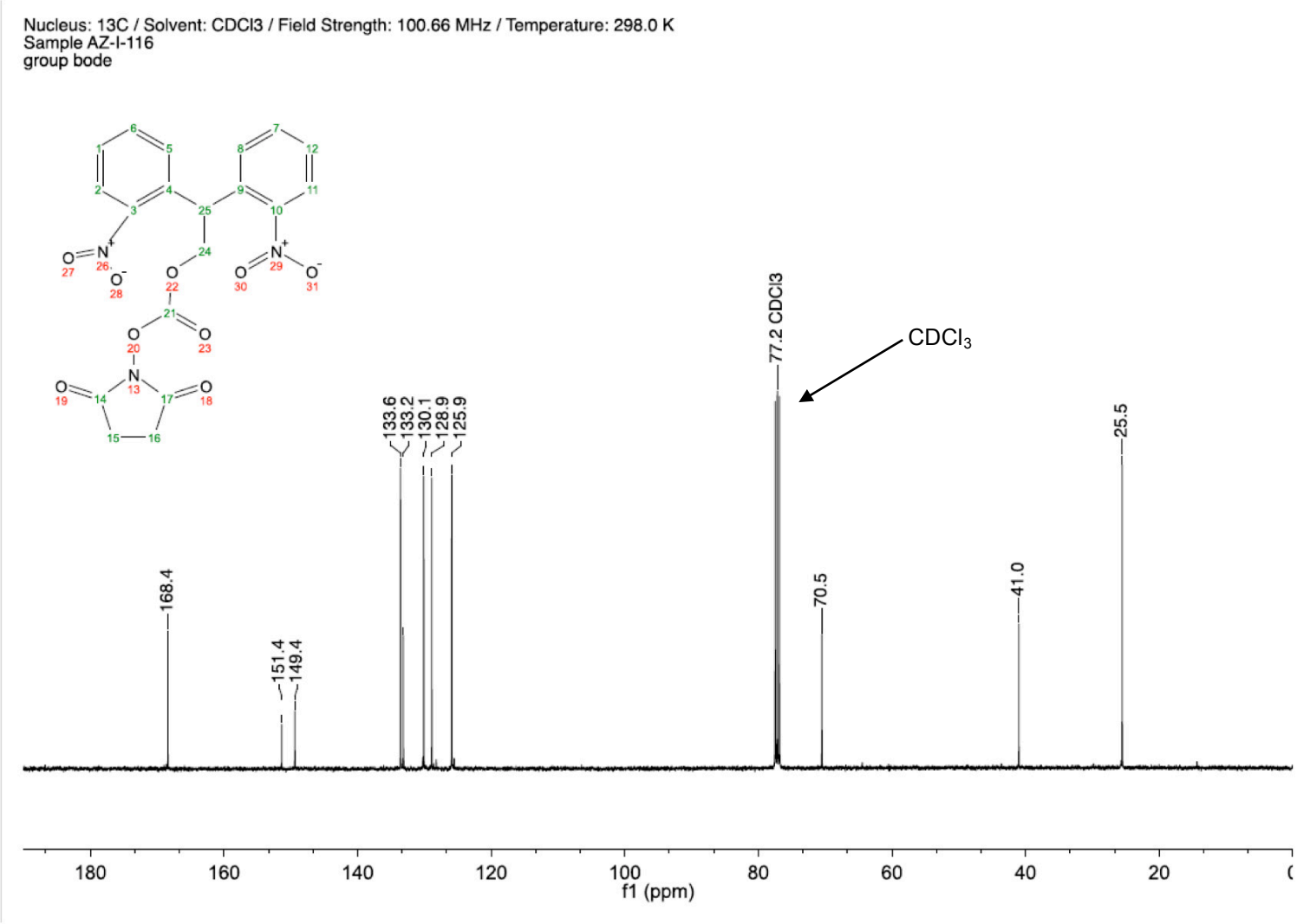
130.2
128.2
125.5
CDCl3
64.4
43.6
149.8
180
160
140
120
100
80
60
40
20
f1 (ppm)

8.4. 2,2-Bis(2-nitrophenyl)ethyl (2,5-dioxopyrrolidin-1-yl) carbonate S4

^{1}H-NMR



^{13}C NMR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group bode
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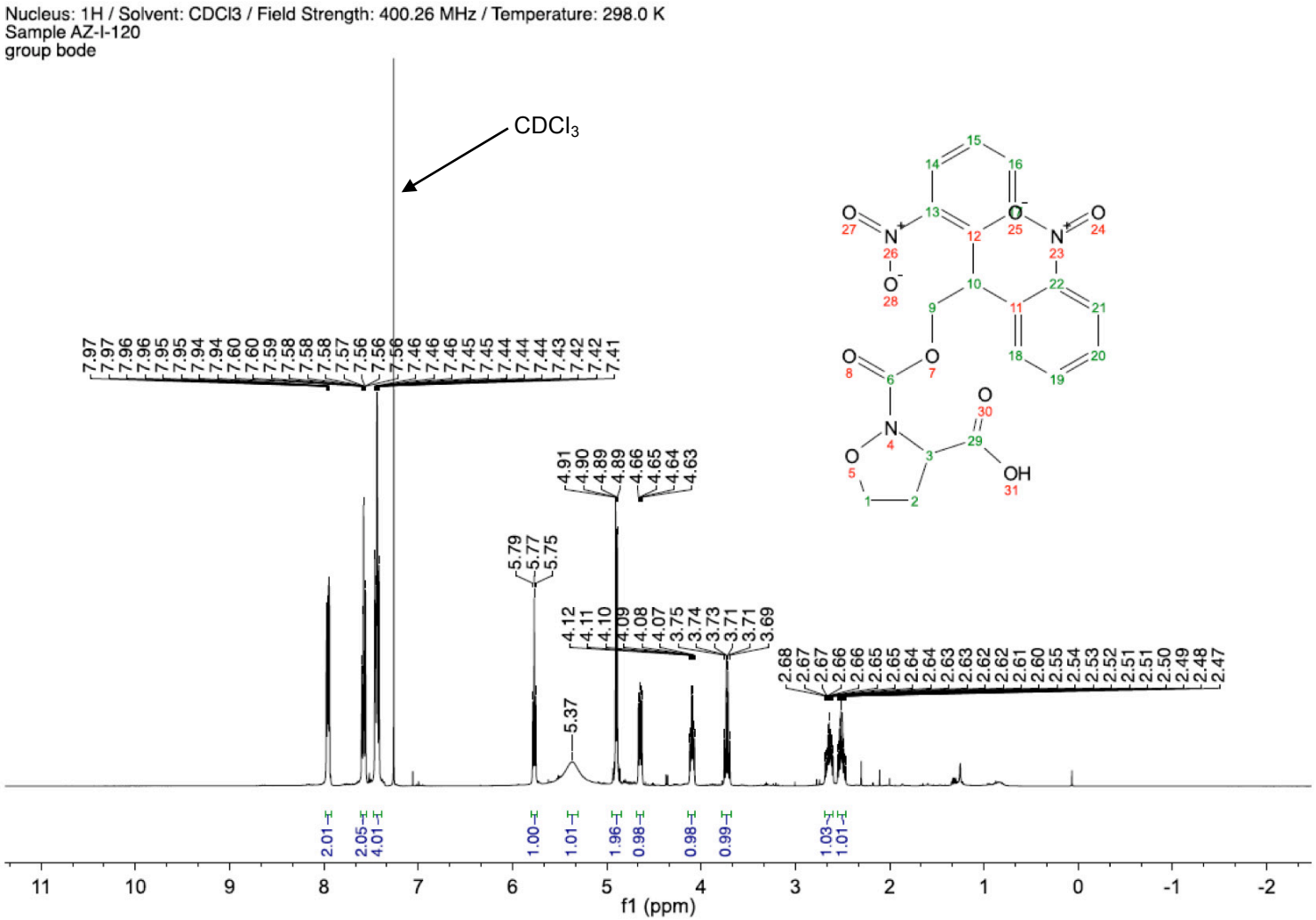
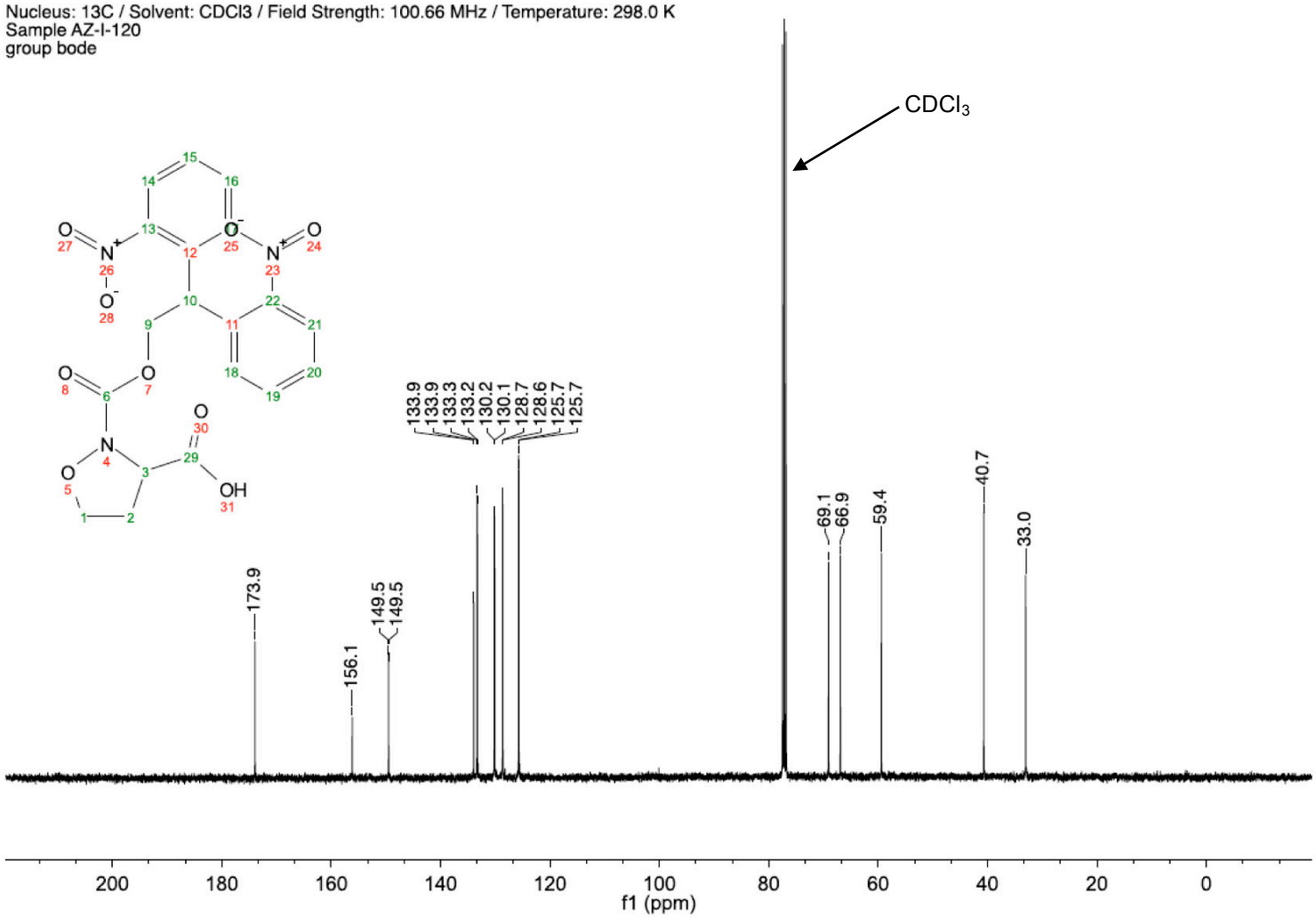
149.4
133.6
133.2
130.1
128.9
125.9
77.2 CDCl3
CDCl3
70.5
41.0
25.5
180
160
140
120
100
80
60
40
20
f1 (ppm)

8.5. (*S*)-((2,2-bis(2-nitrophenyl)ethoxy)carbonyl)-5-oxaproline S5

^{1}H-NMR



^{13}C NMR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149.5
149.5
133.9
133.9
133.3
133.2
130.2
130.1
128.7
128.6
125.7
125.7
69.1
66.9
59.4
40.7
33.0
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)