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Novel chemical probes for the investigation of nonribosomal peptide assembly

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1 General methods

Unless specified otherwise, chemicals were purchased from Sigma Aldrich, Fisher Scientific, Carbosynth and Alfa Aesar and were used without further purification. Dry dichloromethane (DCM), tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) were purchased from VWR International (AR grade) and dried using solvent towers. Dry methanol and dry pyridine were purchased from Fisher Scientific. Reagent grade dichloromethane, ethyl acetate, methanol, cyclohexane, toluene, diethylether, isopropanol, chloroform and tetrahydrofuran were purchased from Fisher Scientific.

Analytical thin-layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel 60 (F_{254}, Merck) and visualized under ultra-violet light (short and long-wave) and using potassium permanganate ($KMnO_4$), vanillin or ninhydrin stains. Silica gel was purchased from Sigma Aldrich (Tech Grade, pore size 60Å, 230-400 mesh).

Infra-red spectra were recorded on a Perkin-Elmer paragon 1000 FT-IR spectrophotometer. Absorption maxima (v_{max}) are quoted in wavenumbers (cm^{-1}) and only structurally significant peaks are quoted.

^{1}H and ^{13}C NMR spectra were recorded in d_4-MeOD, $CDCl_3$ or D_2O on the following Bruker Avance instruments: DPX-300 300 MHz, DPX-400 400 MHz, DRX-500 500 MHz or AV-600 600 MHz.

High-resolution mass spectra (HRMS) were obtained using electrospray ionization (ESI) on a MaXis UHR-TOF (Bruker Daltonics) or on Bruker MaXis (ESI-HR-MS).

Optical rotations were obtained using an AA-1000 Polarimeter from Optical Activity LTD.

General method I

N-(2-aminoethyl) acylamides (1.0 eq.) and *N*-Z protected amino acids (1.0 eq.) were dissolved in dry THF (40 mL), followed by the addition of *N*,*N*-diisopropylethylamine (DIPEA, 2.0 eq.) under argon atmosphere. The reaction mixture was cooled to 0 °C for 15 min before addition of (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU, 1.3 eq.). The mixture was stirred at 0 °C for 30 min and then overnight at room temperature. The reaction was concentrated to give a yellow powder. The crude product was dissolved in dichloromethane and washed with 1.0 M HCl (10 mL), sat. $NaHCO_{3\ (aq)}$ (10 mL) and brine (10 mL). The organic phase was then separated, dried over $MgSO_4$ and filtered. The solvent was removed *in vacuo* and the crude product was purified by silica gel chromatography.

General method II

To a solution of the Cbz-protected compound (1.0 eq.) in anhydrous methanol (20 - 50 mL), palladium on carbon (Pd/C, 0.8 eq.) was added under nitrogen atmosphere. The mixture was then degassed and hydrogen gas was bubbled through it. The reaction was stirred at room temperature under a hydrogen atmosphere until completion. Pd/C was removed by filtration through Celite and washed with acetonitrile. The solvent was removed *in vacuo* to obtain the pure final product without further purification.

General method III

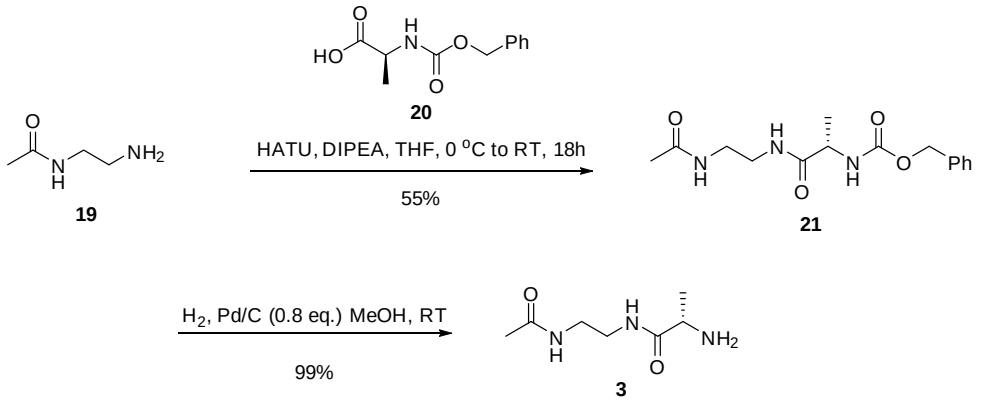
Triethylamine (2.0 eq.) was added to a solution of benzyl (2-aminoethyl) carbamates **24** (1.0 eq.) in dry dichloromethane (25 mL). The reaction mixture was cooled to 0 °C before acyl chloride (1.3 eq.) was slowly added. The mixture was stirred at 0 °C for 30 min, followed by stirring at room temperature overnight. The reaction was washed with aqueous HCl (1 M, 10 mL), saturated aqueous $NaHCO_3$ (10 mL) and brine (10 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by silica gel chromatography.

General method IV

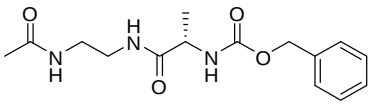
In a three-necked round-bottom-flask, *N*-Z-protected amino acid (e.g. **42**, 1.10 eq.) was dissolved in dry tetrahydrofuran (THF, 10.0 mL) at 0 °C. Oxalyl chloride (1.10 eq.) and dry *N*,*N*-dimethylformamide (DMF, 0.27 eq.) were added and the reaction mixture was stirred for 1 h at 0 °C, followed by 10 min at room temperature. *N*-(2-aminoethyl) acylamide (1.00 eq.) was dissolved in dry THF (20.0 mL) together with anhydrous pyridine (2.30 eq.) in presence of 3 Å molecular sieves. This solution was stirred for 2 h at room temperature and then added dropwise to the first solution containing activated amino acid. The mixture was stirred at room temperature overnight. The solvent was removed *in vacuo* to afford a yellow brown solid, which was purified by silica gel chromatography.

2 Synthesis and characterisation of chemical probes 3-17

2.1 *Synthesis of (S)-N-(2-acetamidoethyl)-2-aminopropanamide (3)*



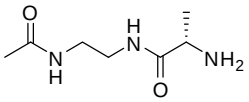
Scheme 1S: Preparation of (*S*)-*N*-(2-acetamidoethyl)-2-aminopropanamide (**3**).



21

Intermediate **21** (benzyl (S)-(1-((2-acetamidoethyl) amino)-1-oxopropan-2-yl) carbamate) was synthesised according to **general method I** from *N*-(2-Aminoethyl) acetamide **19** (200 mg, 1.95 mmol), Z-Ala-OH (**20**, 436 mg, 1.95 mmol), DIPEA (677 µL, 3.90 mmol) and HATU (964 mg, 2.54 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc: methanol 9:1), affording **21** as a white powder (330 mg, 55 %); R_f = 0.17 (MeOH:EtOAc 1:9)**;** $\mathbf{\nu_{max}}$/cm^{-1}: 3290, 1688, 1649, 1560, 1537, 1446, 1372, 1242, 695; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.37 – 7.25 (5H, m, Ar-H), 5.09 (1H, d, *J* = 12.0 Hz, CH_2Ar), 5.05 (1H, d, *J* = 12.0 Hz, CH_2Ar), 4.05 (1H, t, *J* = 7.0 Hz, COC*H*NH), 3.28 – 3.22 (4H, m, NHC*H*$_2$C*H*$_2$NH), 1.89 (3H, s, COC*H*$_3$), 1.30 (3H, d, *J* = 7.0 Hz, CHC*H*$_3$); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.1 (CH_3CO), 173.7 (*C*OCH), 158.4 (COO), 138.2 (Ar), 129.5 (Ar), 129.1 (Ar), 128.9 (Ar), 67.8 (CH_2Ar), 52.3 (COCH), 40.1 (CH_2), 39.9 (CH_2), 22.7 (CH_3CO), 18.3 ($CHCH_3$); **HRMS (ESI)**: calculate for $C_{15}H_{21}N_3NaO_4$ $[M+Na]^+$: 330.1424, found: 330.1423.

Intermediate **21** (300 mg, 0.98 mmol) was then subjected to hydrogenation according to **general method II** using Pd/C (83.0 mg, 0.78 mmol) to obtain **3** as a white powder (168 mg, 99 %).

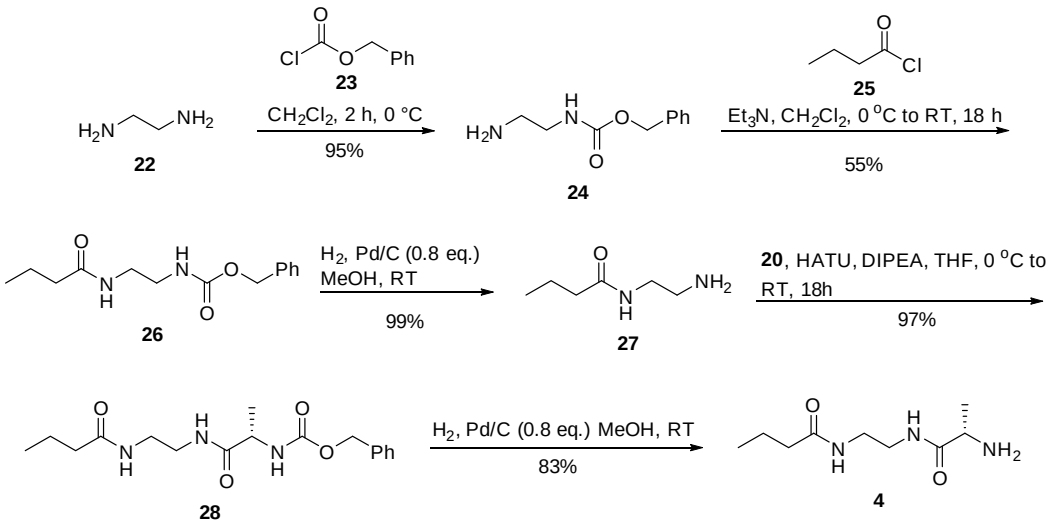


3

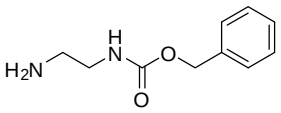
$\mathbf{\nu_{max}}$/cm^{-1}: 3281, 1638, 1541, 1434, 1372, 1200, 1127, 719; **^{1}H NMR** (300 MHz, CD_3OD): δ_H 3.47 (1H, q, *J* = 7.0 Hz, COCH), 3.35 – 3.30 (4H, m, NHC*H*$_2$C*H*$_2$NH), 1.96 (3H, s, C*H*$_3$CO), 1.31 (3H, d, *J* = 7.0 Hz, CHC*H*$_3$); **^{13}C NMR** (75 MHz, CD_3OD): δ_C 178.1 (*C*OCHNH$_2$), 173.7 (CH_3CO), 51.4 (COCHNH$_2$), 40.1 (CH_2), 39.9 (CH_2), 22.6

(*C*H_3CO), 20.9 (*C*H_3CH);**HRMS (ESI)**: calculated for $C_7H_{16}N_3O_2$ $[M+H]^+$: 174.1237, found: 174.1237; $[\alpha]_D^{27}$= +5.24 (c = 0.005, CH_3OH).

2.2 *Synthesis of (S)-N-(2-(2-aminopropanamido)ethyl) butyramide (4)*

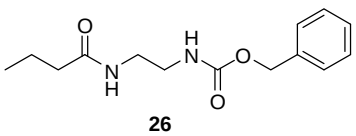


Scheme 2S: Preparation of (*S*)-*N*-(2-(2-aminopropanamido)ethyl) butyramide (**4**).



24

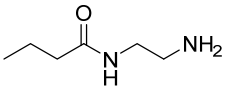
A solution of benzylchloroformate (**23**, 1.3 mL, 9.0 mmol) in dry dichloromethane (25 mL) was added over 1.5 h to a solution of ethylenediamine (**22**, 6.0 mL, 90 mmol) in anhydrous dichloromethane (90 mL) at 0 °C under argon atmosphere. The mixture was stirred at 0 °C for 2 h and then washed with brine, dried over $MgSO_4$, filtered and concentrated in *vacuo* to afford intermediate **24** (benzyl (2-aminoethyl) carbamate) as a white powder (1.70 g, 95 %); this was directly utilised without further purification. **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.35 - 7.26 (5H, m, Ar-H), 5.06 (2H, s, CH_2Ar), 3.19 (2H, t, *J* = 6.0 Hz, NHCH_2), 2.73 (2H, t, *J* = 6.0 Hz, CH_2NH_2); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 159.2 (CO), 138.4 (Ar), 129.6 (Ar), 129.1 (Ar), 128.3 (Ar), 67.6 (CH_2Ar), 41.5 (CH_2NH_2), 43.9 ($NHCH_2$); LRMS: calculated for $C_{10}H_{15}N_2O_2$ $[M+H]^+$: 195.2, found: 195.2. NMR data in accordance with those reported in the literature.[1] This compound is also commercially available.



26

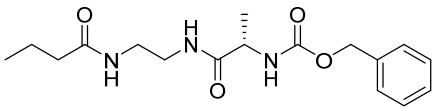
Intermediate **26** (benzyl (2-butyramidoethyl) carbamate) was synthesised using **general method III** from **24** (1.00 g, 5.15 mmol), butyryl chloride (**25**, 0.69 µL, 6.70 mmol) and triethylamine (1.45 mL, 10.3 mmol). The crude product was purified by silica gel chromatography with pure EtOAc. **26** was obtained as a white solid (0.75 g, 55 %); R_f = 0.42 (pure EtOAc)**;** $\mathbf{\nu_{max}}$/cm^{-1}: 3311, 2961, 2872, 1689, 1644, 1543, 1463, 1375, 1274,

1147, 995, 744, 696, 666; **^{1}H NMR** (500 MHz, CD_3OD): δ 7.37 - 7.24 (5H, m, Ar-H), 5.04 (2H, s, CH_2Ar), 3.27 - 3.23 (2H, m, CONHCH_2), 3.22 - 3.17 (2H, m, CH_2NHCOO), 2.13 (2H, t, *J* = 7.5, CH_2CO), 1.58 (2H, sext, *J* = 7.5, CH_3CH_2), 0.90 (3H, t, *J* = 7.5, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ 176.6 (CH_2*C*ONH), 159.1 (NH*C*OO), 138.4 (Ar), 129.5 (Ar), 129.0 (Ar), 128.9 (Ar), 67.5 (CH_2Ar), 41.5 (*C*H_2NHCOO), 40.4 (CONH*C*H_2), 39.1 (*C*H_2CO), 20.3 (CH_2), 14.0 (CH_3); **HRMS (ESI)**: calculated for $C_{14}H_{20}N_2NaO_3$ $[M+Na]^+$: 287.1366, found: 287.1367.



27

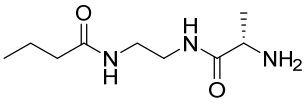
Intermediate **26** (600 mg, 2.61 mmol) was then subjected to hydrogenation according to **general method II** using Pd/C (222 mg, 2.09 mmol) to obtain **27** (*N*-(2-aminoethyl) butyramide) as a white solid (396 mg, 99 %). $\mathbf{v_{max}}$/cm^{-1}: 3289, 2961, 2871, 1639, 1547, 1493, 1342, 1283, 1236, 707, 481; **^{1}H NMR** (400 MHz, CD_3OD): δ_H 3.38 (2H, t, *J* = 6.0 Hz, NHCH_2), 2.98 (2H, t, *J* = 6.0 Hz, CH_2NH_2), 2.15 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.58 (2H, sext, *J* = 7.5 Hz, CH_3CH_2), 0.89 (3H, t, *J* = 7.5 Hz, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.8 (CO), 42.5 (*C*H_2NH_2), 42.0 (*C*H_2NHCO), 39.2 (*C*H_2CO), 20.5 (CH_2), 14.1 (CH_3); **HRMS (ESI)**: calculated for $C_6H_{14}N_2NaO$ $[M+Na]^+$: 153.0998, found: 153.0999.



28

Intermediate **28** (benzyl (*S*)-(1-((2-butyramidoethyl) amino)-1-oxopropan-2-yl) carbamate) was synthesised according to the **general method I** from compound **27** (128 mg, 0.89 mmol), **20** (200 mg, 0.89 mmol), DIPEA (311 µL, 1.78 mmol) and HATU (441 mg, 1.16 mmol). The crude product was purified by column chromatography with a stepwise gradient (pure EtOAc to EtOAc:MeOH 9:1), giving **28** as a white powder (290 mg, 97 %); R_f = 0.32 (EtOAc:MeOH 95:5). $\mathbf{v_{max}}$/cm^{-1}: 3287, 1686, 1649, 1563, 1538, 1445, 1239, 697; **^{1}H NMR** (500 MHz, $CDCl_3$): δ_H 7.38 – 7.30 (5H, m, Ar-H), 6.82 (1H, br s, NH), 6.16 (1H, br s, NH), 5.34 (1H, d, *J* = 6.0 Hz, N*H*COO), 5.13 (1H, d, *J* = 12.0 Hz, CH_2Ar), 5.08 (1H, d, *J* = 12.0 Hz, CH_2Ar), 4.18 (1H, quint, *J* = 7.0 Hz, COC*H*NH), 3.44 – 3.30 (4H, m, NHCH_2CH_2NH), 2.14 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.63 (2H, sext, *J* = 7.5 Hz, CH_2CH_2CO), 1.37 (3H, d, *J* = 7.0 Hz, CHCH_3), 0.92 (3H, t, *J* = 7.5 Hz, CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 174.3 (CH_2*C*O), 173.3 (*C*OCH), 156.0 (COO), 136.1 (Ar), 128.6 (Ar), 128.3 (Ar), 128.1 (Ar), 67.1 (CH_2Ar), 50.8 (CO*C*H), 40.2 (CH_2), 39.5 (CH_2), 38.5 (*C*H_2CO), 19.1 (CH_3CH_2), 18.5 ($CHCH_3$), 13.7 (CH_3CH_2); **HRMS (ESI)**: calculated for $C_{17}H_{25}N_3NaO_4$ $[M+Na]^+$: 358.1737, found: 358.1736.

Intermediate **28** (280 mg, 0.83 mmol) was then subjected to hydrogenation according to **general method II** using Pd/C (71.0 mg, 0.67 mmol) to obtain the final compound **4** as a white powder (138 mg, 83 %).

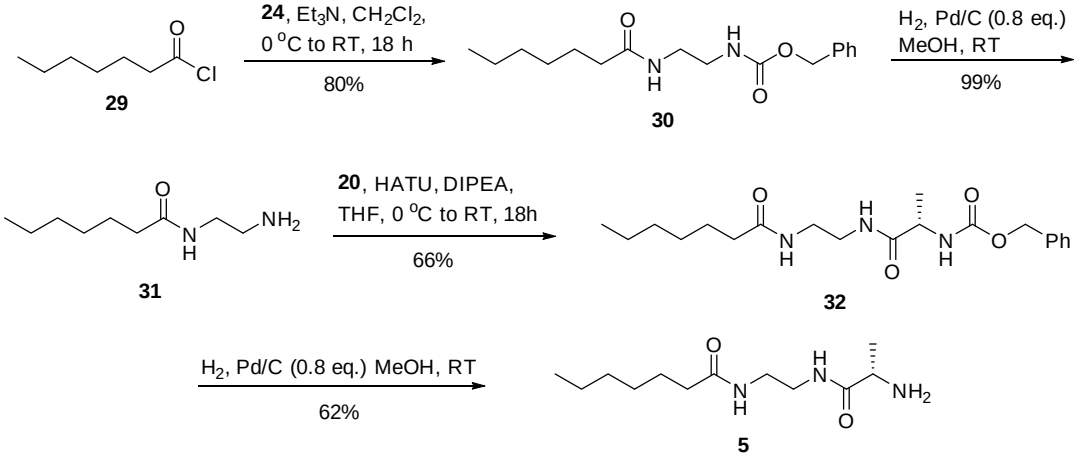


4

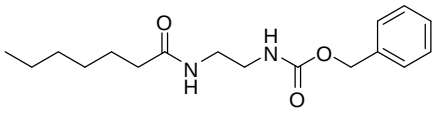
$\mathbf{v_{max}}$/cm^{-1}: 3283, 2965, 1636, 1541, 1446, 1236, 686; **^{1}H NMR** (400 MHz, CD_3OD): δ_H 3.42 – 3.36 (1H, m, COCH), 3.33 – 3.30 (4H, m, NHCH_2CH_2NH), 2.18 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.65 (2H, sext, *J* = 7.5 Hz, CH_2CH_2CO), 1.27 (3H, d, *J* = 7.0 Hz, CHCH_3), 0.96 (3H, t, *J* = 7.5 Hz, CH_3CH_2); **^{13}C NMR** (100 MHz, CD_3OD): δ_C

178.8 (*C*OCHNH$_2$), 176.5 (CH$_2$*C*O), 51.6 (CO*C*HNH$_2$), 40.1 (CH$_2$), 39.9 (CH$_2$), 39.1 (*C*H$_2$CO), 21.5 (*C*H$_3$CH), 20.3 (CH$_3$*C*H$_2$) 14.1 (*C*H$_3$CH$_2$); **HRMS (ESI)**: calculated for $C_9H_{20}N_3O_2$ $[M+H]^+$: 202.1550, found: 202.1552; $[\alpha]_D^{27}$= +1.04 (c = 0.005, CH$_3$OH).

2.3 *Synthesis of (S)-N-(2-(2-aminopropanamido)ethyl) heptanamide (5)*



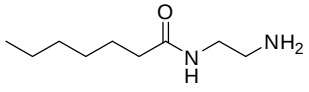
Scheme 3S: Preparation of (*S*)-*N*-(2-(2-aminopropanamido)ethyl) heptanamide (**5**).



30

Intermediate **30** (benzyl (2-heptanamidoethyl) carbamate) was synthesised according to **general method III** from **24** (1.00 g, 5.15 mmol), heptanoyl chloride (**29**, 0.87 mL, 6.70 mmol) and triethylamine (1.22 mL, 10.3 mmol). The crude product was purified by silica gel chromatography with EtOAc: cyclohexane (3:1) to give **30** as a white solid (1.26 g, 80 %); R_f = 0.3 (EtOAc : cyclohexane 3:1); ν_{max}/cm^{-1}: 3316, 2926, 2854, 1692, 1642, 1545, 1277, 995, 710, 710; **^{1}H NMR** (600 MHz, $CDCl_3$): δ_H 7.26-7.20 (5H, m, Ar-H), 5.87 (1H, br s, CON*H*CH$_2$), 5.08 (1H, br s, NHCOO), 4.99 (2H, s, CH$_2$Ar), 3.30 - 3.25 (2H, m, NHC*H*$_2$CH$_2$NH), 3.25 - 3.20 (2H, m, NHCH$_2$C*H*$_2$NH), 2.03 (2H, t, *J* = 7.5 Hz, CH$_2$CO), 1.48 (2H, quint, *J* = 7.5 Hz, C*H*$_2$CH$_2$CO), 1.21 - 1.14 (6H, m, CH$_2$), 0.78 - 0.75 (3H, m, CH$_3$); **^{13}C NMR** (150 MHz, CD_3OD): δ_C 174.1 (CH$_2$*C*O), 157.2 (NH*C*O), 136.3 (Ar), 128.5 (Ar), 128.2 (Ar), 128.1 (Ar), 66.9 (CH$_2$Ar), 41.0 (CONH*C*H$_2$), 40.2 (CH$_2$*C*H$_2$NH), 36.7 (*C*H$_2$CO), 31.5 (CH$_2$), 28.9 (CH$_2$), 25.6 (*C*H$_2$CH$_2$CO), 22.5 (CH$_2$), 14.0 (CH$_3$); **HRMS (ESI)**: calculated for $C_{17}H_{26}N_2NaO_3$ $[M+Na]^+$: 329.1836, found: 329.1835.

Intermediate **30** (650 mg, 2.39 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (202 mg, 1.91 mmol) to afford compound **31** (*N*-(2-aminoethyl) heptanamide) as a white solid (406 mg, 99 %).



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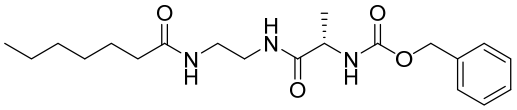
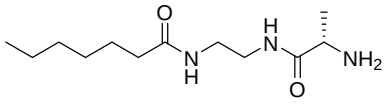
ν_{max}/cm^{-1}: 3286, 2923, 2856, 1636, 1551; **^{1}H NMR** (400 MHz, CD_3OD): δ_H 3.24 (2H, t, *J* = 6.5 Hz, NHC*H*$_2$), 2.73 (2H, t, *J* = 6.0 HZ, C*H*$_2$NH$_2$), 2.19 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.64 - 1.56 (2H, m, C*H*$_2$CH$_2$CO), 1.37 - 1.27 (6H, m, CH_2), 0.93 - 0.87 (3H, m, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$): δ_C 176.7 (CO), 42.8 ($NHCH_2$), 41.9 (CH_2NH), 37.2 (*C*H$_2$CO), 32.7 (CH_2), 30.1 (CH_2), 26.9 (*C*H$_2$CH$_2$CO), 23.6 (CH_2), 14.4 (CH_3); **HRMS (ESI)**: calculated for $C_9H_{21}N_2O$ $[M+H]^+$: 173.1648, found: 173.1649.



32

Intermediate **32** (benzyl (*S*)-(1-((2-heptanamidoethyl) amino)-1-oxopropan-2-yl) carbamate) was synthesised according to the **general method I** from compound **31** (144 mg, 0.84 mmol), **20** (188 mg, 0.84 mmol), DIPEA (293 µL, 1.68 mmol) and HATU (415 mg, 1.09 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc:MeOH 9:1), giving **32** as a white powder (250 mg, 66 %); R_f = 0.32 (EtOAc:MeOH 9:1)**; ν_{max}**/cm^{-1}: 3288, 2929, 2857, 1687, 1650, 1537, 1445, 1238, 1080, 696; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.36 – 7.26 (5H, m, Ar-H), 5.09 (1H, d, *J* = 12.0 Hz, CH_2Ar), 5.05 (1H, d, *J* = 12.0 Hz, CH_2Ar), 4.04 (1H, q, *J* = 7.0 Hz, COC*H*NH), 3.29 – 3.22 (4H, m, NHC*H*$_2$C*H*$_2$NH), 2.14 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.60 – 1.52 (2H, m, C*H*$_2$CH$_2$CO), 1.30 (3H, d, *J* = 7.0 Hz, CHC*H*$_3$), 1.29 – 1.25 (6H, m, CH_2), 0.89 – 0.85 (3H, m, CH_2CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.7 (CH_2CO), 176.1 (*C*OCH), 158.4 (COO), 138.2 (Ar), 129.5 (Ar), 129.1 (Ar), 128.9 (Ar), 67.8 (CH_2Ar), 52.3 (CO*C*H), 40.2 (CH_2), 39.9 (CH_2), 37.3 (*C*H$_2$CO), 32.7 (CH_2), 30.1 (CH_2), 26.9 (*C*H$_2$CH$_2$CO), 23.6 (CH_2), 18.3 (CH*C*H$_3$), 14.4 (*C*H$_3$CH$_2$); **HRMS (ESI)**: calculated for $C_{20}H_{31}N_3NaO_4$ $[M+Na]^+$: 400.2207, found: 400.2213.

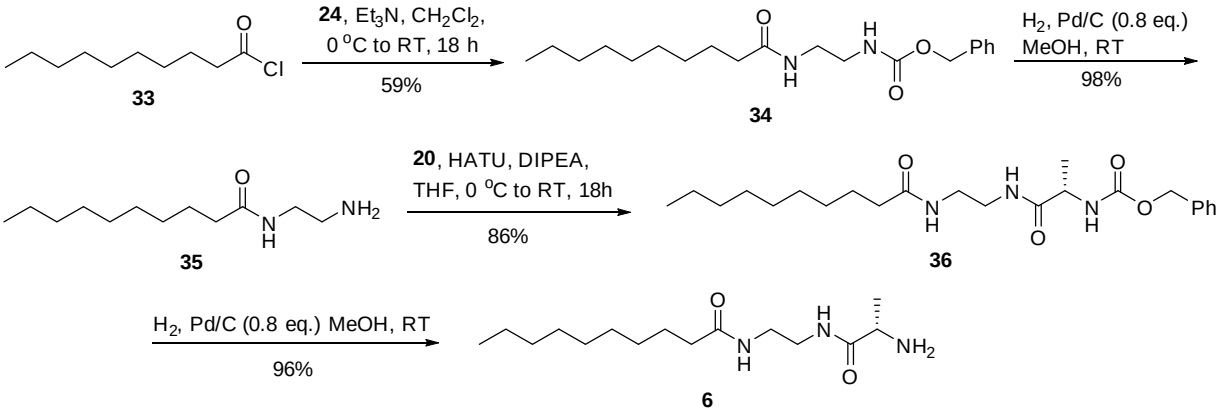
Intermediate **32** (240 mg, 0.64 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (54.0 mg, 0.51 mmol) to obtain the final compound **5** as a white powder (152 mg, 62 %).



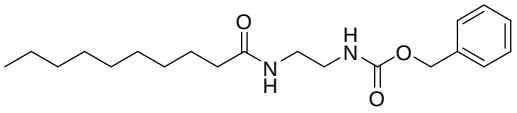
5

ν_{max}/cm^{-1}: 3298, 2926, 1637, 1551, 1445, 1241, 973, 722; **^{1}H NMR** (300 MHz, $CDCl_3$): δ_H 7.70 (1H, br s, NH), 6.28 (1H, br s, NH), 3.49 (1H, q, *J* = 7.0 Hz, COCH), 3.48 – 3.45 (4H, m, NHC*H*$_2$C*H*$_2$NH), 2.16 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.68 – 1.52 (2H, m, C*H*$_2$CH$_2$CO), 1.40 (2H, br s, NH_2), 1.33 (3H, d, *J* = 7.0 Hz, CHC*H*$_3$), 1.31 – 1.19 (6H, m, CH_2), 0.92 – 0.82 (3H, m, C*H*$_3$CH$_2$); **^{13}C NMR** (100 MHz, CD_3OD): δ_C 178.7 (*C*OCHNH$_2$), 176.7 (CH_2CO), 51.7 (CO*C*HNH$_2$), 40.1 (CH_2), 39.9 (CH_2), 37.2 (*C*H$_2$CO), 32.7 ($CH_3CH_2CH_2$), 30.0 (CH_2), 26.9 (*C*H$_2$CH$_2$CO), 23.6 (CH_3CH_2), 21.4 (*C*H$_3$CH), 14.4 (*C*H$_3$CH$_2$); **HRMS (ESI)**: calculated $C_{12}H_{26}N_3O_2$ $[M+H]^+$: 244.2020, found: 244.2019; **$[\alpha]_D^{28}$**= +4.29 (c = 0.005, CH_3OH).

2.4 *Synthesis of (S)-N-(2-(2-aminopropanamido)ethyl) decanamide (6)*

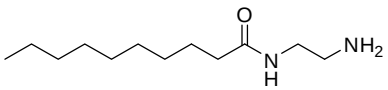


Scheme 4S: Preparation of (*S*)-*N*-(2-(2-aminopropanamido)ethyl) decanamide (**6**).



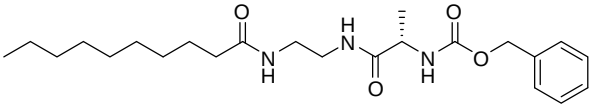
34

Intermediate **34** (benzyl (2-decanamidoethyl) carbamate) was synthesised according to **general method III** from **24** (1.00 g, 5.15 mmol) and decanoyl chloride (**33**, 1.38 mL, 6.70 mmol). The crude product was purified by column chromatography with EtOAc : cyclohexane (1:1) yielding **34** as a white solid (1.05 g, 59 %); R_f = 0.22 (EtOAc : cyclohexane 1:1)**;** $\mathbf{\nu_{max}}$/cm^{-1}: 3321, 3305, 2916, 2849, 1688, 1638, 1545, 1446, 1276, 1246, 1147, 997, 727, 667; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.34 – 7.25 (5H, m, Ar-H), 5.04 (2H, s, $COOCH_2$), 3.24 (2H, t, *J* = 5.5 Hz, $CONHCH_2$), 3.18 – 3.21 (2H, m, CH_2NHCOO), 2.13 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.60 – 1.52 (2H, m, CH_2CH_2CO), 1.33- 1.23 (12H, m, CH_2), 0.89 – 0.86 (3H, m, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.9 (CH_2CONH), 159.2 ($NHCOCH$), 138.5 (Ar), 129.6 (Ar), 129.1 (Ar), 129.0 (Ar), 67.6 (OCH_2Ph), 41.6 (CH_2NHCOO), 40.5 ($CONHCH_2$), 37.3 (CH_2CO), 33.2 (CH_2), 30.7 (CH_2), 30.6 (CH_2), 30.6 (CH_2), 30.5 (CH_2), 27.1 (CH_2), 23.9 (CH_2), 14.2 (CH_3); **HRMS (ESI)**: calculated for $C_{20}H_{32}N_2NaO_3$ $[M+Na]^+$: 371.2305, found: 371.2305.



35

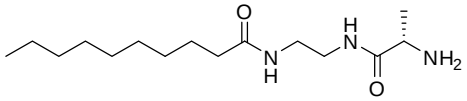
Intermediate **34** (660 mg, 1.90 mmol) was then subjected to hydrogenation according to **general method II** using Pd/C (161 mg, 1.52 mmol) to obtain the compound **35** (*N*-(2-aminoethyl) decanamide) as a white powder (160 mg, 98 %); R_f = 0.2 (CH_2Cl_2: MeOH 3:7); $\mathbf{\nu_{max}}$/cm^{-1}: 3285, 2918, 2849, 1635, 1552, 1466, 967, 927, 719; **^{1}H NMR** (400 MHz, CD_3OD): δ_H 3.27 (2H, t, *J* = 6.0 Hz, $NHCH_2$), 2.78 (2H, t, *J* = 6.5 Hz, CH_2NH_2), 2.18 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.64 – 1.54 (2H, m, CH_2CH_2CO), 1.35 – 1.24 (12H, m, CH_2), 0.91 – 0.85 (3H, m, CH_3) ; **^{13}C NMR** (100 MHz, CD_3OD): δ_C 173.2 (CO), 41.9 ($NHCH_2$), 41.6 (CH_2NH_2), 37.0 (CH_2CO), 33.9 (CH_2), 29.6 (CH_2), 29.5(CH_2), 29.5 (CH_2), 26.4 (CH_2CH_2CO), 25.9 (CH_2), 22.8 (CH_2), 14.3 (CH_3); **HRMS (ESI)**: calculated for $C_{12}H_{27}N_2O$ $[M+H]^+$: 215.2118, found: 215.2121.



36

Intermediate **36** (benzyl (*S*)-(1-((2-decananamidoethyl) amino)-1-oxopropan-2-yl) carbamate) was synthesised according to the **general method I** from compound **35** (200 mg, 0.78 mmol), **20** (174 mg, 0.78 mmol), DIPEA (272 µL, 1.56 mmol) and HATU (388 mg, 1.02 mmol). The crude product was purified by silica gel chromatography with a stepwise gradient (EtOAc: MeOH 99:1), affording **36** as a white powder (280 mg, 86 %); R_f = 0.3 (EtOAc: MeOH 99:1).**^{1}H NMR** (500 MHz, $CDCl_3$): δ_H 7.38 – 7.29 (5H, m, Ar-H), 6.85 (1H, br s, NH), 6.16 (1H, br s, NH), 5.39 (1H, d, *J* = 7.0 Hz, NHCOO), 5.13 (1H, d, *J* = 12.0 Hz, CH_2Ar), 5.07 (1H, d, *J* = 12.0 Hz, CH_2Ar), 4.18 (1H, quint, *J* = 7.0 Hz, COC*H*NH), 3.44 – 3.28 (4H, m, NHC*H*$_2$C*H*$_2$NH), 2.18 – 2.11 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.64 – 1.54 (2H, m, C*H*$_2$CH$_2$CO), 1.37 (3H, d, *J* = 7.0 Hz, CHC*H*$_3$), 1.32 – 1.22 (12H, m, CH_2), 0.90 – 0.85 (3H, m, CH_2CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 174.7 (CH_2*C*O), 173.4 (*C*OCH), 156.1 (COO), 136.3 (Ar), 128.7 (Ar), 128.4 (Ar), 128.2 (Ar), 67.2 (CH_2Ar), 50.9 (CO*C*H), 40.4 (CH_2), 39.7 (CH_2), 36.8 (CH_2CO), 31.9 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 25.8 (*C*H$_2$CH$_2$CO), 22.8 (CH_2), 18.7 (CH*C*H$_3$), 14.3 (CH_2*C*H$_3$); **HRMS (ESI)**: calculated for $C_{23}H_{37}N_3NaO_4$ $[M+Na]^+$: 442.2676, found: 442.2678.

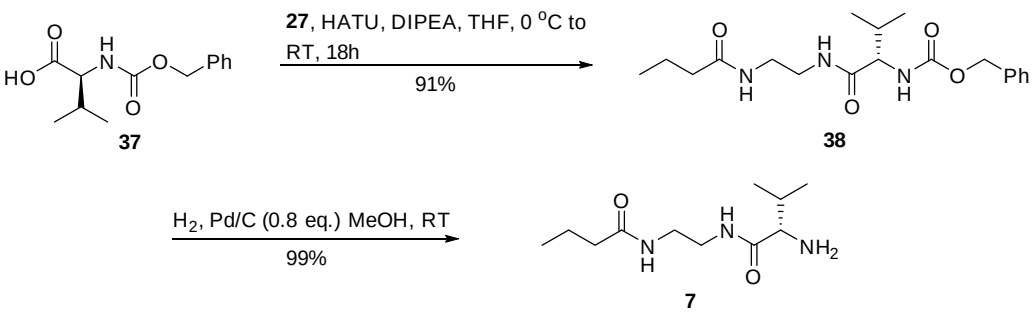
Intermediate **36** (260 mg, 0.62 mmol) was then subjected to hydrogenation according to **general method II** using Pd/C (53.0 mg, 0.50 mmol) to obtain the final compound **6** as a white powder (170 mg, 96 %).



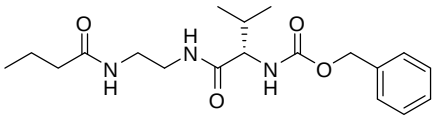
6

ν_{max}/cm^{-1}: 3298, 2918, 2850, 1637, 1552, 1445, 1241, 937, 721; **^{1}H NMR** (400 MHz, CD_3OD): δ_H 3.40 (1H, m, COCH), 3.33 – 3.28 (4H, m, NHC*H*$_2$C*H*$_2$NH), 2.20 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.69 – 1.55 (2H, m, C*H*$_2$CH$_2$CO), 1.39 – 1.29 (12H, m, CH_2), 1.28 (3H, d, *J* = 7.0, CHC*H*$_3$), 0.99 – 0.86 (3H, m, C*H*$_3$CH$_2$); **^{13}C NMR** (100 MHz, CD_3OD): δ_C 178.8 (*C*OCHNH$_2$), 176.7 (CH_2*C*O), 51.6 (CO*C*HNH$_2$), 40.1 (CH_2), 39.9 (CH_2), 37.2 (*C*H$_2$CO), 33.0 (CH_2), 30.6 (CH_2), 30.5 (CH_2), 30.4 (CH_2), 30.3 (CH_2), 26.9 (*C*H$_2$CH$_2$CO), 23.7 (CH_2), 21.5 (*C*H$_3$CH), 14.4 (*C*H$_3$CH$_2$); **HRMS (ESI)**: calculated for $C_{15}H_{32}N_3O_2$ $[M+H]^+$: 286.2489, found: 286.2488; **$[\alpha]_D^{28}$**= +3.49 (c = 0.0045, CH_3OH).

2.5 *Synthesis of (S)-2-amino-N-(2-butyramidoethyl)-3-methylbutanamide (7)*



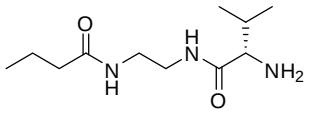
Scheme 5S: Preparation of (*S*)-2-amino-*N*-(2-butyramidoethyl)-3-methylbutanamide (**7**).



38

Intermediate **38** (benzyl (*S*)-(1-((2-butyramidoethyl)amino)-3-methyl-1-oxobutan-2-yl)carbamate) was synthesised according to the **general method I** from amine **27** (52 mg, 0.40 mmol), Z-Val-OH (**37,** 100 mg, 0.40 mmol), DIPEA (139 µL, 0.80 mmol) and HATU (198 mg, 0.52 mmol). The crude product was purified by column chromatography with an isocratic elution of EtOAc : MeOH (98:2), affording **38** as a white powder (140 mg, 91 %); R_f = 0.32 (EtOAc : MeOH 98:2)**;** $\mathbf{\nu_{max}}$/cm^{-1}:3292, 3919, 2851, 1720, 1689, 1646, 1248, 1138, 732; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7. 36 – 7.24 (5H, m, Ar-H), 5.09 (1H, d, *J* = 12.5 Hz, CH_2Ar), 5.06 (1H, d, *J* = 12.5 Hz, CH_2Ar), 3.83 (1H, d, *J* = 7.0 , COC*H*NH), 3.28 – 3.23 (4H, m, NHCH_2CH_2NH), 2.12 (2H, t, *J* = 7.5 Hz, CH_2CO), 2.06 – 1.97 (1H, m, C*H*$(CH_3)_2$), 1.59 (2H, sext, *J* = 7.5 Hz, CH_3CH_2), 0.93 – 0.87 (9H, m, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.5 (CH_2CO), 174.73 (*C*OCHNH), 158.7 (COO), 138.2 (Ar), 129.5 (Ar), 129.1 (Ar), 128.9 (Ar), 67.8 (CH_2Ar), 62.5 (CO*C*HNH), 40.0 (CH_2), 39.9 (CH_2), 39.1 (CH_2CO), 31.8 (*C*H$(CH_3)_2$), 20.3 (CH_3CH_2), 19.8 (CH(*C*$H_3)_2$), 18.6 (CH(*C*$H_3)_2$), 14.1 (CH_2CH_3); **HRMS (ESI)**: calculated for $C_{19}H_{29}N_3NaO_4$ $[M+Na]^+$: 386.2050, found: 386.2058.

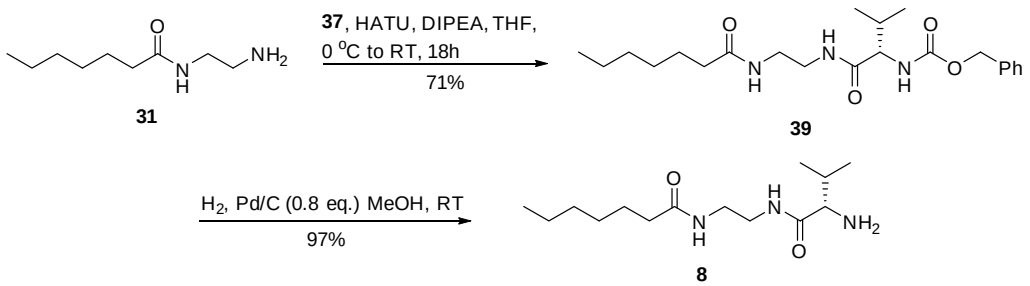
Intermediate **38** (130 mg, 0.36 mmol) was then subjected to hydrogenation according to **general method II** using Pd/C (30 mg, 0.29 mmol) to obtain the final compound **7** as a white powder (82 mg, 99 %).



7

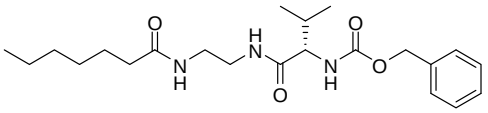
$\mathbf{\nu_{max}}$/cm^{-1}: 3286, 2958, 2930, 2871, 1633, 1593, 1552, 1466, 1361, 1259, 683; **^{1}H NMR** (500 MHz, $CDCl_3$): δ_H 7.74 (1H, br s, NH), 6.40 (1H, br s, NH), 3.47 - 3.36 (4H, m, NHCH_2CH_2NH), 3.24 (1H, d, *J* = 4.0 Hz, COC*H*NH_2), 2.29 (1H, sept. d, *J* = 4.0, 7.0 Hz, C*H*$(CH_3)_2$), 2.14 (2H, t, *J* = 7.5, CH_2CO), 1.64 (2H, sext, *J* = 7.5 Hz, CH_3CH_2), 1.55 (2H, br s, NH_2), 0.99 (3H d, *J* = 7.0 Hz, CHCH_3), 0.93 (3H, t, *J* = 7.5 Hz, CH_2CH_3), 0.81 (3H, d, *J* = 7.0 Hz, CHCH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 176.4 (*C*OCHNH_2), 173.9 (CH_2CO), 60.2 (CO*C*H), 41.2 (CH_2), 39.2 (CH_2), 38.8 (CH_2CO), 30.9 (*C*H$(CH_3)_2$), 19.8 (CH(*C*$H_3)_2$), 19.3 (CH_3CH_2), 16.1 (CH(*C*$H_3)_2$), 13.9 (CH_2CH_3); **HRMS (ESI)**: calculated for $C_{11}H_{24}N_3O_2$ $[M+H]^+$: 230.1863, found: 230.1861; $\mathbf{[\alpha]_D}^{28}$= +14.03 (c = 0.005, CH_3OH).

2.6 *Synthesis of (S)-N-(2-(2-amino-3-methylbutanamido)ethyl)heptanamide (8)*



31 **39** **8**

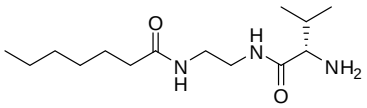
Scheme 6S: Preparation of (*S*)-*N*-(2-(2-amino-3-methylbutanamido)ethyl)heptanamide (**8**).



39

Intermediate **39** (benzyl (S)-(1-((2-heptanamidoethyl)amino)-3-methyl-1-oxobutan-2-yl)carbamate) was synthesised according to the **general method I** from compound **31** (74.0 mg, 0.43 mmol), **37** (141 mg, 0.56 mmol), DIPEA (225 µL, 1.29 mmol) and HATU (213 mg, 0.56 mmol). The crude product was purified by column chromatography with an isocratic elution of EtOAc, affording **39** as a white powder (123 mg, 71 %); R_f = 0.3 (EtOAc); $\mathbf{\nu_{max}}$/cm^{-1}: 3290, 2919, 2851, 1687, 1641, 1536, 1245, 1139, 1039, 677; **^{1}H NMR** (500 MHz, $CDCl_3$): δ_H 7.33 - 7.29 (5H, m, Ar-H), 6.94 (1H, br s, NH), 6.32 (1H, br s, NH), 5.48 (1H, m, NH), 5.48 (1H, d, *J* = 12.0 Hz, CH_2Ar), 5.12 (1H, d, *J* = 12.0 Hz, CH_2Ar), 4.01 – 3.93 (1H, m, COC*H*NH), 3.43 – 3.26 (4H, m, NHC*H*$_2$C*H*$_2$NH), 2.19 - 2.05 (3H, m, CH_2CO, C*H*$(CH_3)_2$), 1.64 – 1.52 (2H, m, C*H*$_2$$CH_2CO$), 1.34 – 1.19 (6H, m, CH_2), 0.95 (3H, d, *J* = 7.0 Hz, CH(C*H*$_3$)$_2$), 0.92 – 0.80 (6H, m, CH_2C*H*$_3$, CH(C*H*$_3$)$_2$); **^{13}C NMR** (100 MHz, $CDCl_3$): δ_C 164.6 (*C*OCHNH$_2$), 172.5 (CH_2*C*O), 156.6 (COO), 136.3 (Ar), 128.7 (Ar), 128.4 (Ar), 128.1 (Ar), 67.2 (CH_2Ar), 60.8 (COC*H*NH), 40.2 (CH_2), 39.8 (CH_2), 36.8 (*C*H_2CO), 31.6 (CH_2), 31.0 (*C*H$(CH_3)_2$), 29.1 (CH_2), 25.8 (*C*H_2CH_2CO), 22.6 (CH_2), 19.4 (CH(*C*H_3)$_2$), 17.9 (CH(*C*H_3)$_2$), 14.2 (CH_2*C*H_3); **HRMS (ESI)**: calculated for $C_{22}H_{35}N_3NaO_4$ [M+Na]$^+$: 428.2520, found: 428.2520.

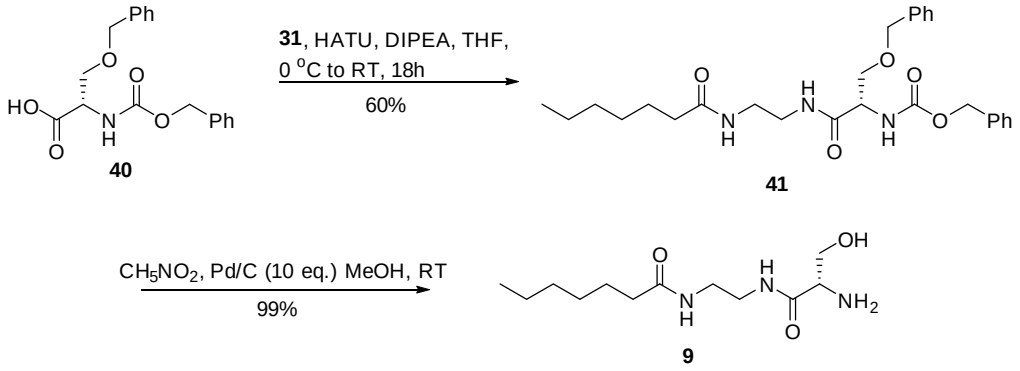
Intermediate **39** (89 mg, 0.22 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (19 mg, 0.18 mmol) to obtain the final compound **8** as a white powder (57 mg, 97 %).



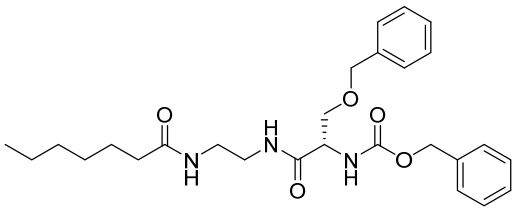
8

$\mathbf{\nu_{max}}$/cm^{-1}: 3292, 2926, 2871, 1634, 1549, 1446, 1245, 709; **^{1}H NMR** (500 MHz, D_2O): δ_H 3.44 – 3.28 (4H, m, NHC*H*$_2$C*H*$_2$NH), 3.10 (1H, d, *J* = 6.5 Hz, , COC*H*NH_2), 2.22 (2H, t, *J* = 7.5, CH_2CO), 1.92 – 1.83 (1H, m, C*H*$(CH_3)_2$), 1.61 – 1.51 (2H, m, C*H*$_2$$CH_2CO$), 1.33 – 1.23 (6H, m, CH_2), 0.92 – 0.83 (9H, m, CH_3); **^{13}C NMR** (125 MHz, D_2O): δ_C 177.6 (CH_2*C*O), 177.3 (*C*OCH), 60.5 (CO*C*H), 38.5 (CH_2), 38.4 (CH_2), 35.8 (*C*H_2CO), 31.8 (*C*H$(CH_3)_2$), 30.7 (CH_2), 27.9 (CH_2), 25.2 (*C*H_2CH_2CO), 21.8 (CH_2), 18.5 (CH(*C*H_3)$_2$), 16.9 (CH(*C*H_3)$_2$), 13.3 (CH_3); **HRMS (ESI)**: calculated for $C_{14}H_{30}N_3O_2$ [M+H]$^+$: 272.2333, found: 272.2333; **$[\alpha]_D^{28}$**= +9.79 (c = 0.029, CH_3OH).

2.7 *Synthesis of (S)-N-(2-(2-amino-3-hydroxypropanamido)ethyl)heptanamide (9)*



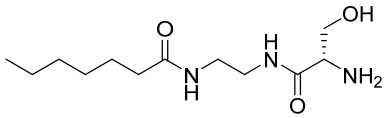
Scheme 7S: Preparation of (*S*)-*N*-(2-(2-amino-3-hydroxypropanamido)ethyl)heptanamide (**9**).



41

Intermediate **41** (benzyl (*S*)-(1-((2-heptanamidoethyl)amino)-3-hydroxy-1-oxopropan-2-yl)carbamate) was synthesised according to the **general method I** from compound **31** (360 mg, 2.09 mmol), Z-L-Ser(Bzl)-OH (**40,** 500 mg, 2.09 mmol), DIPEA (728 µL, 4.18 mmol) and HATU (1.00 g, 2.71 mmol). The crude product was purified by column chromatography with an isocratic elution of EtOAc: petroleum ether (3:1), affording **41** as a white powder (498 mg, 60 %); R_f = 0.3 (EtOAc: petroleum ether 3:1)**;** $\mathbf{\nu_{max}}$/cm^{-1}: 3288, 2927, 2858, 1688, 1640, 1537, 1451, 1237, 1027, 732, 693; **^{1}H NMR** (500 MHz, $CDCl_3$): δ_H 7.30 - 7.18 (10H, m, Ar-H), 6.82 (1H, br s, NH), 5.97 (1H, br s, NH), 5.64 – 5.54 (1H, m, NH), 5.07 (1H, d, *J* = 12.0 Hz, $COOCH_2$), 5.03 (1H, d, *J* = 12.0 Hz, $COOCH_2$), 4.45 (2H, m, *J* = 12.0 Hz, CH_2OCH_2), 4.24 (1h, s, COCH), 3.89 – 3.79 (1H, m, $CHCH_2$), 3.52 (1H, dd, *J* = 9.5, 3.5 Hz, $CHCH_2$), 3.36 – 3.22 (4H, m, $NHCH_2CH_2NH$), 2.01 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.53 – 1.45 (2H, m, CH_2CH_2CO), 1.25 – 1.13 (6H, m, CH_2), 0.82 – 0.77 (3H, m, CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 174.3 (CH_2CONH), 171.0 (*C*OCH), 156.3 (COO), 137.4 (Ar), 136.1 (Ar), 128.7 (Ar), 128.5 (Ar), 128.3 (Ar), 128.2 (Ar), 127.9 (Ar), 73.6 (CH_2OCH_2), 69.8 ($CHCH_2$), 67.4 ($COOCH_2$), 54.9 (COCH), 40.2 (CH_2), 39.8 (CH_2), 36.7 (CH_2CO), 31.6 (CH_2), 29.1 ($CH_3CH_2CH_2CH_2$), 25.7 (CH_2CH_2CO), 22.6 (CH_2), 14.2 (CH_3); **HRMS (ESI)**: calculated for $C_{27}H_{37}N_3NaO_5$ $[M+Na]^+$: 506.2625 found: 506.2625.

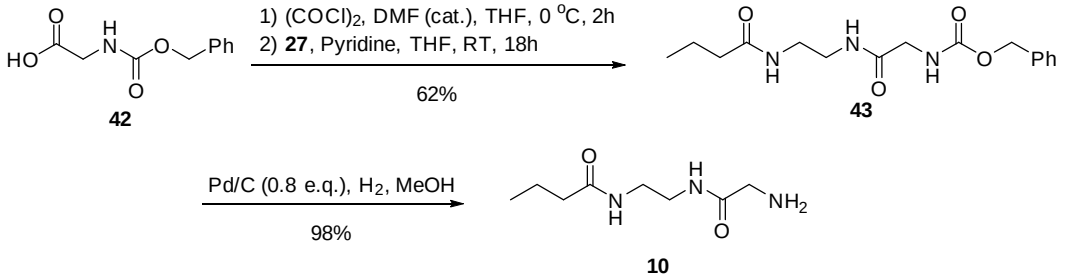
To a solution of **41** (51 mg, 0.11 mmol) in anhydrous methanol, Pd/C (112 mg, 1.05 mmol) and ammomium formate (41 mg, 0.63 mmol) were added under argon atmosphere. The reaction was heated at reflux and stirred overnight. The reaction was then filtered through Celite and the solvent was removed *in vacuo* to obtain the final compound **9** as a white powder (28 mg, 99 %) without further purification.



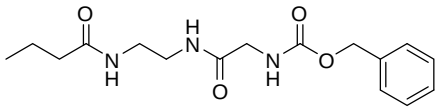
9

$\mathbf{\nu_{max}}$/cm^{-1}: 3284, 2926, 2856, 1638, 1554, 1445, 1243, 1059, 585; **^{1}H NMR** (400 MHz, $CDCl_3$): δ_H 7.75 (1H, br s, NH), 6.05 (1H, s, NH), 3.89 (1H, dd, *J* = 11.0, 5.0 Hz, $CHCH_2OH$), 3.68 (1H, dd, *J* = 11.0, 6.0 Hz, $CHCH_2OH$) 3.50 – 3.33 (5H, m, $CHCH_2OH$, $NHCH_2CH_2NH$), 2.17 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.60 – 1.53 (4H, m, CH_2CH_2CO, NH_2), 1.34 – 1.23 (6H, m, CH_2), 0.91 – 0.85 (3H, m, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$): δ_C 175.2 (CONH), 167.0 (COCH), 65.5 (CH_2OH), 55.6 (COCH), 40.1 (CH_2), 39.6 (CH_2), 36.8 (CH_2CO), 31.5 (CH_2), 28.9 (CH_2), 25.6 (CH_2CH_2CO), 22.5 (CH_2), 14.1 (CH_3); **HRMS (ESI)**: calculated for $C_{12}H_{26}N_3O_3$ $[M+H]^+$: 260.1896, found: 260.1896. $\mathbf{[\alpha]_D}^{30}$= -8.51 (c = 0.026, CH_3OH)

2.8 *Synthesis of N-(2-(2-aminoacetamido)ethyl)butyramide (10)*



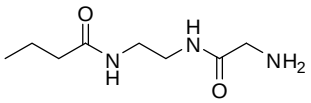
Scheme 8S: Preparation of *N*-(2-(2-aminoacetamido)ethyl)butyramide (**10**).



43

Intermediate **43** (benzyl (2-((2-butyramidoethyl)amino)-2-oxoethyl)carbamate) was synthesised according to **general method IV** from **27** (316 mg, 2.43 mmol), Z-Gly-OH (**42**, 559 mg, 2.67 mmol), oxalyl chloride (229 µL, 2.67 mmol), dry DMF (51 µL, 0.66 mmol) and anhydrous pyridine (417 µL, 5.18 mmol). The crude product was purified by silica gel chromatography with an isocratic elution of EtOAc : MeOH (96:4). The product **43** was obtained as a white solid (200 mg, 62 %); R_f = 0.18 (EtOAc : MeOH 96:4). **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.38 - 7.25 (5H, m, Ar-H), 5.09 (2H, s, CH_2Ph), 3.76 (2H, s, $COCH_2NH$), 3.30 - 3.20 (4H, m, $NHCH_2CH_2NH$), 2.14 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.60 (2H, sext, *J* = 7.5 Hz, CH_2CH_2CO), 0.91 (3H, t, *J* = 7.5 Hz, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.6 (CH_2CONH), 172.7 ($NHCOCH_2$), 159.2 (NHCOO), 138.1 (Ar), 129.5 (Ar), 129.1 (Ar), 128.9 (Ar), 67.9 (CH_2Ar), 45.1 ($COCH_2NH$), 40.2 (CH_2), 39.9 (CH_2), 39.1 (CH_2CO), 20.3 (CH_3CH_2), 14.1 (CH_3); **HRMS (ESI)**: calculated for $C_{16}H_{23}N_3NaO_4$ $[M+Na]^+$: 344.1581, found: 344.1583.

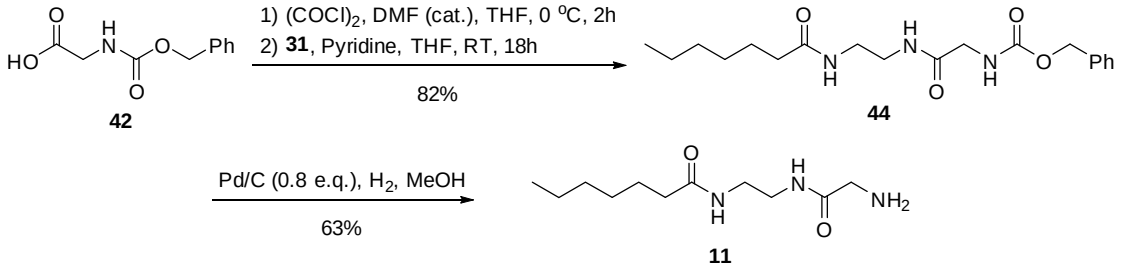
Intermediate **43** (280 mg, 0.87 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (74 mg, 0.70 mmol)to obtain **10** as a white powder (160 mg, 98 %). R_f = 0.2 (CH_2Cl_2: MeOH 3:7).



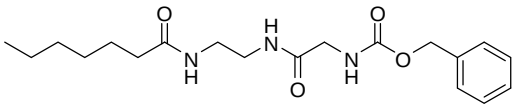
10

ν_{max}/cm^{-1}: 3279, 2960, 1634, 1544, 1457, 1256, 682; **^{1}H NMR** (500 MHz, $CDCl_3$): δ_H 7.69 (1H, br s, NH), 6.32 (1H, br s, NH), 3.47 – 3.37 (4H, m, $NHCH_2CH_2NH$), 3.35 (2H, s, $COCH_2NH_2$), 2.15 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.65 (2H, sext, *J* = 7.5 Hz, CH_3CH_2), 1.59 (2H, br s, NH_2), 0.93 (3H, t, *J* = 7.5 Hz, CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 174.5 (CH_2CONH), 174.0 ($COCH_2NH_2$), 44.8 ($COCH_2NH_2$), 40.8 (CH_2), 39.3 (CH_2), 38.8 (CH_2CO), 19.3 (CH_3CH_2), 13.9 (CH_3); **HRMS (ESI)**: calculated for $C_8H_{17}N_3NaO_2$ $[M+Na]^+$: 210.1213, found: 210.1214.

2.9 *Synthesis of N-(2-(2-aminoacetamido)ethyl) heptanamide (11)*



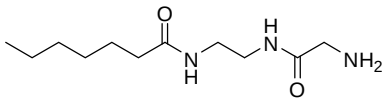
Scheme 9S: Preparation of *N*-(2-(2-aminoacetamido)ethyl) heptanamide (**11**).



44

Intermediate **44** (benzyl (2-((2-heptanamidoethyl)amino)-2-oxoethyl) carbamate) was synthesised according to **general method IV** from **31** (300 mg, 1.74 mmol), **42** (402 mg, 1.92 mmol), oxalyl chloride (165 µL, 1.92 mmol), dry DMF (36.0 µL, 0.47 mmol) and anhydrous pyridine (299 µL, 3.71 mmol). The crude product was purified by silica gel chromatography with an isocratic elution of EtOAc : MeOH (96:4). The product **44** was obtained as a white solid (520 mg, 82 %); R_f = 0.29 (EtOAc : MeOH 96:4); $\mathbf{\nu_{max}}$/cm^{-1}: 3333, 3285, 2922, 2853, 1689, 1656, 1639, 1541, 1293, 1249, 694; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.30 - 7.26 (5H, m, Ar-H), 5.09 (2H, s, CH_2Ar), 3.72 (2H, s $COCH_2NH$), 3.28 – 3.24 (4H, m, $NHCH_2CH_2NH$), 2.15 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.60 – 1.53 (2H, m, CH_2CH_2CO), 1.33 – 1.26 (6H, m, CH_2), 0.89 – 0.86 (3H, m, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.8 (CH_2CONH), 172.7 ($COCH_2NH$), 159.2 (COO), 138.1 (Ar), 129.5 (Ar), 129.1 (Ar), 129.0 (Ar), 68.0 (CH_2Ar), 45.1 ($COCH_2NH$), 40.2 (CH_2), 39.9 (CH_2), 37.2 (CH_2CO), 32.7 (CH_2), 30.1 (CH_2), 26.9 (CH_2CH_2CO), 23.6 (CH_2), 14.4 (CH_3); **HRMS (ESI)**: calculate for $C_{19}H_{29}N_3NaO_4$ $[M+Na]^+$: 386.2050, found: 386.2055.

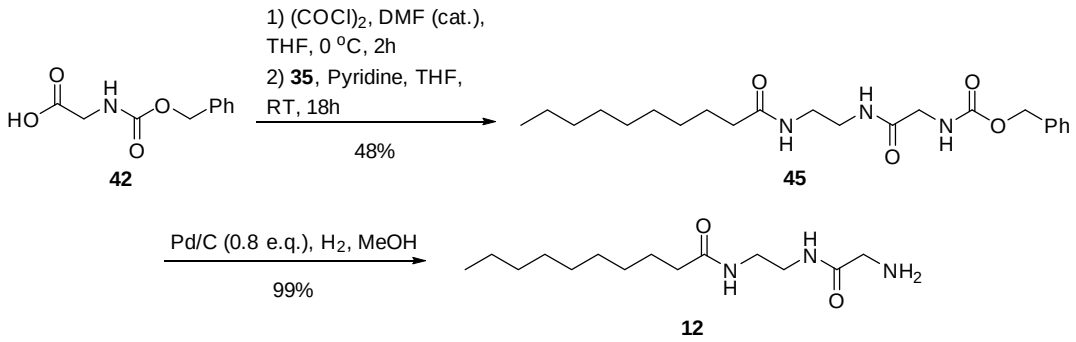
Intermediate **44** (300 mg, 0.83 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (70.0 mg, 0.66 mmol) to obtain the final compound **11** as a white powder (120 mg, 63 %). R_f = 0.09 (CH_2Cl_2: MeOH 3:7).



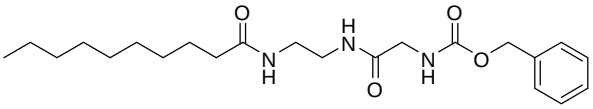
11

$\mathbf{\nu_{max}}$/cm^{-1}: 3275, 2921, 2854, 1633, 1557, 1445, 1257, 713; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 3.33 - 3.29 (4H, m, $NHCH_2CH_2NH$), 3.25 (2H, s, $COCH_2NH_2$), 2.19 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.64 - 1.56 (2H, m, CH_2CH_2CO), 1.37 – 1.29 (6H, m, CH_2), 0.94 - 0.89 (3H, m, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.7 (CH_2CONH), 175.7 ($COCH_2NH_2$), 45.2 ($COCH_2NH_2$), 40.0 (CH_2), 39.9 (CH_2), 37.2 (CH_2CO), 32.7 (CH_2), 30.0 (CH_2), 26.9 (CH_2), 23.6 (CH_2), 14.4 (CH_3); **HRMS (ESI)**: calculated for $C_{11}H_{23}N_3NaO_2$ $[M+Na]^+$: 230.1863, found: 230.1867.

2.10 *Synthesis of N-(2-(2-aminoacetamido)ethyl) decanamide (12)*



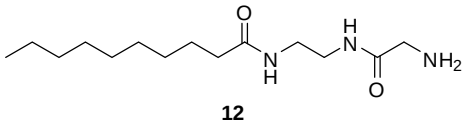
Scheme 10S: Preparation of *N*-(2-(2-aminoacetamido)ethyl) decanamide (**12**).



45

Intermediate **45** (benzyl (2-((2-decanamidoethyl)amino)-2-oxoethyl)carbamate) was synthesised according to **general method IV** from **35** (319 mg, 1.49 mmol), **42** (342 mg, 1.64 mmol), oxalyl chloride (140 µL, 1.64 mmol), dry DMF (31.0 µL, 0.40 mmol) and anhydrous pyridine (256 µL, 3.17 mmol). The crude product was purified by suspending in cold MeOH (2 mL) and filtering the solid, followed by washing of it with diethyl ether. Pure **45** was obtained as a white solid (289 mg, 48 %); R_f = 0.44 (EtOAc : MeOH 9:1); **^{1}H NMR** (400 MHz, $CDCl_3$): δ_H 7.33 - 7.38 (5H, m, Ar-H), 6.70 (1H, br s, NH), 5.99 (2H, s, C*H*$_2$Ar), 5.36 (1H, br s, NH), 3.85 (2H, *J* = 6.0 Hz, COC*H*$_2$NH), 3.41 (4H, s, NHC*H*$_2$C*H*$_2$NH), 2.17 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.64 – 1.57 (2H, m, C*H*$_2$CH$_2$CO), 1.32 – 1.20 (12H, m, CH_2), 0.88 (3H, t, *J* = 7.0 Hz, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.9 (CH_2*C*ONH), 172.7 (*C*OCH$_2$NH), 159.5 (COO), 138.3 (Ar), 129.6 (Ar), 129.2 (Ar), 129.0 (Ar), 68.1 (CH_2Ar), 45.4 (CO*C*H$_2$NH), 40.5 (CH_2), 40.1 (CH_2), 37.4 (*C*H$_2$CO), 33.1 (CH_2), 30.6 (CH_2), 30.5 (2 x CH_2), 30.5 (CH_2), 27.0 (*C*H$_2$CH$_2$CO), 23.7 (CH_2), 14.4 (CH_3); **HRMS (ESI)**: calculate for $C_{22}H_{35}N_3NaO_4$ $[M+Na]^+$: 428.2520, found: 428.2523.

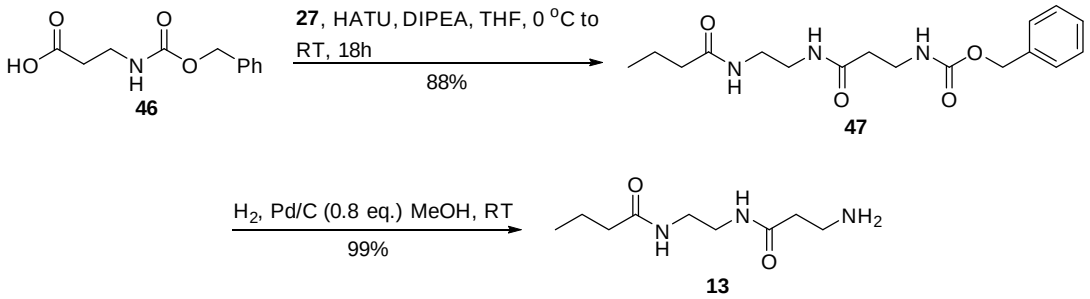
Intermediate **45** (493 mg, 0.49 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (52.0 mg, 0.05 mmol) to obtain the final compound **12** as a white powder (132 mg, 99 %). R_f = 0.53 (CH_2Cl_2: MeOH 9:1).



12

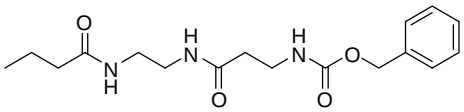
ν_{max}/cm^{-1}: 3285, 2915, 2847, 1635, 1549, 1464, 1258, 719; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 3.34 – 3.32 (4H, m, NHC*H*$_2$C*H*$_2$NH), 3.27 (2H, s, COC*H*$_2$NH$_2$), 2.19 (2H, t, *J* = 5.0 Hz, CH_2CO), 1.66 – 1.58 (2H, m, C*H*$_2$CH$_2$CO), 1.38 – 1.26 (12H, m, CH_2), 0.92 (3H, t, *J* = 5.5 Hz, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.7 (CH_2*C*ONH), 175.7 (*C*OCH$_2$NH), 45.2 (CO*C*H$_2$NH), 40.0 (CH_2), 39.9 (CH_2), 37.2 (*C*H$_2$CO), 33.0 (CH_2), 30.6 (CH_2), 30.5 (CH_2), 30.4 (CH_2), 30.3 (CH_2), 27.1 (*C*H$_2$CH$_2$CO), 23.9 (CH_2), 14.6 (CH_3); **HRMS (ESI)**: calculate for $C_{14}H_{29}N_3NaO_2$ $[M+Na]^+$: 294.2152, found: 294.2155.

2.11 *Synthesis of N-(2-(3-aminopropanamido)ethyl) butyramide (13)*



46 47 13

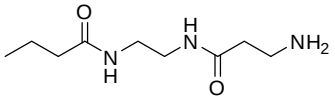
Scheme 11S: Preparation of *N*-(2-(3-aminopropanamido)ethyl) butyramide (**13**).



47

Intermediate **47** (benzyl (3-((2-butyramidoethyl)amino)-3-oxopropyl) carbamate) was synthesised according to the **general method I** from compound **27** (128 mg, 0.89 mmol), Z-β-Ala-OH (**46**, 200 mg, 0.89 mmol), DIPEA (311 µL, 1.78 mmol) and HATU (441 mg, 1.16 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc : MeOH 99:1), giving **47** as a white powder (261 mg, 88 %); R_f = 0.52 (EtOAc : MeOH 99:1). **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.42 – 7.26 (5H, m, Ar-H), 5.07 (2H, s, CH_2Ar), 3.39 (2H, t, *J* = 6.5 Hz, $COCH_2CH_2NH$), 3.25 – 3.23 (4H, m, $NHCH_2CH_2NH$), 2.38 (2H, t, *J* = 6.5 Hz, $COCH_2CH_2NH$), 2.16 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.62 (2H, sext, *J* = 7.5 Hz, CH_3CH_2), 0.93 (3H, t, *J* = 7.5 Hz, CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 176.6 (CH_2CONH), 174.2 ($NHCOCH$), 158.8 (COO), 138.4 (Ar), 129.5 (Ar), 129.0 (Ar), 128.9 (Ar), 67.5 (CH_2Ar), 40.2 (CH_2), 39.9 (CH_2), 39.9 (CH_2CO), 38.6 ($COCH_2CH_2$), 37.5 ($COCH_2CH_2$), 20.3 (CH_3CH_2), 14.1 (CH_3); **HRMS (ESI)**: calculated for $C_{17}H_{25}N_3NaO_4$ $[M+Na]^+$: 358.1737, found: 358.1736.

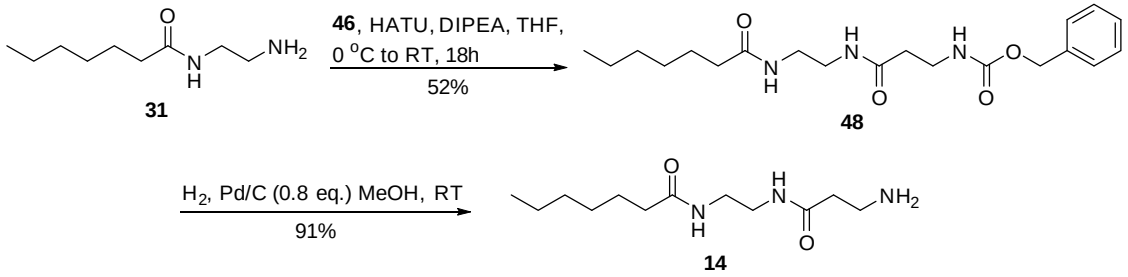
Intermediate **47** (200 mg, 0.60 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (51.0 mg, 0.48 mmol) to obtain the final compound **13** as a white powder (120 mg, 99 %). R_f = 0.07 (CH_2Cl_2: MeOH 3:7)



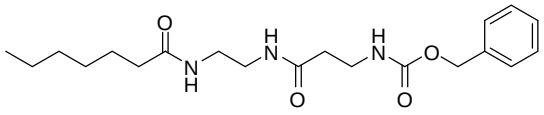
13

ν_{max}/cm^{-1}: 3291, 2960, 2872, 1635, 1549, 1241, 838; **^{1}H NMR** (400 MHz, CD_3OD): δ_H 3.32 – 3.25 (4H, m, $NHCH_2CH_2NH$), 2.95 (2H, t, *J* = 6.5, $COCH_2CH_2NH_2$), 2.40 (2H, t, *J* = 6.5 Hz, $COCH_2CH_2NH_2$), 2.18 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.65 (2H, sext, *J* = 7.5 Hz, CH_2CH_2CO), 0.96 (3H, t, *J* = 7.5, CH_3); **^{13}C NMR** (100 MHz, CD_3OD): δ_C 176.5 (CH_2CONH_2), 174.5 ($COCH_2CH_2NH$), 40.1 (CH_2), 39.9 (CH_2), 39.1 (CH_2CO), 38.8 ($COCH_2CH_2NH_2$), 38.6 ($COCH_2CH_2NH_2$), 20.3 (CH_3CH_2), 14.0 (CH_3); **HRMS (ESI)**: calculated for $C_9H_{20}N_3O_2$ $[M+H]^+$: 202.1550, found: 202.1552.

2.12 *Synthesis of N-(2-(3-aminopropanamido)ethyl) heptanamide (14)*



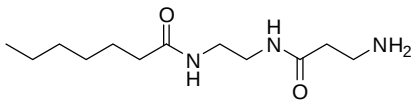
Scheme 12S: Preparation of *N*-(2-(3-aminopropanamido)ethyl) heptanamide (**14**).



48

Intermediate **48** (benzyl (3-((2-heptanamidoethyl)amino)-3-oxopropyl) carbamate) was synthesised according to **general method I** from compound **31** (153 mg, 0.89 mmol), **46** (200 mg, 0.89 mmol), DIPEA (310 µL, 1.78 mmol) and HATU (441 mg, 1.16 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure ethyl acetate to EtOAc : methanol 9:1), affording **48** as a white powder (176 mg, 52 %); R_f = 0.07 (pure EtOAc). $\mathbf{\nu_{max}}$/cm^{-1}: 3325, 2925, 2857, 1680, 1638, 1538, 1234, 728, 692; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.40 – 7.24 (5H, m, Ar-H), 5.05 (2H, s, CH_2Ar), 3.37 (2H, t, *J* = 7.0 Hz, $COCH_2CH_2NH$), 3.27 – 3.20 (4H, m, $NHCH_2CH_2NH$), 2.36 (2H, t, *J* = 7.0 Hz, $COCH_2CH_2NH$), 2.15 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.63 – 1.51 (2H, m, CH_2CH_2CO), 1.38 – 1.21 (6H, m, CH_2), 0.91 – 0.86 (3H, m, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.8 (CH_2CONH), 174.2 ($NHCOCH_2$), 159.1 (COO), 138.7 (Ar), 129.5 (Ar), 129.0 (Ar), 128.8 (Ar), 67.5 (CH_2Ar), 40.2 (CH_2), 39.9 (CH_2), 38.6 ($COCH_2CH_2$), 37.5 ($COCH_2CH_2$) 37.2 (CH_2CO), 32.7 (CH_2), 30.1 (CH_2), 26.9 (CH_2CH_2CO), 23.6 (CH_2), 14.4 (CH_3); **HRMS (ESI)**: calculated for $C_{20}H_{31}N_3NaO_4$ $[M+Na]^+$: 400.2207, found: 400.2204.

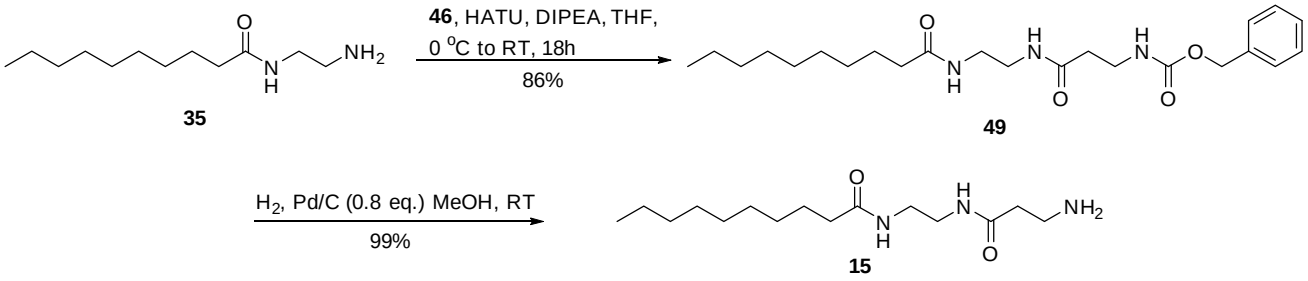
Intermediate **48** (217 mg, 0.64 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (36.0 mg, 0.51 mmol) to obtain the final compound **14** as a white powder (100 mg, 91 %).



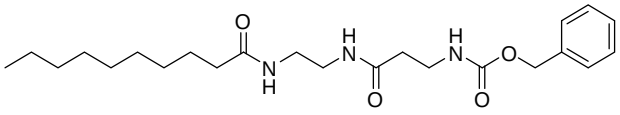
14

$\mathbf{\nu_{max}}$/cm^{-1}: 3289, 2923, 2856, 1634, 1553, 1279, 722; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 3.30 – 3.26 (4H, m, $NHCH_2CH_2NH$), 2.90 (2H, t, *J* = 7.0 Hz, $COCH_2CH_2NH_2$), 2.35 (2H, t, *J* = 6.5 Hz, $COCH_2CH_2NH_2$), 2.18 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.64 – 1.57 (2H, m, CH_2CH_2CO), 1.38 – 1.27 (6H, m, CH_2), 0.95 – 0.88 (3H, m, CH_3); **^{13}C NMR** (125 MHz, CD_3OD): δ_C 176.7 (CH_2CONH), 174.8 ($NHCOCH_2$), 40.1 (CH_2), 39.9 (CH_2), 39.3 (CH_2CO), 38.9 ($CH_2CH_2NH_2$), 37.2 ($COCH_2CH_2NH_2$), 32.7 ($CH_3CH_2CH_2$), 30.0 (CH_2), 26.9 (CH_2CH_2CO), 23.6 (CH_3CH_2), 14.4 (CH_3); **HRMS (ESI)**: calculated for $C_{12}H_{26}N_3O_2$ $[M+H]^+$: 244.2020, found: 244.2020.

2.13 *Synthesis of N-(2-(3-aminopropanamido)ethyl) decanamide (15)*



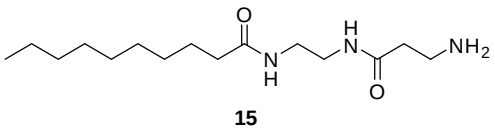
Scheme 13S: Preparation of *N*-(2-(3-aminopropanamido)ethyl) decanamide (**15**).



49

Intermediate **49** (benzyl (3-((2-decanamidoethyl)amino)-3-oxopropyl) carbamate) was synthesised according to the **general method I** from compound **35** (125 mg, 0.58 mmol), **46** (130 mg, 0.58 mmol), DIPEA (202 µL, 1.16 mmol) and HATU (285 mg, 0.75 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc:MeOH 96:4) to afford **49** as a white powder (120 mg, 86 %); R_f = 0.10 (pure EtOAc). $\mathbf{\nu_{max}}$/cm^{-1}: 3300, 2923, 2853, 1689, 1644, 1531, 1286, 1238, 1044, 731, 693, 560, 458; **^{1}H NMR** (300 MHz, CD_3OD): δ_H 7.41 – 7.25 (5H, m, Ar-H), 5.05 (2H, s, CH_2Ar), 3.37 (2H, t, *J* = 7.0 Hz, $COCH_2CH_2NH$), 3.28 – 3.21 (4H, m, $NHCH_2CH_2NH$), 2.36 (2H, t, *J* = 6.5 Hz, $COCH_2CH_2NH$), 2.19 – 2.11 (2H, m, CH_2CO), 1.64 – 1.50 (2H, m, CH_2CH_2CO), 1.36 – 1.20 (12H, m, CH_2), 0.91 – 0.85 (3H, m, CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 176.8 (CH_2CONH), 174.2 ($NHCOCH_2$), 159.1 (COO), 138.7 (Ar), 129.5 (Ar), 129.0 (Ar), 128.8 (Ar), 67.5 (CH_2Ar), 40.2 (CH_2), 39.9 (CH_2), 38.6 ($COCH_2CH_2NH_2$), 37.5 ($COCH_2CH_2NH_2$) 37.2 (CH_2CO), 32.7 (CH_2), 30.1 (CH_2), 29.4 (CH_2), 29.3 (2 x CH_2), 26.9 (CH_2CH_2CO), 23.6 (CH_2), 14.4 (CH_3); **HRMS (ESI)**: calculated for $C_{23}H_{37}N_3NaO_4$ $[M+Na]^+$: 442.2676, found: 442.2678.

Intermediate **49** (100 mg, 0.24 mmol) was subjected to hydrogenation according to **general method II** using Pd/C (20.0 mg, 0.19 mmol) to obtain the final compound **15** as a white powder (68.0 mg, 99 %). R_f = 0.04 (CH_2Cl_2: MeOH 3:7)

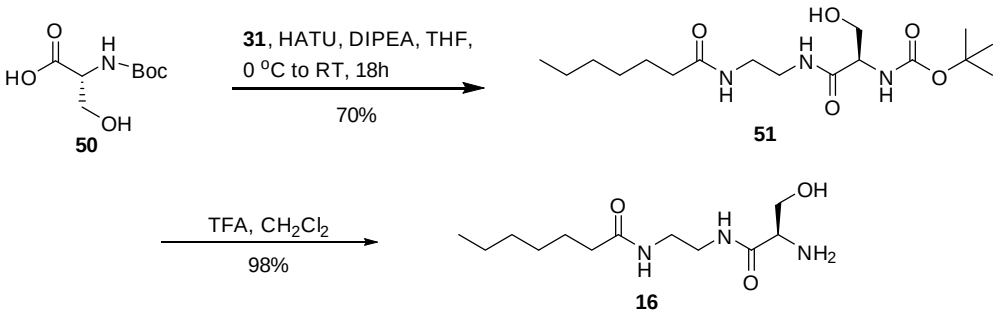


15

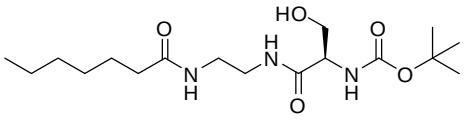
$\mathbf{\nu_{max}}$/cm^{-1}: 3293, 2917, 2847, 1628, 1533, 1467, 1247, 722, 522; **^{1}H NMR** (400 MHz, CD_3OD): δ_H 3.31 – 3.26 (4H, m, $NHCH_2CH_2NH$), 2.92 (2H, t, *J* = 6.5 Hz, $COCH_2CH_2NH_2$), 2.37 (2H, t, *J* = 6.5 , $COCH_2CH_2NH_2$), 2.19 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.66 – 1.56 (2H, m, CH_2CH_2CO), 1.37 – 1.27 (12H, m, CH_2), 0.91 (3H, t, *J* = 6.5 Hz, CH_3); **^{13}C NMR** (100 MHz, CD_3OD): δ_C 176.7 ($COCH_2CH_2$), 174.6 (CH_2CO), 40.1 (CH_2), 39.9 (CH_2), 38.9 ($COCH_2CH_2NH_2$), 38.9 ($COCH_2CH_2NH_2$), 37.2 (CH_2CO), 33.1 (CH_2), 30.6 (CH_2), 30.6 (CH_2), 30.5 (CH_2), 30.4

(CH_2), 26.9 (CH_2CH_2CO), 23.7 (CH_2), 14.4 (CH_3CH_2); **HRMS (ESI)**: calculated for $C_{15}H_{32}N_3O_2$ $[M+H]^+$: 286.2489, found: 286.2490.

2.14 *Synthesis of (R)-N-(2-(2-amino-3-hydroxypropanamido)ethyl)heptanamide (16)*



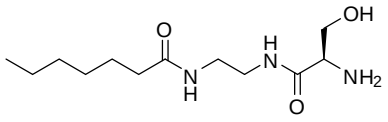
Scheme 14S: Preparation of (*R*)-*N*-(2-(2-amino-3-hydroxypropanamido)ethyl)heptanamide (**16**).



51

Intermediate **51** (*tert*-butyl (*R*)-(1-((2-heptanamidoethyl)amino)-3-hydroxy-1-oxopropan-2-yl) carbamate) was synthesised according to **general method I** from **31** (360 mg, 2.09 mmol), **50** (500 mg, 2.09 mmol), DIPEA (728 µL, 4.18 mmol) and HATU (1.00 g, 2.71 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc : MeOH 97:3), affording **51** as a white powder (502 mg, 70 %); R_f = 0.25 (EtOAc : MeOH 98:2). $\mathbf{\nu_{max}}$/cm^{-1}: 3295, 2827, 2858, 1635, 1527, 1241, 1169, 620; **^{1}H NMR** (500 MHz, $CDCl_3$): δ_H 7.34 (1H, br s, N*H*COCH), 6.69 (1H, br s, CON*H*CH_2), 5.82 – 5.79 (1H, m, N*H*COO), 4.15 (1H, br s, COC*H*NH), 3.93 (1H, dd, *J* = 11.0 Hz, 3.5 Hz, C*H*$_2$OH), 3.67 (1H, s, C*H*$_2$OH), 3.43 – 3.30 (4H,m, NHC*H*$_2$C*H*$_2$NH), 2.14 (2H, t, *J* = 7.0 Hz, CH_2CO), 1.61 – 1.52 (2H, m, C*H*$_2$$CH_2CO$), 1.42 (9H, s, $(CH_3)_3$), 1.30 – 1.22 (6H, m, CH_2), 0.88 – 0.82 (3H, m, CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 175.0 (CH_2*C*O), 172.3 (*C*OCH), 156.1 (COO), 80.4 (*C*$(CH_3)_3$), 63.1 (CH_2OH), 56.2 (CO*C*H), 39.8 (CH_2), 39.5 (CH_2), 36.7 (CH_2CO), 31.6 (CH_2), 29.1 (CH_2), 28.5 ($(CH_3)_3$), 25.7 (*C*H_2CH_2CO), 22.6 (CH_2), 14.1 (*C*H_3CH_2); **HRMS (ESI)**: calculated for $C_{17}H_{33}N_3NaO_5$ $[M+Na]^+$: 382.2312, found: 382.2312.

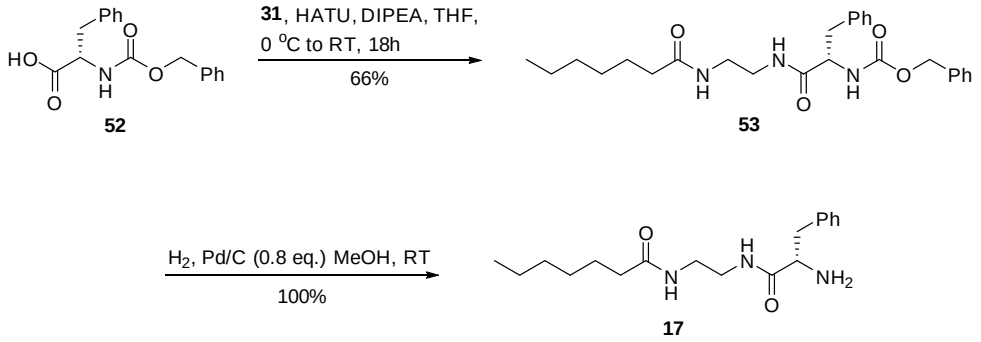
TFA (2.0 mL, 6.53 mol) was added to a solution of **51** (116 mg, 0.32 mmol) in dry dichlomethane (2.0 mL). The reaction mixture was stirred at room temperature for 1.5 h before dichloromethane and TFA were removed *in vacuo*. The crude material was dissolved in water and saturated aqueous $NaHCO_3$ added. The aqueous layer was extracted with a mixture of 2-isopropanol and chloroform (1:4, 5 x 10 mL). The organic phases were dried over $MgSO_4$ and filtered. The solvent was removed *in vacuo* to afford the final compound **16** as a white powder (70 mg, 84 %).



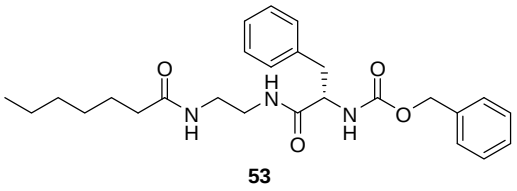
16

$\mathbf{v_{max}}$/cm^{-1}: 3278, 2924, 2855, 1639, 1558, 1446, 1297, 1508, 696; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 3.80 – 3.61 (2H, m, *CH*$_2$OH), 3.55 – 3.39 (1H, m , COCH), 3.39 – 3.22 (4H, m, NHC*H*$_2$C*H*$_2$NH), 2.19 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.67 – 1.52 (2H, m, C*H*$_2$CH$_2$CO), 1.43 – 1.18 (6H, m, CH_2), 0.92 (3H, t, *J* = 7.0 Hz, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$): δ_C 176.9 (CH_2*C*O), 168.4 (*C*OCH), 61.6 (CH_2OH), 56.3 ($CHNH_2$), 40.6 (CH_2), 39.6 (CH_2), 37.2 (CH_2), 32.7 (CH_2), 30.0 (CH_2), 26.9 (CH_2), 23.6 (CH_2), 14.4 (CH_3); **HRMS (ESI)**: calculated for $C_{12}H_{26}N_3O_3$ $[M+H]^+$: 260.1896, found: 260.1903. **$[\alpha]_D^{30}$**= +11.5 (c = 0.032, CH_3OH).

2.15 *Synthesis of (S)-N-(2-(2-amino-3-phenylpropanamido)ethyl)heptanamide (17)*

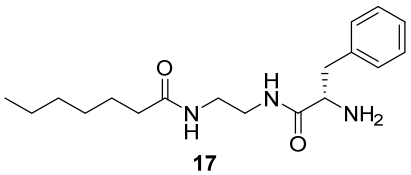


Scheme 15S: Preparation of (*S*)-*N*-(2-(2-amino-3-phenylpropanamido)ethyl)heptanamide (**17**).



Intermediate **53** (benzyl (*S*)-(1-((2-heptanamidoethyl)amino)-1-oxo-3-phenylpropan-2-yl) carbamate) was synthesised according to **general method I** from **31** (360 mg, 2.09 mmol), Z-Phe-OH (**52**, 630 mg, 2.09 mmol), DIPEA (728 µL, 4.18 mmol) and HATU (1.00 g, 2.71 mmol). The crude product was purified by column chromatography with an elution of EtOAc: petroleum ether (3:1), affording **53** as a white powder (250 mg, 66 %); R_f = 0.32 (EtOAc: petroleum ether 3:1); $\mathbf{v_{max}}$/cm^{-1}: 3295, 2927, 2853, 1686, 1644, 1533, 1259, 1042, 749, 694; **^{1}H NMR** (500 MHz, $CDCl_3$): δ_H 7.32 - 7.06 (10H, m, Ar-H), 6.26 (1H, br s, NH), 5.80 (1H, br s, NH), 5.30 – 5.18 (1H, m, COCHN*H*), 5.01 (2H, dd, *J* = 12.5, 6.5 Hz, O*CH*$_2$Ar), 4.28 (1H, dd, *J* =7.0, 6.5 Hz, C*H*CH$_2$), 3.75 – 3.10 (4H, m, NHC*H*$_2$C*H*$_2$NH), 3.06 – 2.93 (2H, m, CHC*H*$_2$), 2.03 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.55 – 1.46 (2H, m, C*H*$_2$CH$_2$CO), 1.26 – 1.10 (6H, m, CH_2), 0.83 – 0.78 (3H, m, CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 174.1 (CH_2*C*ONH), 171.7 (*C*OCH), 155.9 (COO), 136.4 (Ar), 136.1 (Ar), 129.3 (Ar), 128.8 (Ar), 128.6 (Ar), 128.3 (Ar), 128.1 (Ar), 127.2 (Ar), 67.2 (OCH_2Ar), 67.3 (CH*C*H$_2$Ar), 56.5 (CO*C*H), 40.1 (CH_2), 39.5 (CH_2), 38.6 (CH_2*C*H), 36.7 (*C*H$_2$CO), 31.5 (CH_2), 29.0 (CH_2), 25.6 (*C*H$_2$CH$_2$CO), 22.5 (*C*H$_2$CH$_3$), 14.1 (CH_3); **HRMS (ESI)**: calculated for $C_{26}H_{36}N_3NaO_4$ $[M+Na]^+$: 476.2607, found: 476.2609.

Intermediate **53** (70 mg, 0.154 mmol) was then subjected to hydrogenation according to **general method II** using Pd/C (213 mg, 0.123 mmol) to obtain the final compound **17** as a white powder (49 mg, 100 %).



17

ν_{max}/cm^{-1}: 2917, 2849, 1722, 1365, 1247, 1142, 845, 716; **^{1}H NMR** (500 MHz, CD_3OD): δ_H 7.32 - 7.19 (5H, m, Ar-H), 3.50 (1H, t, *J* = 7.0 Hz, CH), 3.25 - 3.15 (4H, m, NHC*H*$_2$C*H*$_2$NH), 2.99 (1H, dd, *J* = 13.5, 6.5 Hz, CHC*H*$_2$), 2.80 (1H, dd, *J* = 13.5, 7.5 Hz, CHC*H*$_2$), 2.15 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.62 – 1.54 (2H, m, C*H*$_2$CH$_2$CO), 1.36 – 1.26 (6H, m, CH_2), 0.91 – 0.89 (3H, m, CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ_C 177.1 (*C*OCH), 176.6 (CH_2*C*O), 138.9 (Ar), 130.4 (Ar), 129.6 (Ar), 127.8 (Ar), 57.9 (CH), 42.5 (CH*C*H$_2$), 39.9 (CH_2), 39.8 (CH_2), 37.2 (*C*H$_2$CO), 32.7 (*C*H$_2$CH$_2$CH$_2$CO), 30.0 (CH_2), 26.9 (*C*H$_2$CH$_2$CO), 23.6 (CH_2), 14.4 (*C*H$_3$CH$_2$); **HRMS (ESI)**: calculated for $C_{18}H_{30}N_3O_2$ $[M+H]^+$: 319.2020, found: 319.2019; **$[\alpha]_D^{33}$**= +8.14 (c = 0.018, CH_3OH).

3 Feeding experiments

3.1 *General microbiology methods*

All glassware and media were prepared and sterilised by autoclaving (Astell) according to reported procedures.[2] Liquid cultures were grown with shaking in Innova 44 incubator/Shaker (New Brunswick scientific), solid cultures were grown in Hearaeus incubator (Thermo). *S. lasaliensis* ACP12(S970A) was grown and maintained as previously described.[3]

Table 1S: List and composition of media utilised. [3]

Media	**Ingredient**
M79 media	2.5 g glucose, 2.5 g peptone, 0.5 g yeast extract, 1.5 g NaCl, 2.5 g casamino acids, and 250 mL deionised water (final volume), adjusted to pH 7.0.
MYM Agar	0.8 g maltose, 0.8 g yeast extract, 2.0 g malt extract, 4.0 g agar, and 200 mL deionised water (final volume), adjusted to pH 7.2.
MYM Liquid	0.8 g maltose, 0.8 g yeast extract, 2.0 g malt extract, and 200 mL deionised water (final volume), adjusted to pH 7.2.

S. lasaliensis ACP12(S970A) strain (100 µL glycerol spore stock) was grown in M79 medium (10 mL) for 3 days, 250 rpm at 30 °C in 50 mL Erlenmeyer flasks with spring. Seed cultures (100 µL) were used to either inoculate MYM liquid cultures (5 mL, in duplicates, in 50 mL Erlenmeyer flasks with spring) or MYM agar plates (5 mL, in duplicates, in 10 mL petri dishes) with varying concentrations (0.04 – 4.0 mM) of probes (**3 – 17**) dissolved in methanol (100 µL). Liquid cultures were incubated at 30 °C, 250 rpm for 5 days. After the first day of fermentation, the probes were added portionwise (10 µmol dissolved in 10 µL of MeOH per day) to liquid cultures over days 2 - 5 to reach the final concentration, whereas solid cultures on MYM agar already containing the probes at variable concentrations were incubated at 30 °C for 5 days. Both control liquid and solid cultures in the absence of the probes were also prepared (in duplicates). After 5 days, all cultures were extracted with ethyl acetate (10 mL) or methanol (agar plates only, 10 mL). The extracts were filtered and concentrated, and the residues were redissolved in HPLC-grade methanol (500 µL) for mass spectrometry analysis.

3.2 *High-resolution LC-MS analyses of extracts*

HR-ESI-MS analyses of *S. lasaliensis* ACP12 (S970A) strains were performed on a MaXis Impact UHR-TOF (Bruker Daltonics) and on an Orbitrap Fusion with UltiMate 3000 RSLCnano System (Thermo Scientific). % of concentrations indicated in HPLC solvents/conditions are v/v.

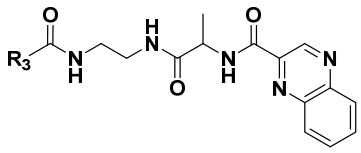
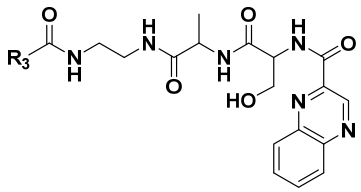
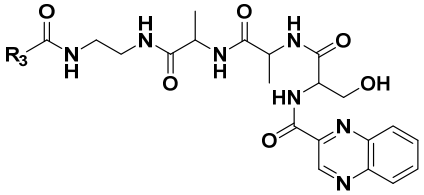
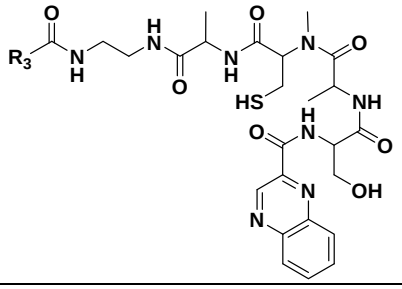
UPLC-HR-ESI-MS analyses of extracts on a MaXis Impact UHR-TOF (Bruker Daltonics): samples (5 µL) were injected onto an Acquity UPLC HSS T3 (150 mm x 1.0 mm, 1.8 µm) or Agilent Eclipse C18 (1.8um, 100 mm x 2.1 mm). The mobile phase consisted of a gradient of water and acetonitrile (HPLC grade, each with 0.1 % trifluoroacetic acid). The following solvent (A =0.1% TFA in H_2O, B =0.1% TFA in MeCN) gradient was applied: 10% B 0-2.7 min; 10-100 % B 2.7-42.7 min; 100 % B 42.7-52.7 min; 100-10 % B 52.7-55.7 min; 10 % B 55.7-67.7 min, using an Acquity UPLC HSS T3 column at a flow rate of 0.05 mL/min. Spectra were recorded in positive ionisation mode, scanning from *m/z* 100 to 3000, with the resolution set at 45K. Selected ion search within 5 ppm was performed.

Further high resolution analyses for extracts obtained from *N*-butyryl to *N*-decanoyl probes were performed on a *Thermo Orbitrap Fusion* (Q-OT-qIT, Thermo) instrument. Reversed phase chromatography was used to separate the mixtures prior to MS analysis. Two columns were utilised: an Acclaim PepMap µ-precolumn cartridge 300 µm i.d. x 5 mm 5 µm 100 Å and an Acclaim PepMap RSLC 75 µm x 15 cm 2 µm 100 Å (Thermo Scientific). The columns were installed on an Ultimate 3000 RSLCnano system (Dionex). Mobile phase buffer A was composed of 0.1 % aqueous formic acid and mobile phase B was composed of 100 % acetonitrile containing 0.1 % formic acid. Samples were loaded onto the µ-precolumn equilibrated in 2 % aqueous acetonitrile containing 0.1 % trifluoroacetic acid for 5 min at 10 µL min^{-1} after which compounds were eluted onto the analytical column following a gradient for which the mobile phase B concentration was increased from 3 % to 90 % over 15 min, then maintained at 90 % B for 5 min, then decreasing to 3 % B over 16 min, followed by a 9 min wash at 3 % B. Eluting cations were converted to gas-phase ions by electrospray ionization and analysed. Survey scans of precursors from 150 to 1500 *m/z* were performed at 60K resolution (at 200 *m/z*) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 0.7 Th with the quadrupole, HCD fragmentation with normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS^2 ion count target was set to 10^4 and the maximum injection time was 35 ms. A filter targeted inclusion mass list was used to select the precursor ions. The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 5 s cycles, meaning the instrument would continuously perform MS^2 events until the list of non-excluded precursors diminishes to zero or 5 s, whichever is shorter. Fusion runs were performed with Survey scans of

precursors from 150 to 1500 *m*/*z* 60K resolution (at 200 *m*/*z*) with a 1 × 10^6 ion count target. Tandem MS was performed by isolation at 1.8 Th with the ion-trap, CAD fragmentation with normalized collision energy of 32, and 15 K resolution scan MS analysis in the Orbitrap. MS^2 ion count target was set to 4 × 10^6 and the max injection time was 50 ms. The dynamic exclusion duration was set to 40 s with a 10 ppm tolerance around the selected precursor and its isotopes.

3.3 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A)* via *L-alanine-based chain termination probes 3-6*

Table 2S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) *via* alanine-based probes **3-6** (analysed on a MaXis Impact instrument[a] and on an Orbitrap Fusion instrument[b]).

Putative intermediate structures	**Probe 3**[a] $R_3 = CH_3$	**Probe 4** [a],[b] $R_3 = (CH_2)_2CH_3$	**Probe 5** [b] $R_3 = (CH_2)_5CH_3$	**Probe 6**[b] $R_3 = (CH_2)_8CH_3$
	n.d.	✓ Modest abundance	✓ Low abundance	n.d.
	n.d.	n.d.	✓ Low abundance	n.d.
	n.d.	n.d.	✓ Low abundance	n.d.
	n.d.	n.d.	n.d.	n.d.

3.3.1 Putative echinomycin intermediate capture *via* (*S*)-*N*-(2-(2-aminopropanamido)ethyl) butyramide probe (4)

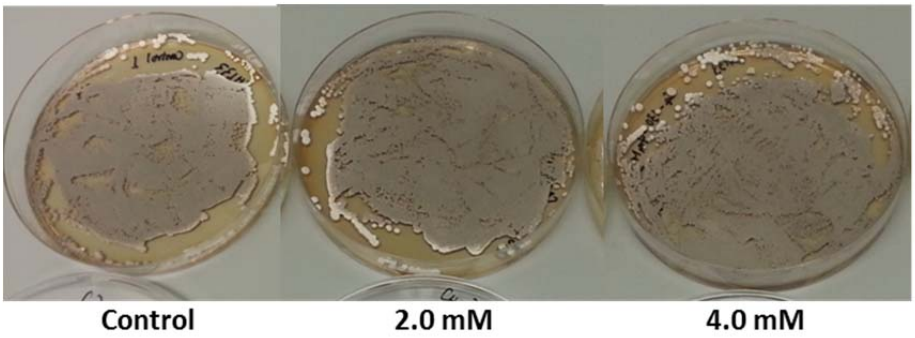


Figure 1S: Growth of *S. lasaliensis* ACP12(S970A) in the absence (control) and in the presence of 2.0 mM and 4.0 mM of probe **4** on MYM agar plates.

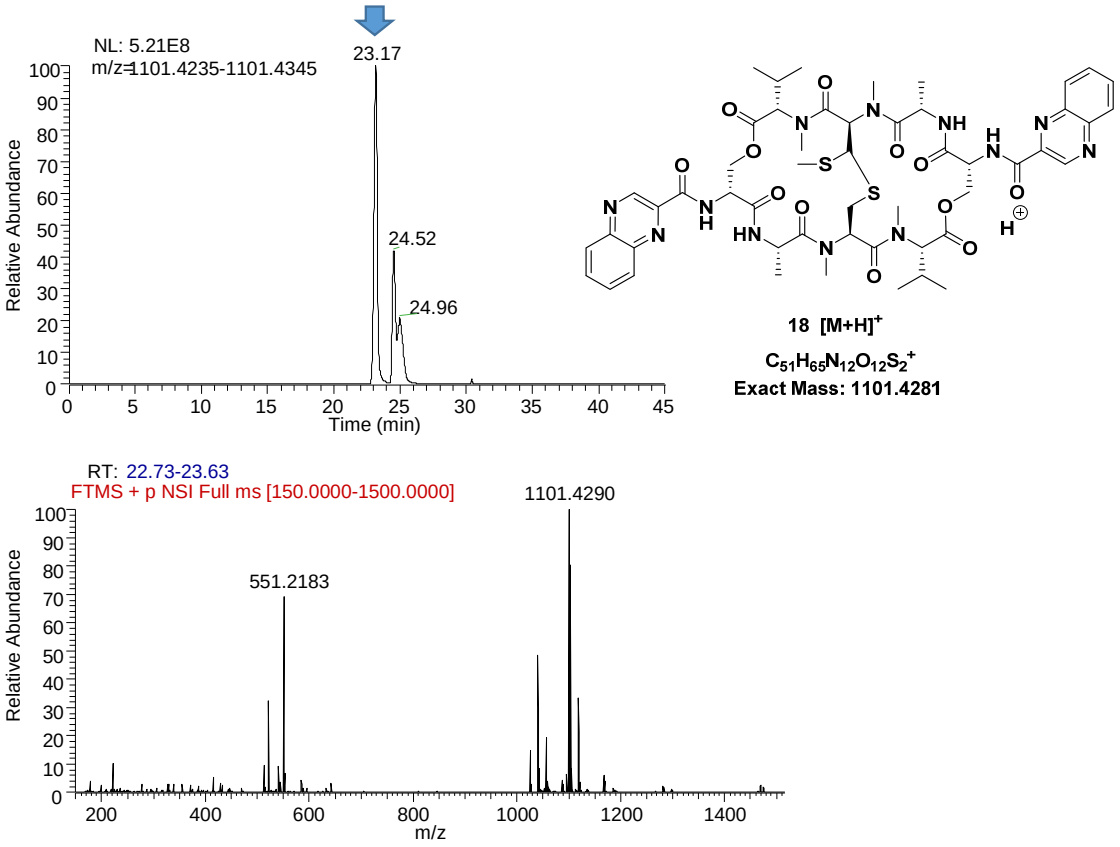


Figure 2S: Echinomycin (**18**) production in *S. lasaliensis* ACP12(S970A) grown on MYM agar.

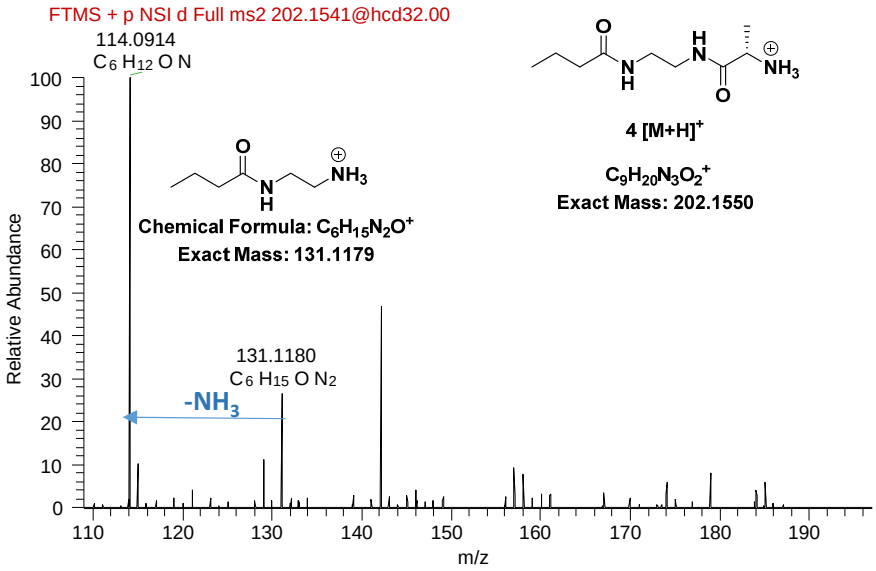


Figure 3S: HR-MS^2 characterisation of probe **4** (Orbitrap Fusion) from the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **4** (4.0 mM on MYM agar).

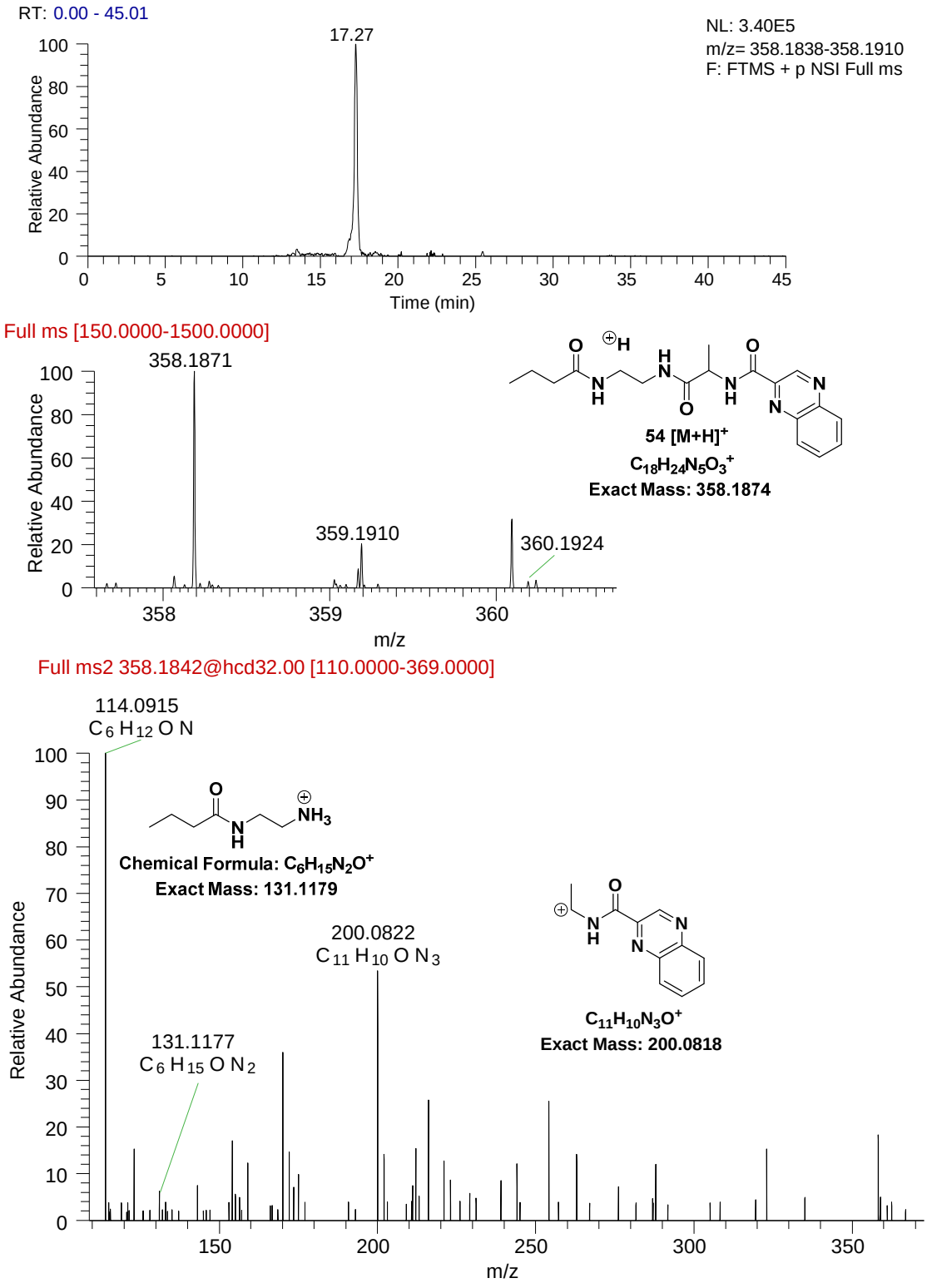


Figure 4S: Detection and characterisation of putative dipeptide intermediate **54**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **4** (MYM agar, 4.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **54** (bottom). This species was not found in control samples (data not shown).

3.3.2 Putative echinomycin intermediate capture *via* (*S*)-*N*-(2-(2-aminopropanamido)ethyl) heptanamide probe (5)

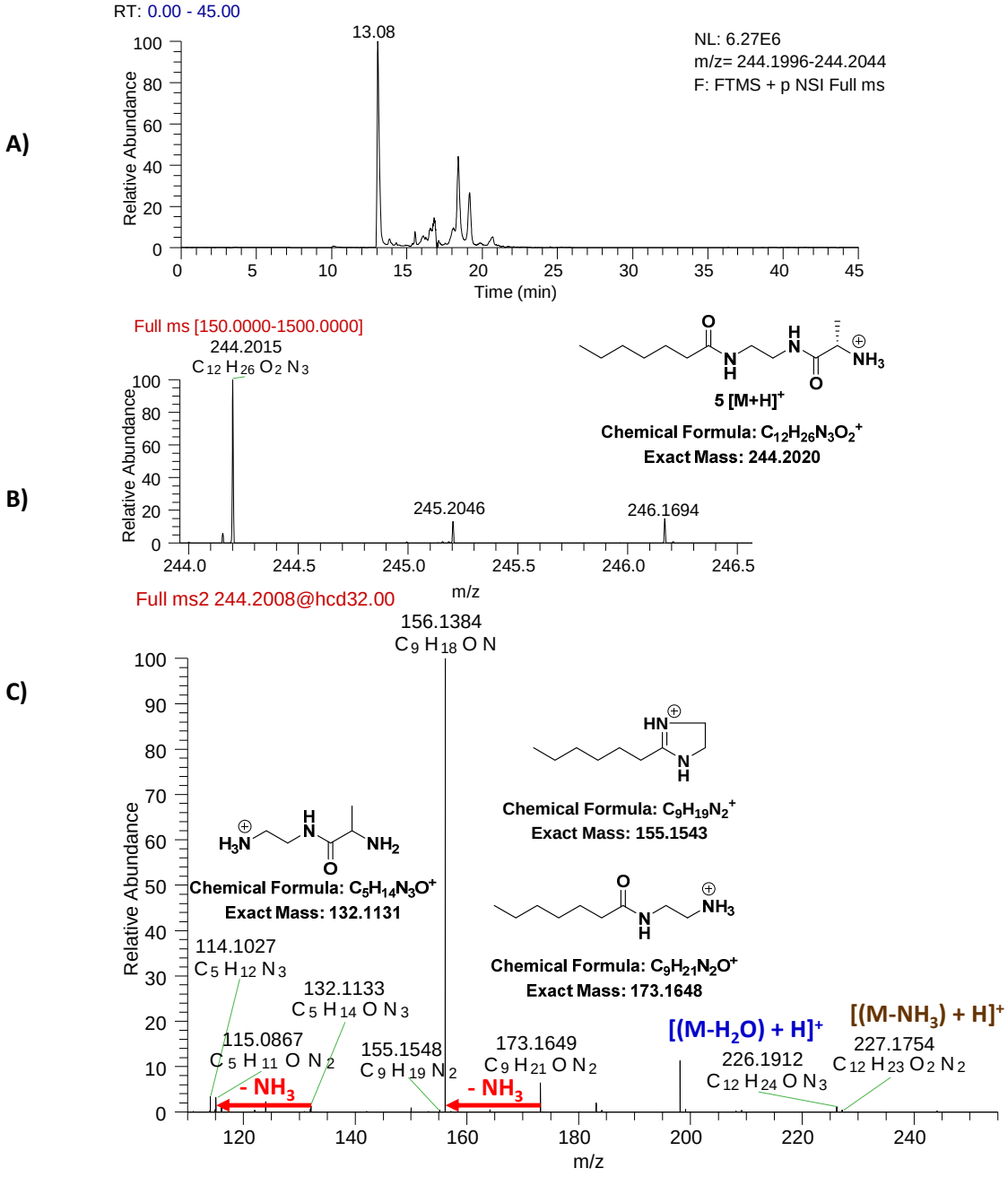


Figure 5S: Detection and characterisation of probe **5**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **5** (2.0 mM on MYM agar): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **5**.

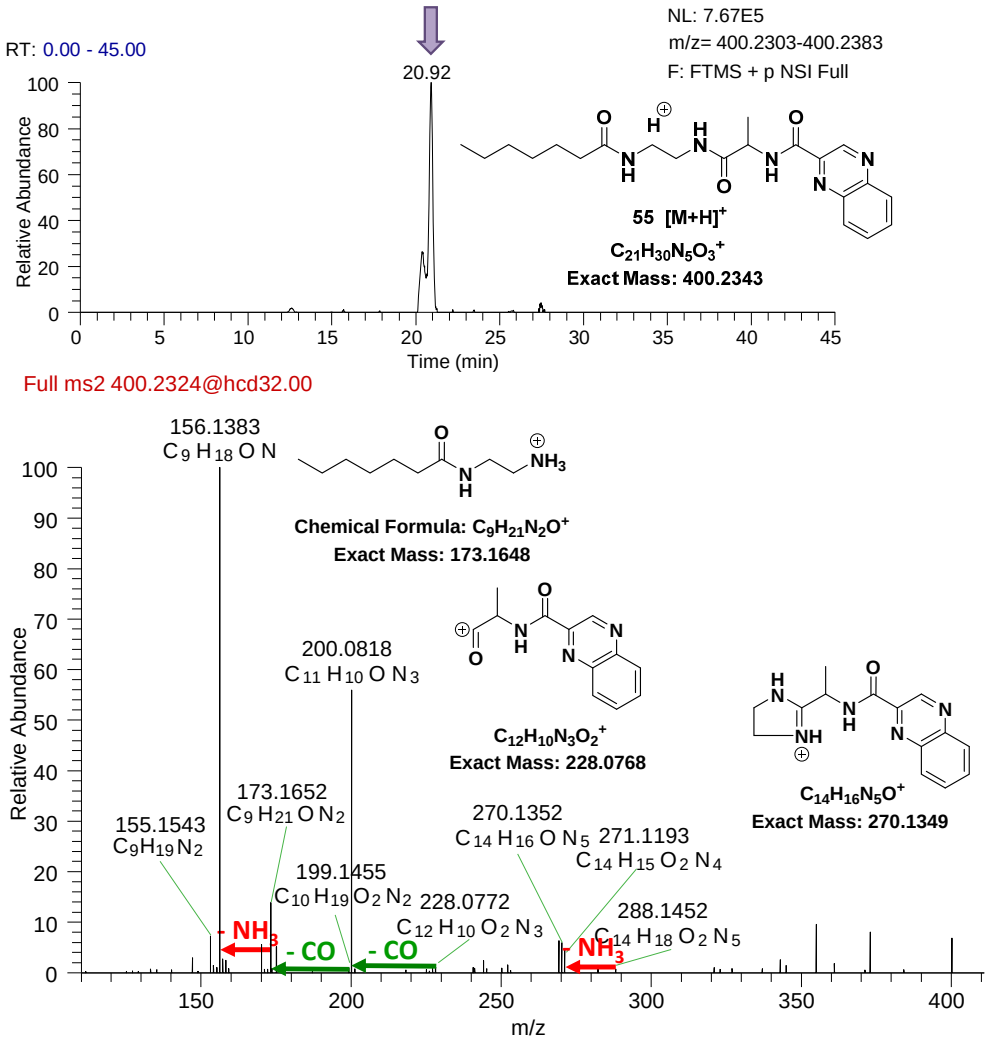


Figure 6S: Detection and characterisation of putative dipeptide intermediate **55**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **5** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **55** (bottom). This species was not found in control samples (data not shown).

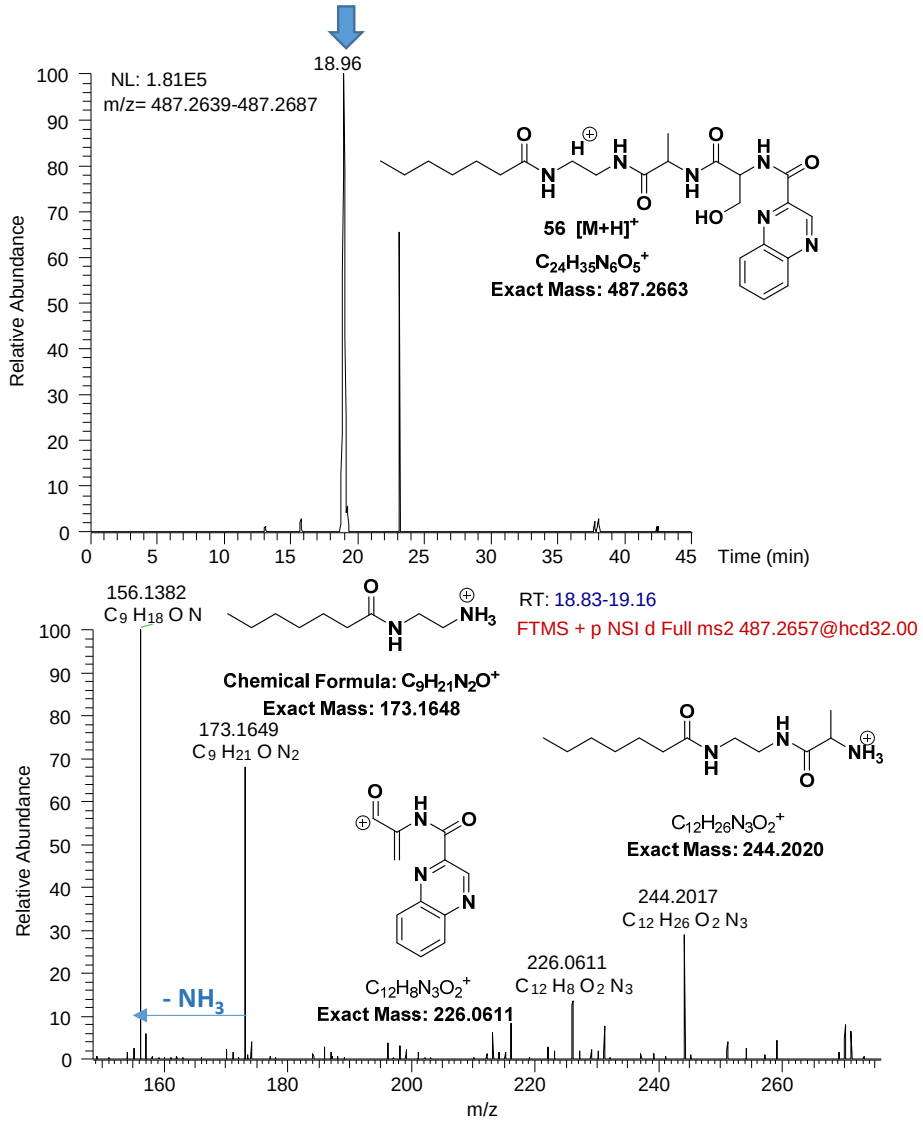


Figure 7S: Detection and characterisation of putative tripeptide intermediate **56**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **5 (**MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **56** (bottom). This species was not found in control samples (data not shown).

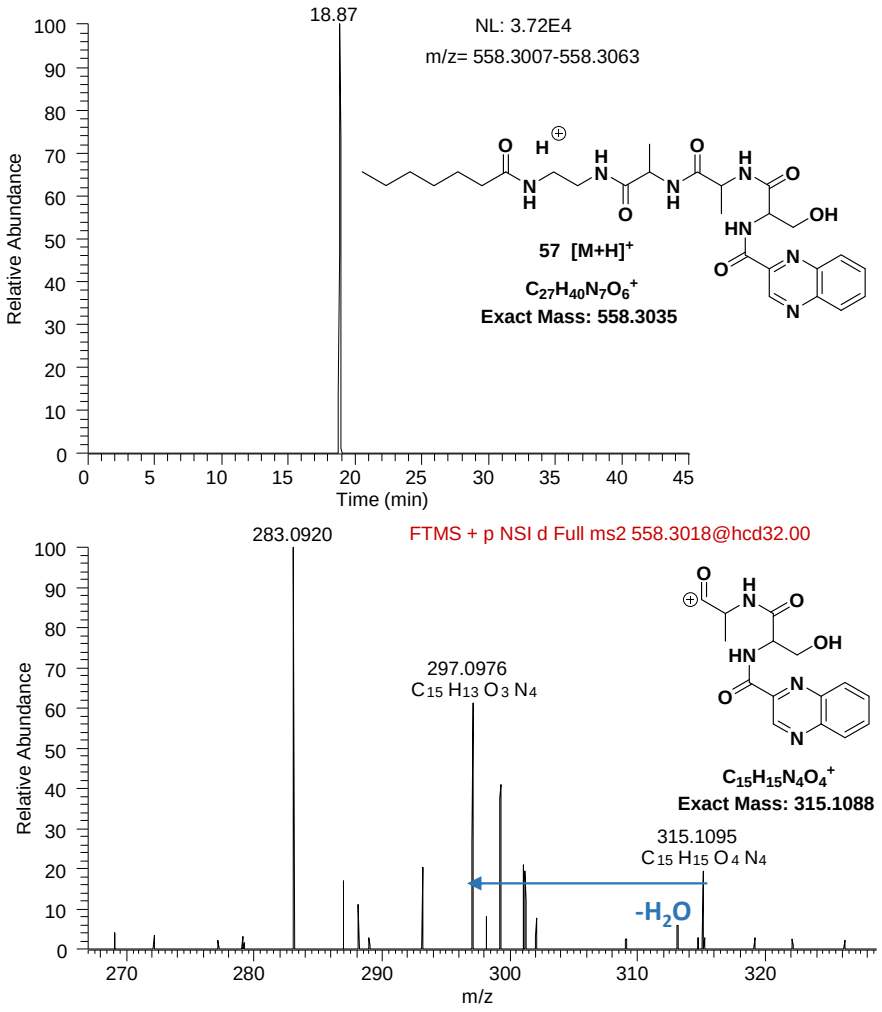


Figure 8S: Detection and characterisation of putative tetrapetide intermediate **57**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **5** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **57** (bottom, expansion). Further fragments related to **57** (m/z= 244, 226, 173 and 156, as shown in Figure 7S) were found (here not shown). **57** was not found in control samples (data not shown).

3.3.3 Putative echinomycin intermediate capture *via* (*S*)-*N*-(2-(2-aminopropanamido)ethyl) decanamide probe (6)

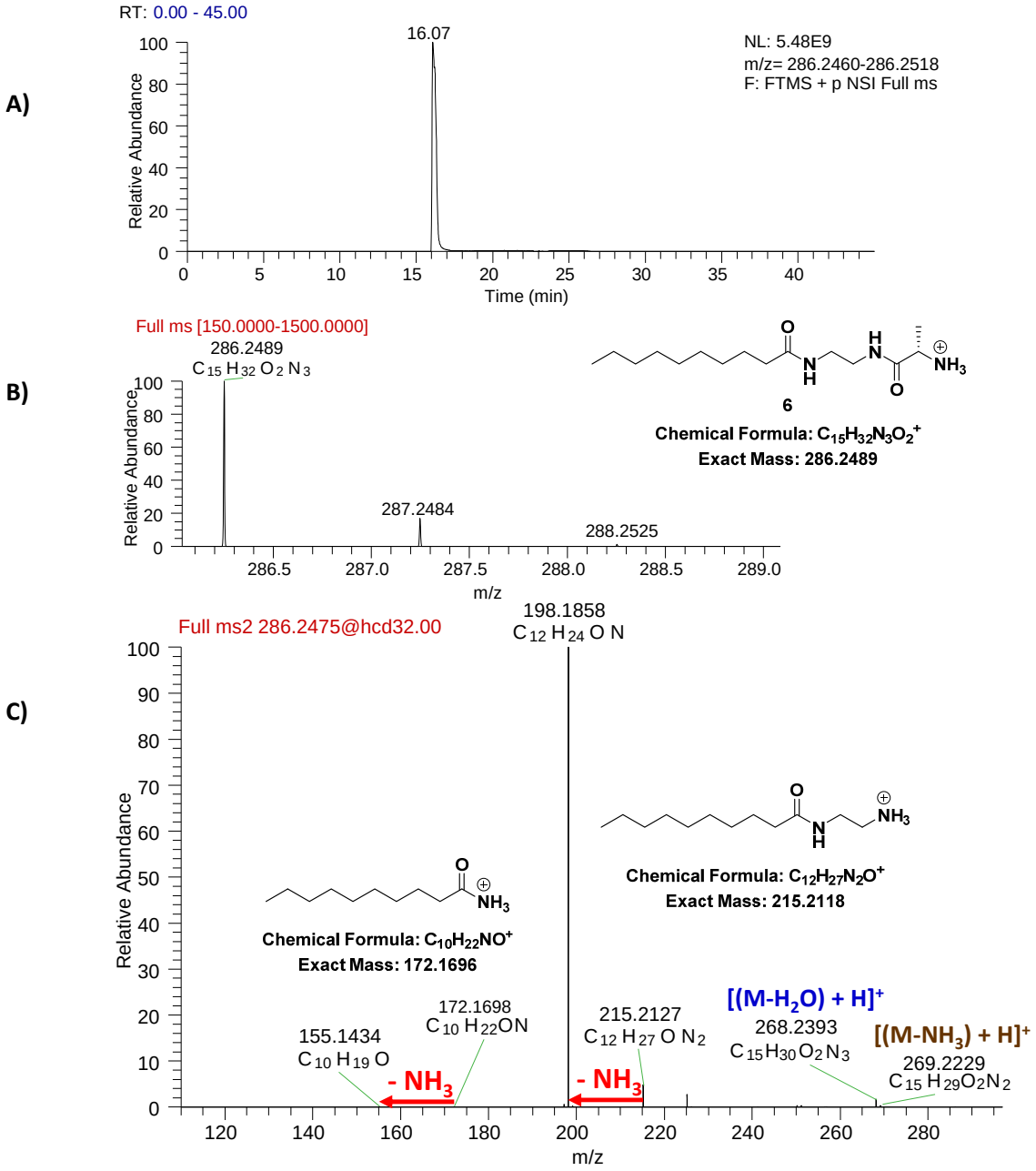
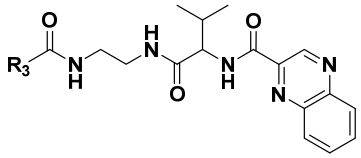
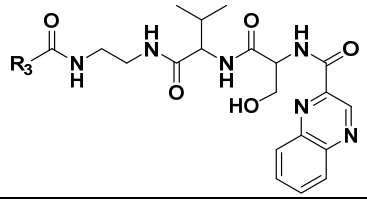
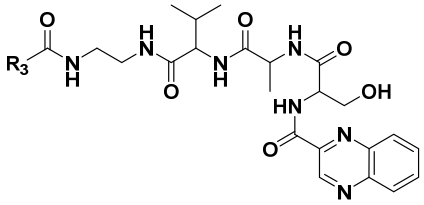
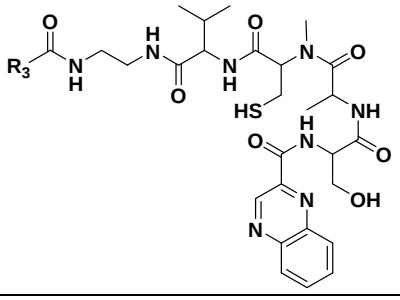


Figure 9S: Detection and characterisation of probe **6**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **6** on MYM agar (2.0 mM): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **6**. Probe **6** proved cytotoxic at concentrations above 1.0 mM.

No peptide intermediates were found/characterised from experiments with **6**.

3.4 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A)* via *L-valine-based chain termination probes 7-8*

Table 3S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) *via* valine-based probes **7-8** (analysed on a MaXis Impact instrument[a] and on an Orbitrap Fusion instrument[b]).

Putative intermediate structures	**Probe 7** [a],[b] $R_3 = (CH_2)_2CH_3$	**Probe 8** [b] $R_3 = (CH_2)_5CH_3$
	✓ modest abundance	✓ modest abundance
	✓ traces	✓ low abundance
	n.d.	✓ low abundance
	n.d.	n.d.

3.4.1 Putative echinomycin intermediate capture *via* (*S*)-2-amino-*N*-(2-butyramidoethyl)-3-methylbutanamide probe (11)

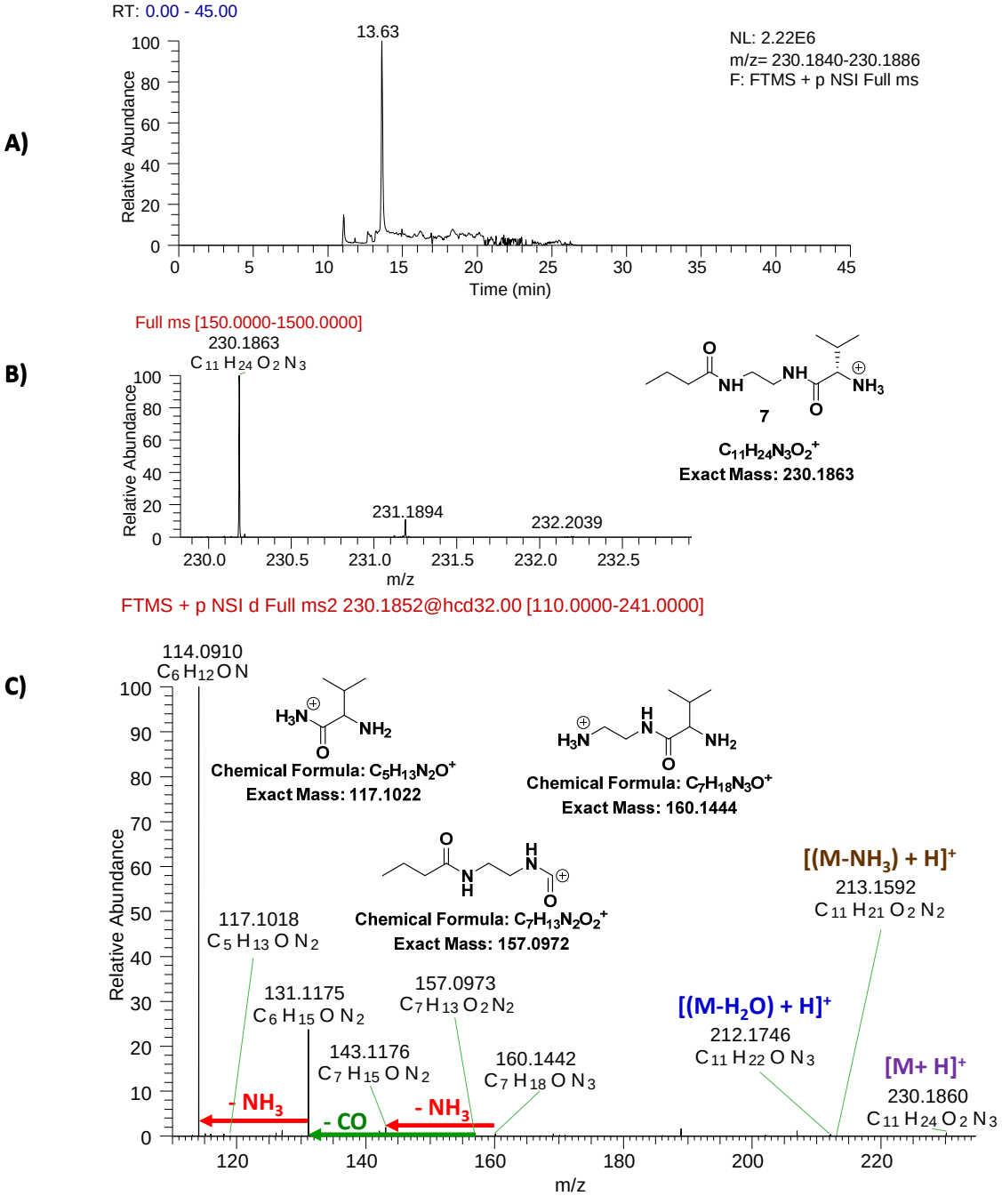


Figure 10S: Detection and characterisation of probe **7**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **7** (2.0 mM on MYM agar): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **7**.

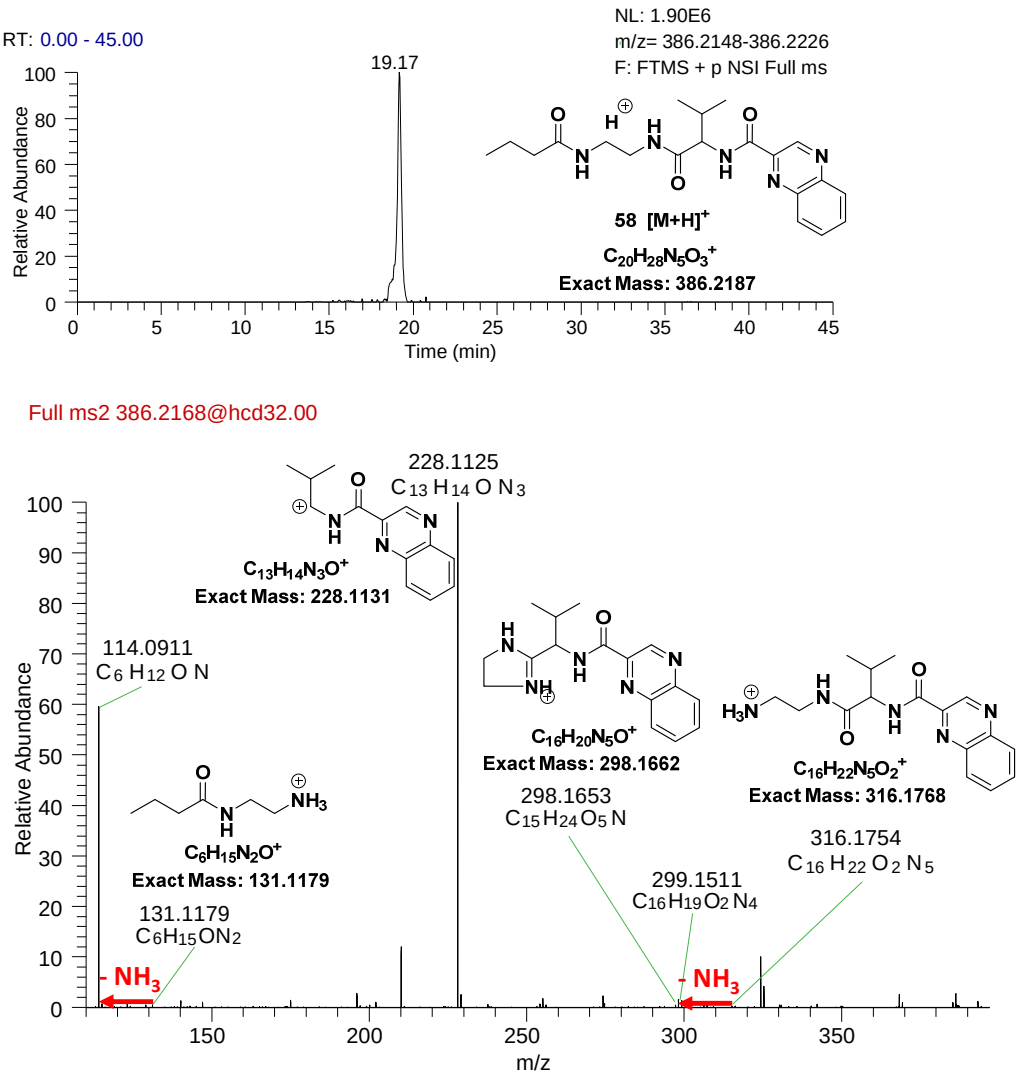
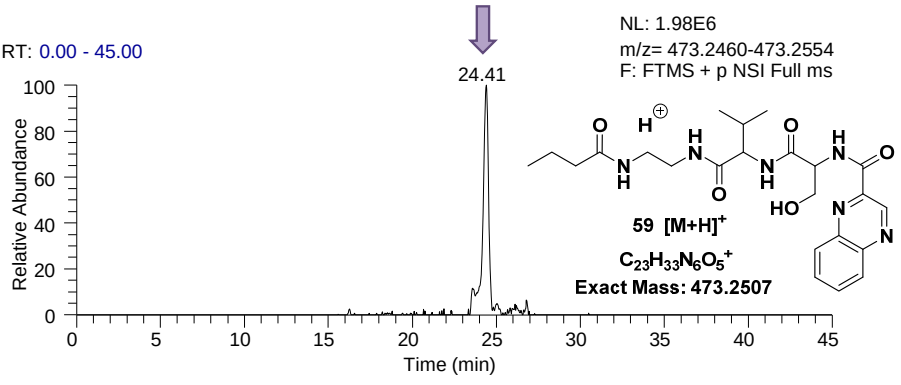


Figure 11S: Detection and characterisation of putative dipeptide intermediate **58**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **7** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **58** (bottom). This species was not found in control samples (data not shown). A putative tripeptide of *m/z* 473 was also observed in the same sample (see **59** below) however the very low abundance abundance of MS^2 fragments for this species (not shown) did not allow his unequivocal identification.



3.4.1 Putative echinomycin intermediate capture *via* (*S*)-*N*-(2-(2-amino-3-methylbutanamido) ethyl)heptanamide probe (8)

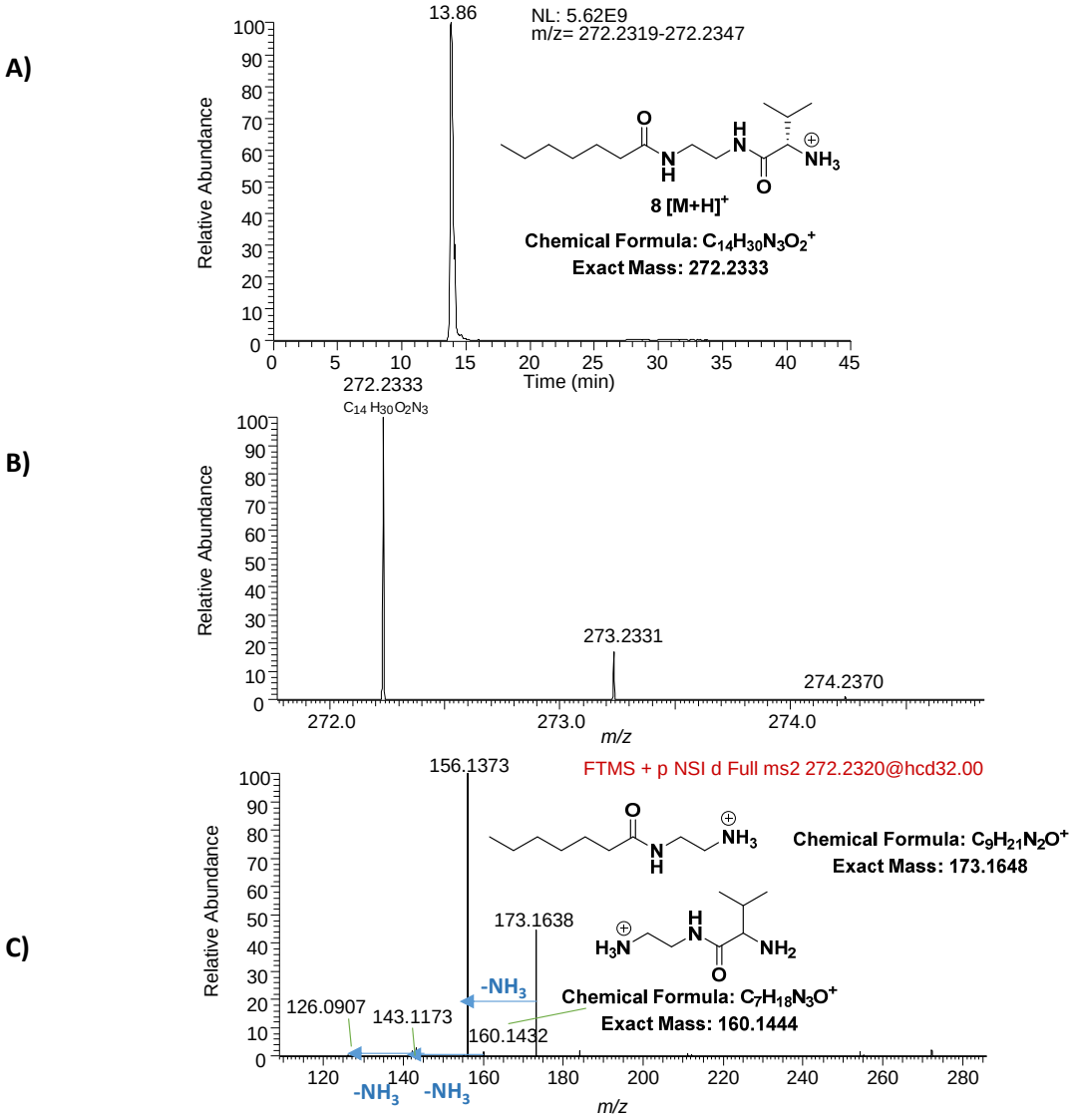


Figure 12S: Detection and characterisation of probe **8**. LC-HRMS analysis (Orbitrap Fusion) of the organic extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **8** (2.0 mM on MYM agar): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **8**.

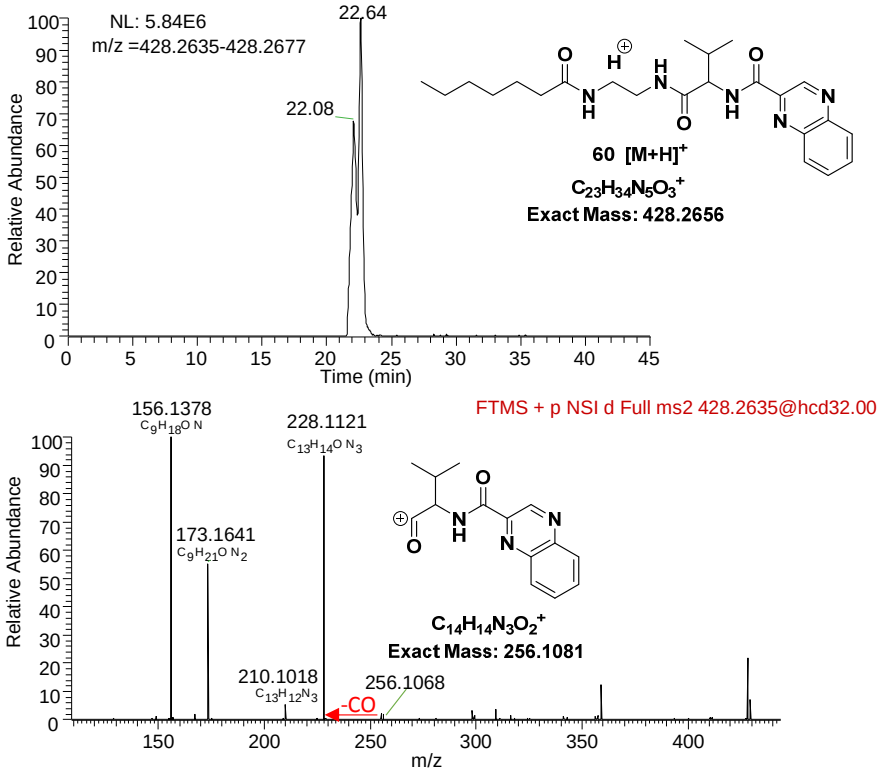


Figure 13S: Detection and characterisation of putative dipeptide intermediate **60**. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **8** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **60** (bottom). This species was not found in control samples (data not shown).

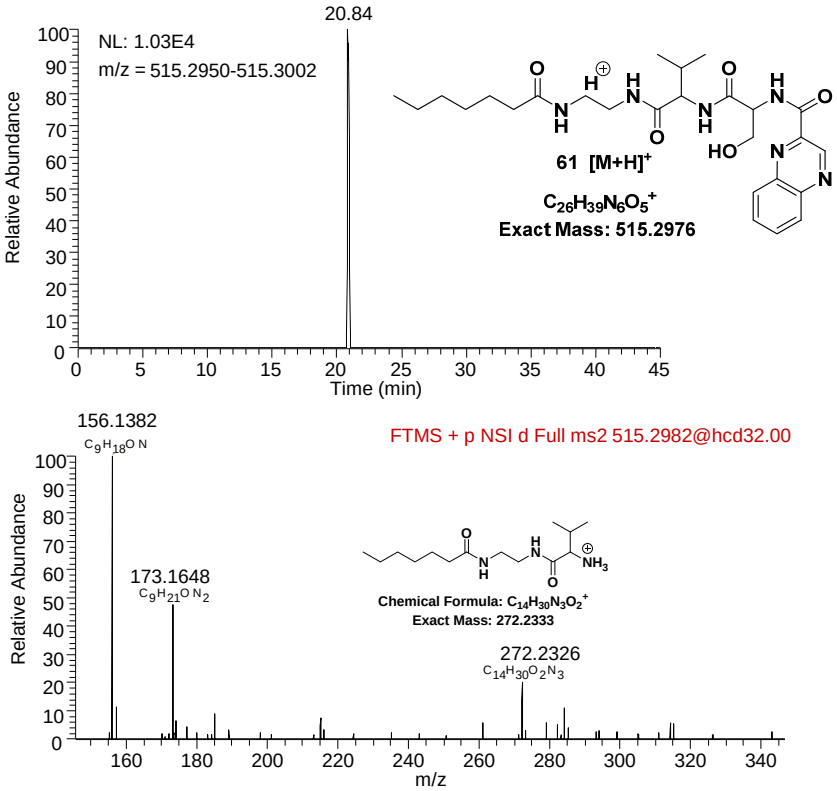


Figure 14S: Detection and characterisation of putative tripeptide intermediate **61**. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **8** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **61** (bottom). This species was not found in control samples (data not shown).

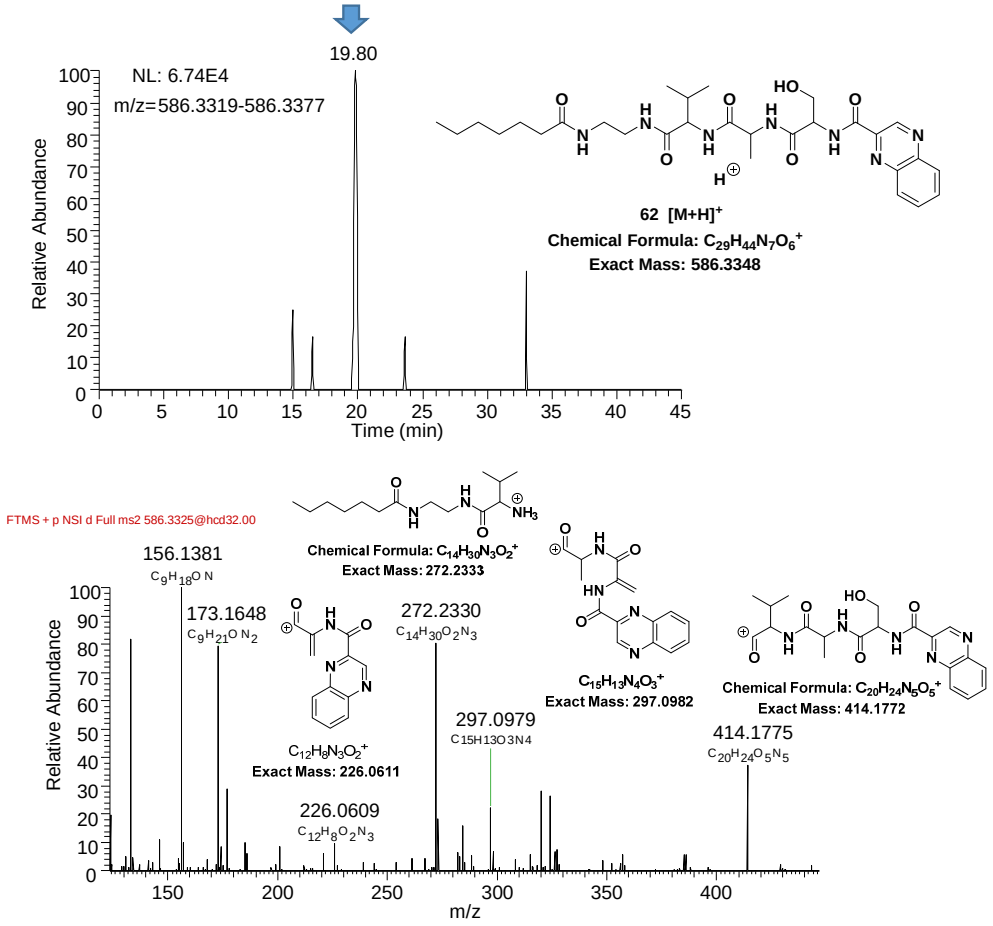
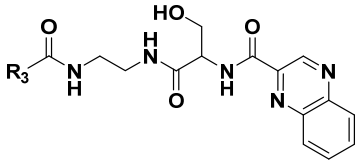
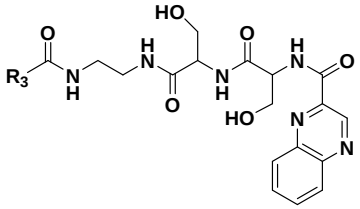
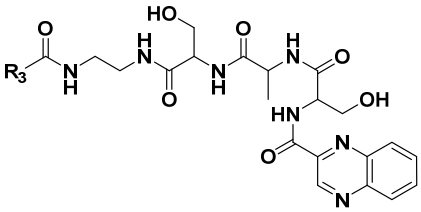
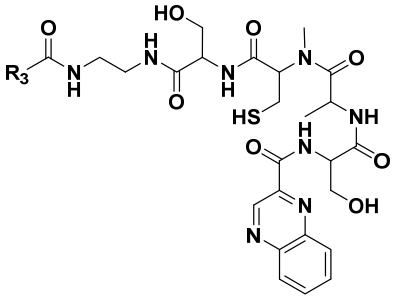


Figure 15S: Detection and characterisation of putative tetrapeptide intermediate **62**. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **8** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **62** (bottom). This species was not found in control samples (data not shown).

3.5 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A)* via *L-serine-based chain termination probe 9*

Table 4S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) *via* L-Ser-based probe **9** (analysed on an Orbitrap Fusion instrument).

Putative intermediate structures	**Probe 9** $R_3 = (CH_2)_5CH_3$
	✓ moderate abundance
	✓ low abundance
	n.d.
	n.d.

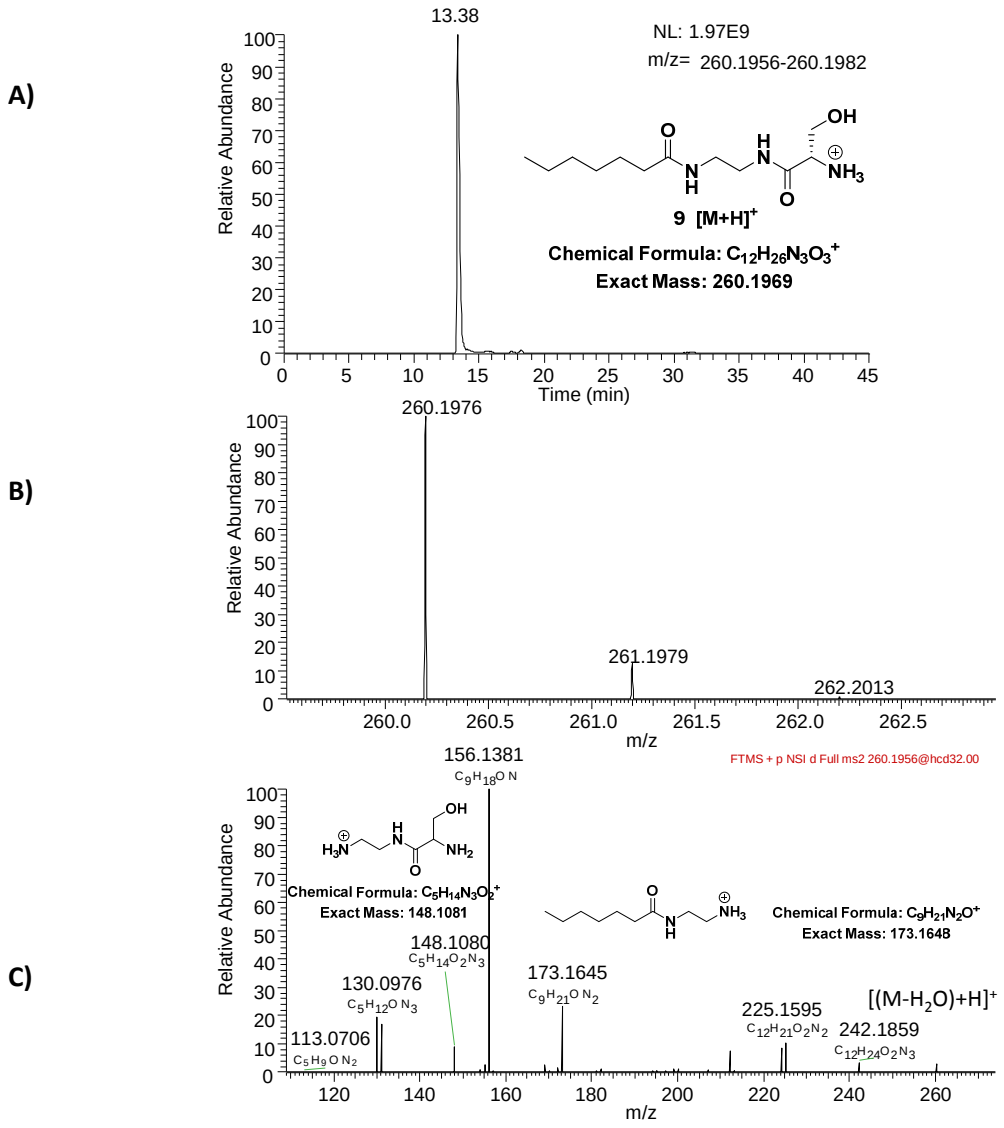


Figure 16S: Detection and characterisation of probe **9**. LC-HRMS analysis (Orbitrap Fusion) of the organic extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **9** (2.0 mM on MYM agar): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **9**.

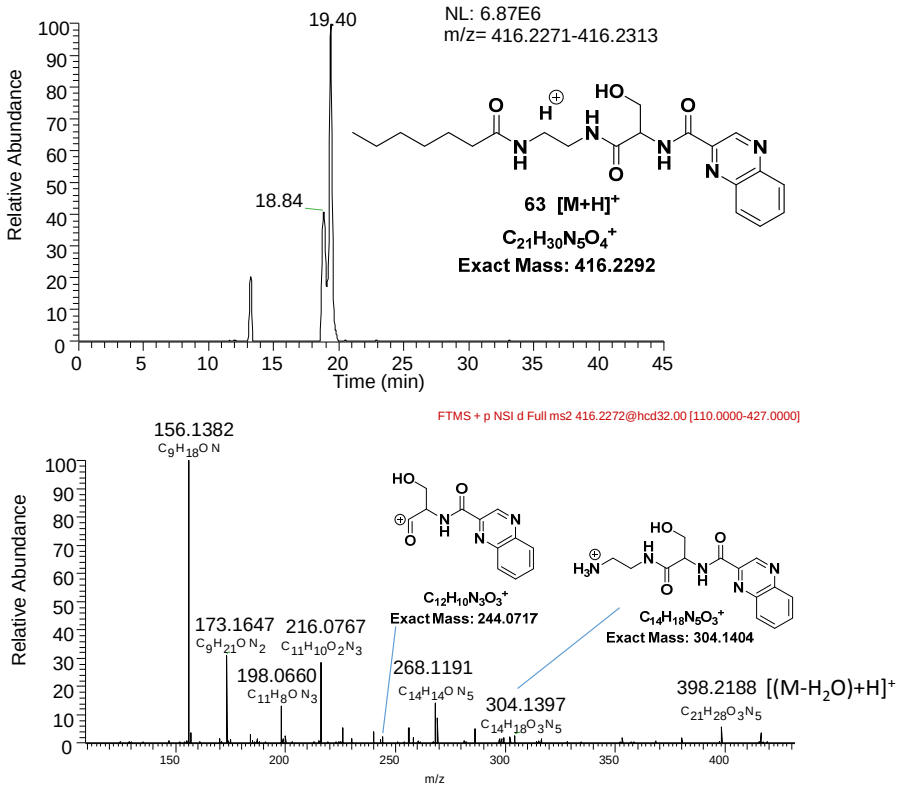


Figure 17S: Detection and characterisation of putative dipeptide intermediate **63**. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **9** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **63** (bottom). This species was not found in control samples (data not shown).

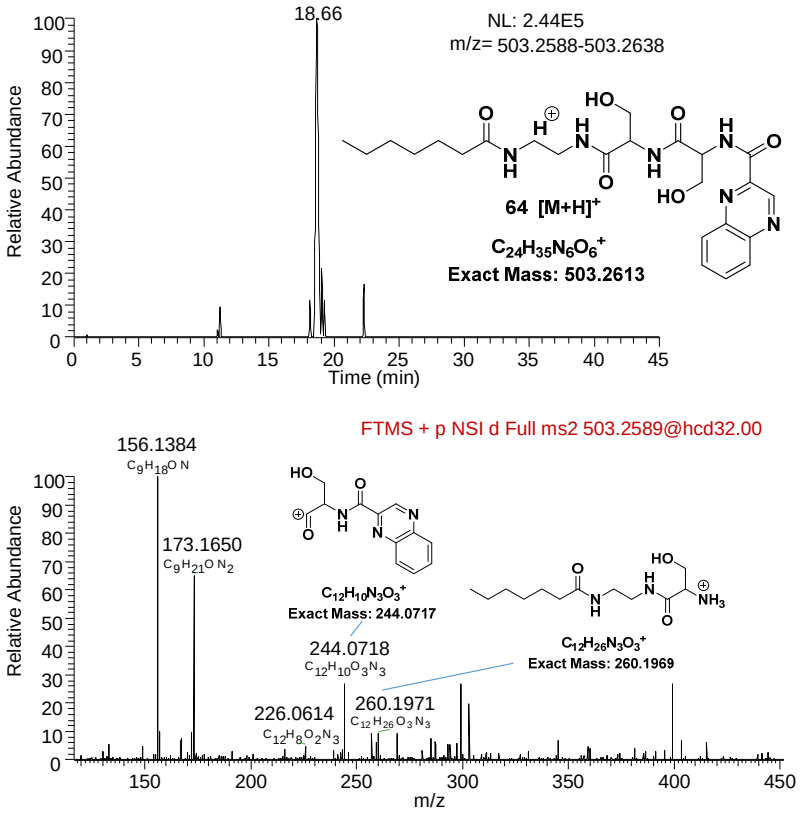
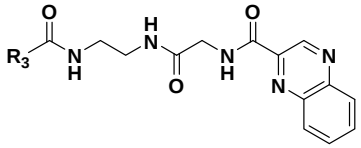
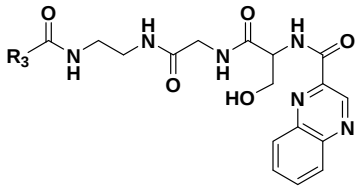
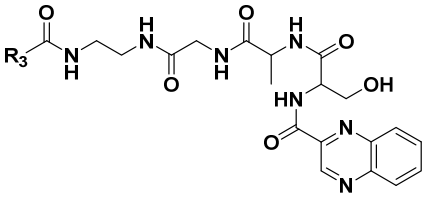
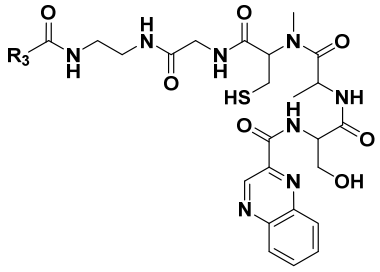


Figure 18S: Detection and characterisation of putative tripeptide intermediate **64**. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **9** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **64** (bottom). This species was not found in control samples (data not shown).

3.6 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A)* via *glycine-based chain termination probes 10-12*

Table 5S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) *via* glycine-based probes **10-12** (analysed on an Orbitrap Fusion instrument).

Putative intermediate structures	**Probe 10** $R_3 = (CH_2)_2CH_3$	**Probe 11** $R_3 = (CH_2)_5CH_3$	**Probe 12** $R_3 = (CH_2)_8CH_3$
	✓ High abundance	✓ High abundance	✓ Low abundance
	✓ Low abundance	✓ Low abundance	n.d.
	✓ Low abundance	✓ Low abundance	n.d.
	✓ Low abundance	n.d.	n.d.

3.6.1 Putative echinomycin intermediate capture *via* 2-(2-aminoacetamido)ethyl) butyramide probe (10)

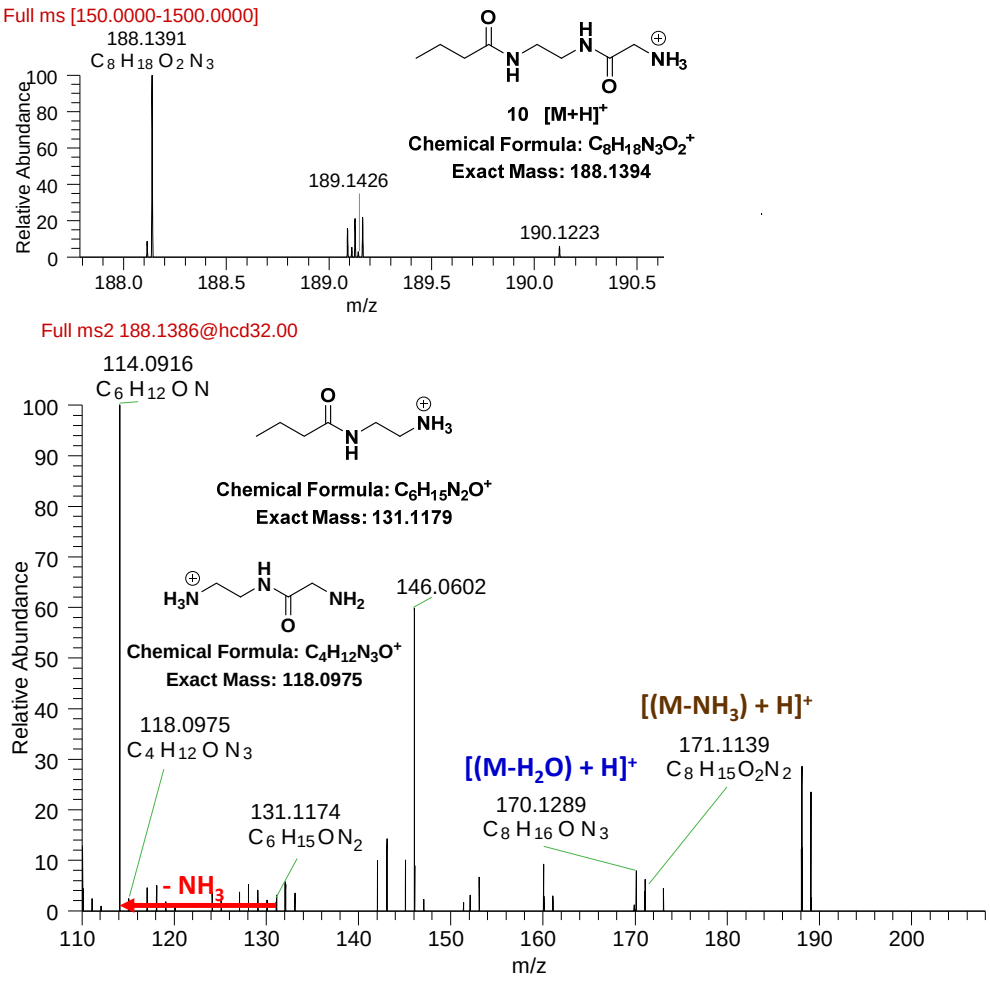


Figure 19S: LC-HRMS characterisation of probe **10**: accurate mass isotopic distribution ($[M+H]^+$, top) and MS^2 fragmentation (bottom).

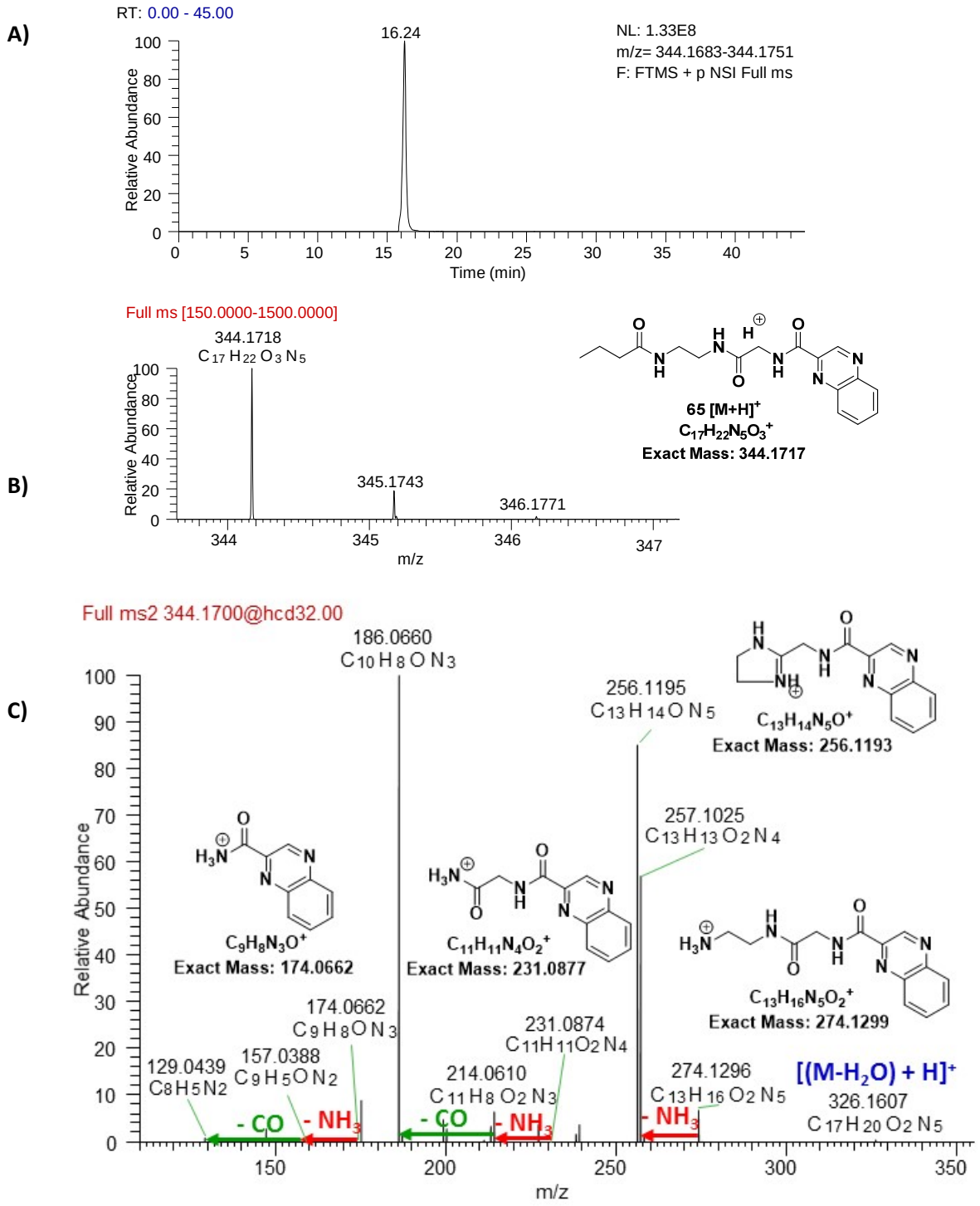


Figure 20S: Detection and characterisation of a putative dipeptide intermediate (**65**) from echinomycin biosynthesis. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **10** (liquid culture, final concentration 4.0 mM): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **65**. This species was not found in control samples (data not shown).

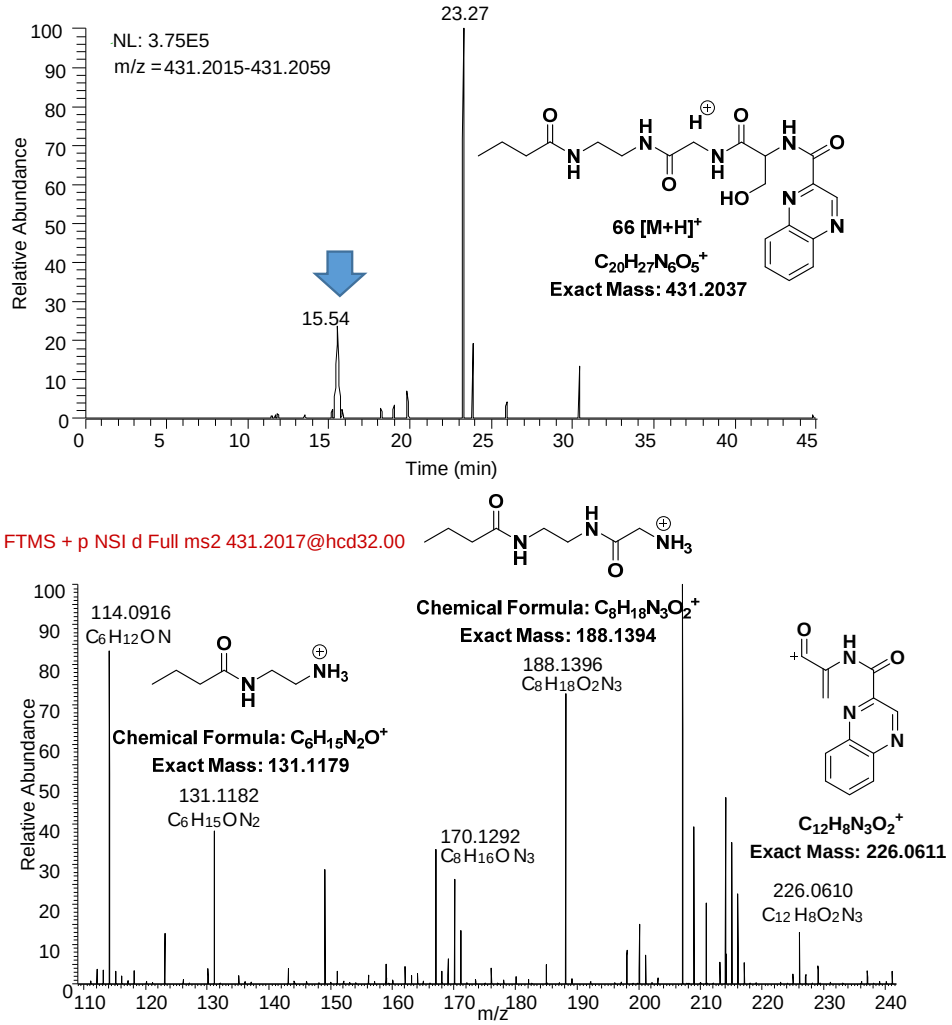


Figure 21S: Detection and characterisation of putative tripeptide intermediate **66**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **10** (agar plate, 2.0 mM concentration). Top: extracted ion chromatogram ($[M+H]^+$) and MS^2 fragmentation for **66** (bottom). This species was not found in control samples (data not shown).

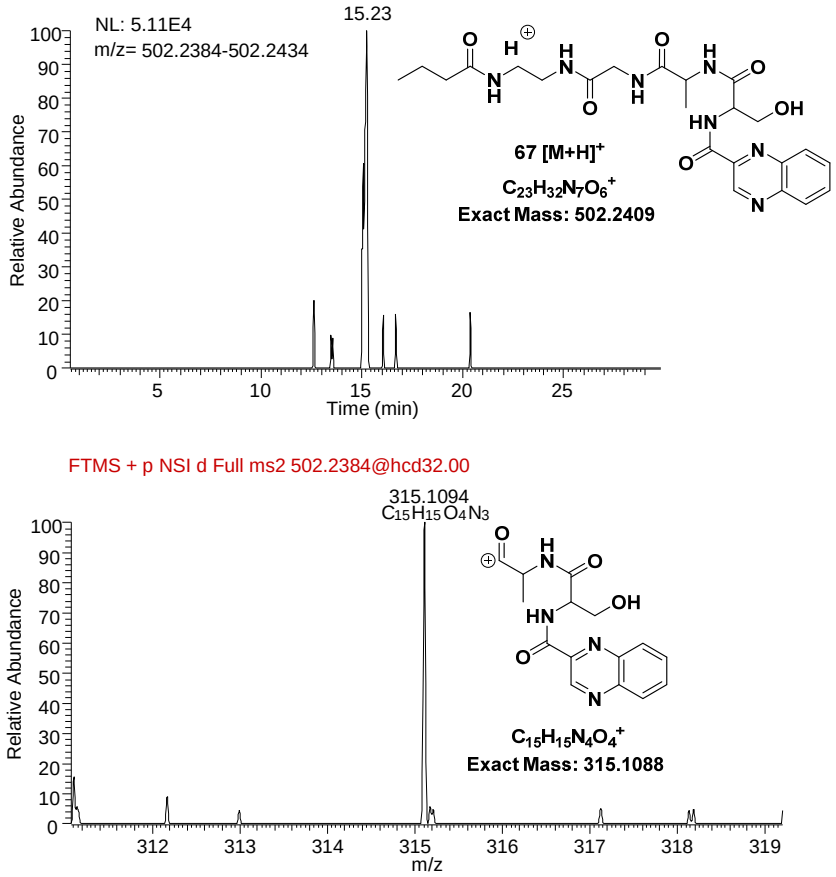


Figure 22S: Detection and characterisation of putative tetrapeptide intermediate **67**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **10** (agar plate, 2.0 mM concentration). Top: extracted ion chromatogram ($[M+H]^+$) and MS^2 fragmentation for **67** (bottom, expansion). Further fragments related to **67** (*m/z*= 226, 188, 170, 131 and 114, as shown in Figure 21S) were found (here not shown). **67** could not be found in control samples (data not shown).

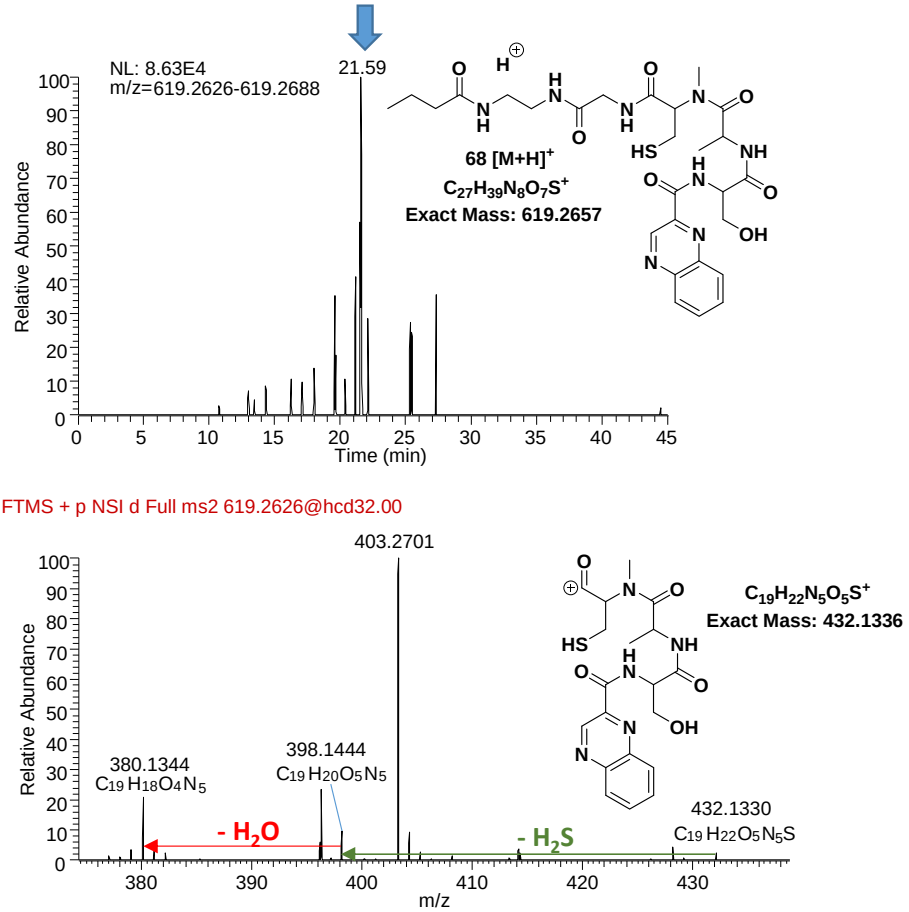


Figure 23S: Detection and characterisation of putative pentapeptide intermediate **68**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **10** (agar plate, 2.0 mM concentration). Top: extracted ion chromatogram ($[M+H]^+$) and MS^2 fragmentation for **68** (bottom, expansion). Further fragments related to **68** (m/z= 226, 188, 170, 131 and 114, as shown in Figure 21S) were found (here not shown). **68** could not be found in control samples (data not shown).

3.6.2 Putative echinomycin intermediate capture *via* *N*-(2-(2-aminoacetamido)ethyl) heptanamide probe (11)

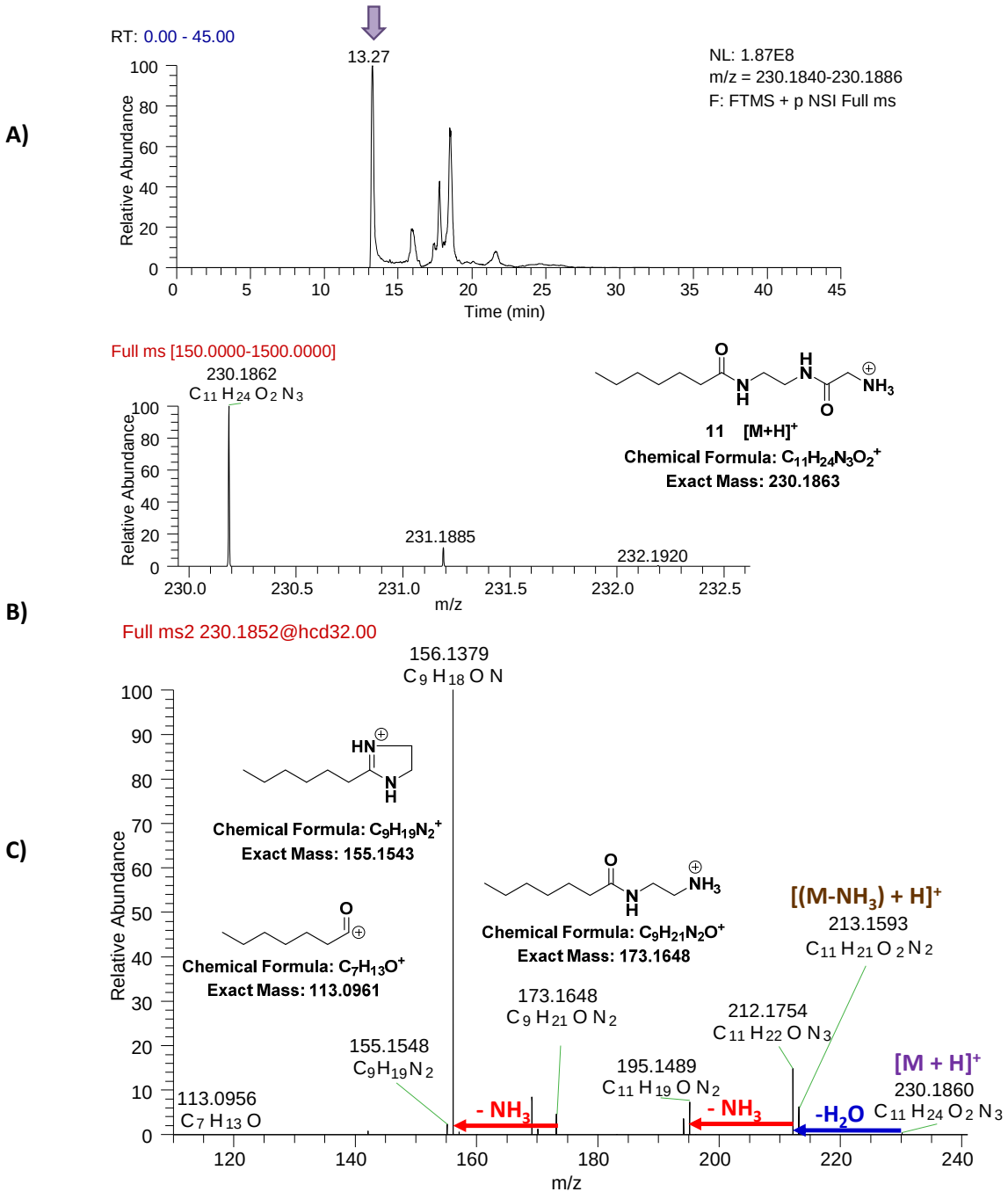


Figure 24S: Detection and characterisation of probe **11**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **11** (liquid culture, final concentration 4.0 mM): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **11**.

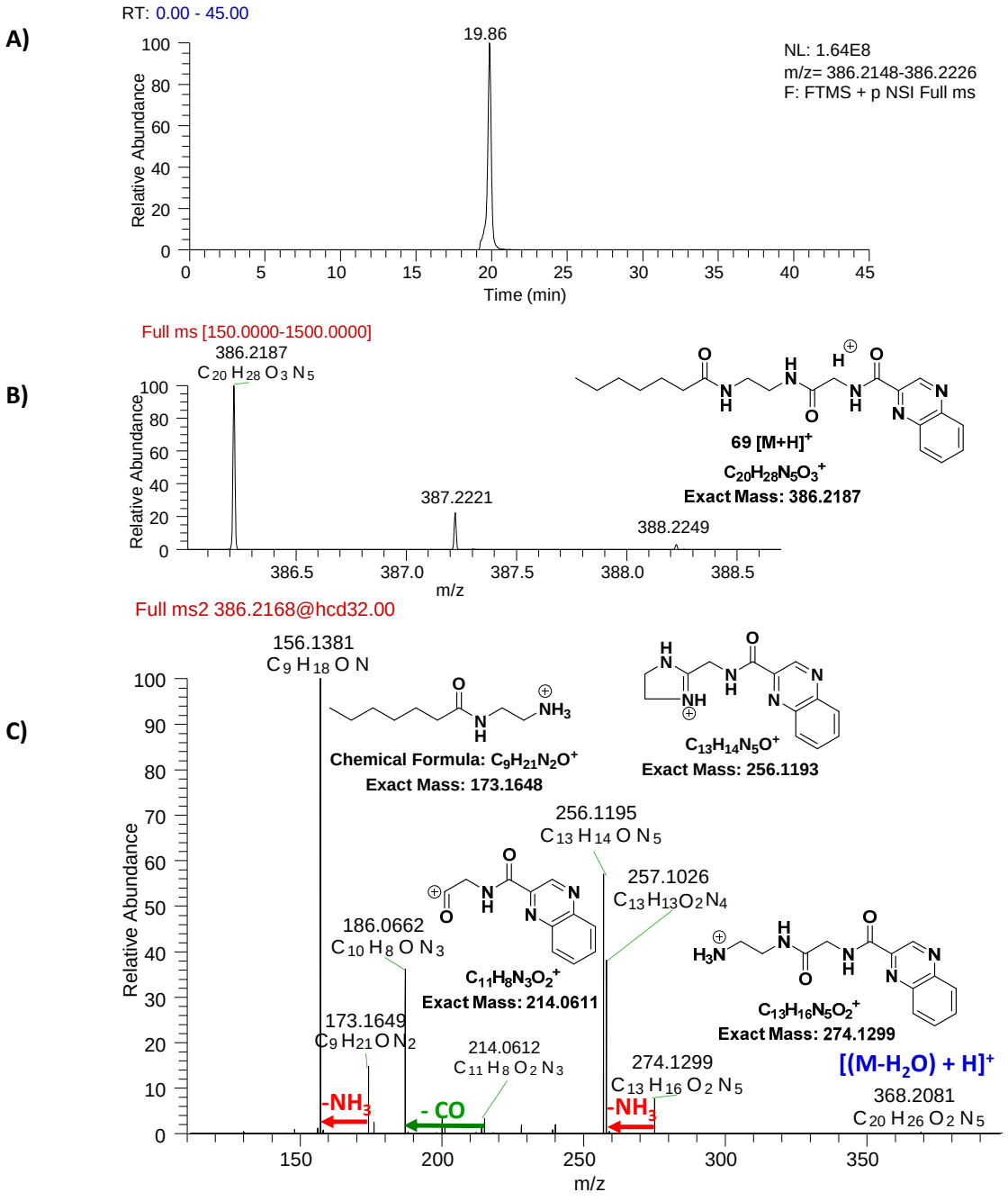


Figure 25S: Detection and characterisation of putative dipeptide intermediate **69**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **11** (liquid culture, final concentration 4.0 mM): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **69**. This species was not found in control samples (data not shown).

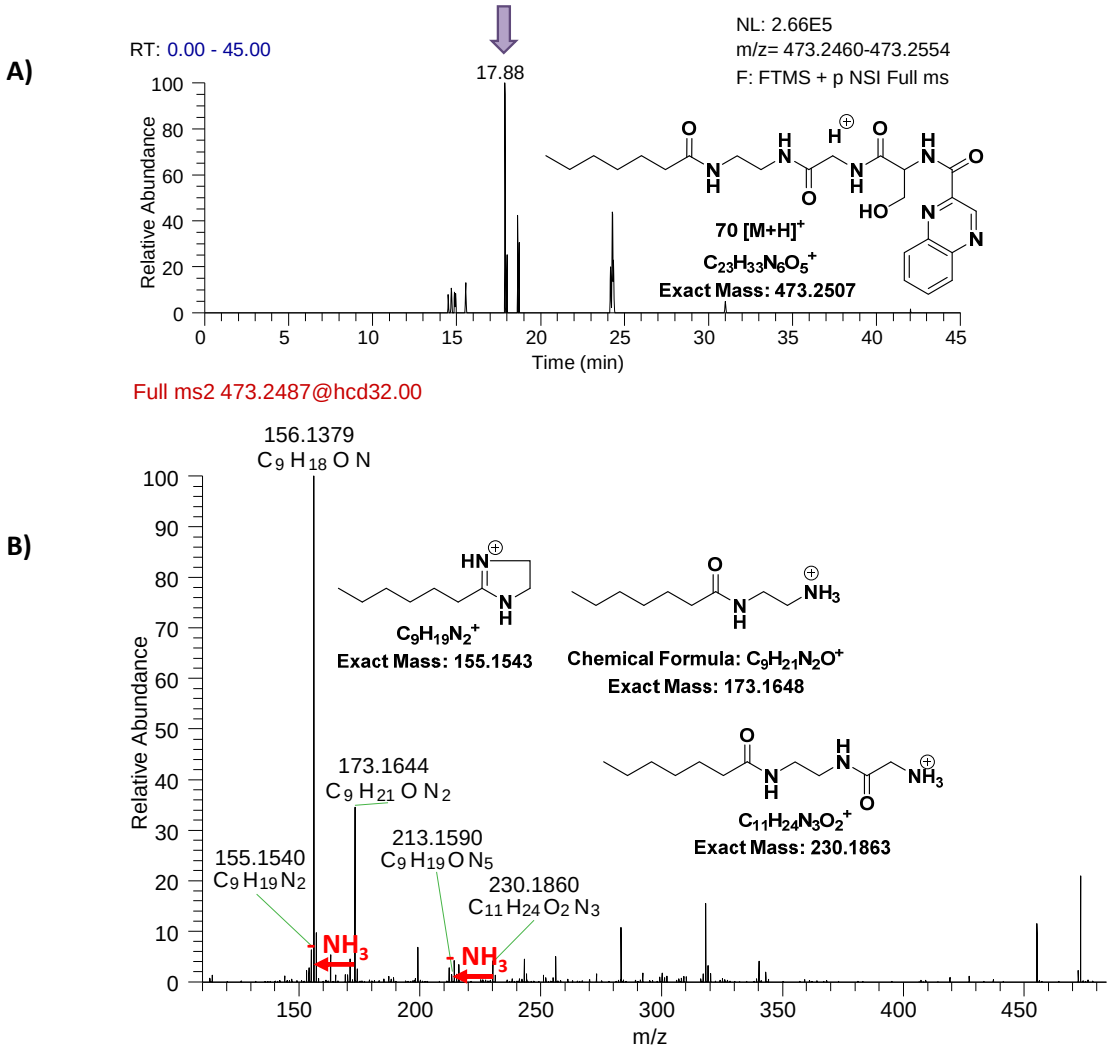


Figure 26S: Detection and characterisation of putative tripeptide intermediate **70**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **11** (liquid culture, final concentration 4.0 mM): (A) extracted ion chromatogram ($[M+H]^+$) and (B) MS^2 fragmentation for **70**. This species was not found in control samples (data not shown).

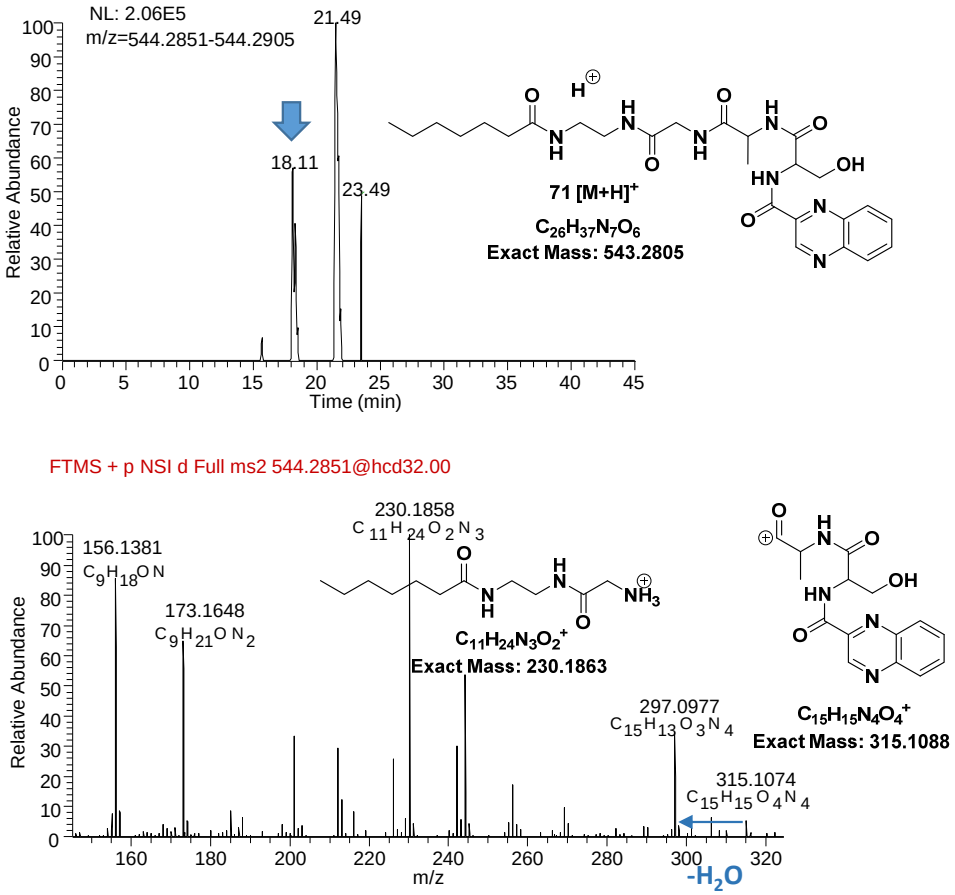


Figure 27S: Detection and characterisation of the putative tetrapeptide **71**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **11** (on MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **71** (bottom, expansion). **71** could not be found in control samples (data not shown).

3.6.3 Putative echinomycin intermediate capture *via* *N*-(2-(2-aminoacetamido)ethyl) decanamide probe (12)

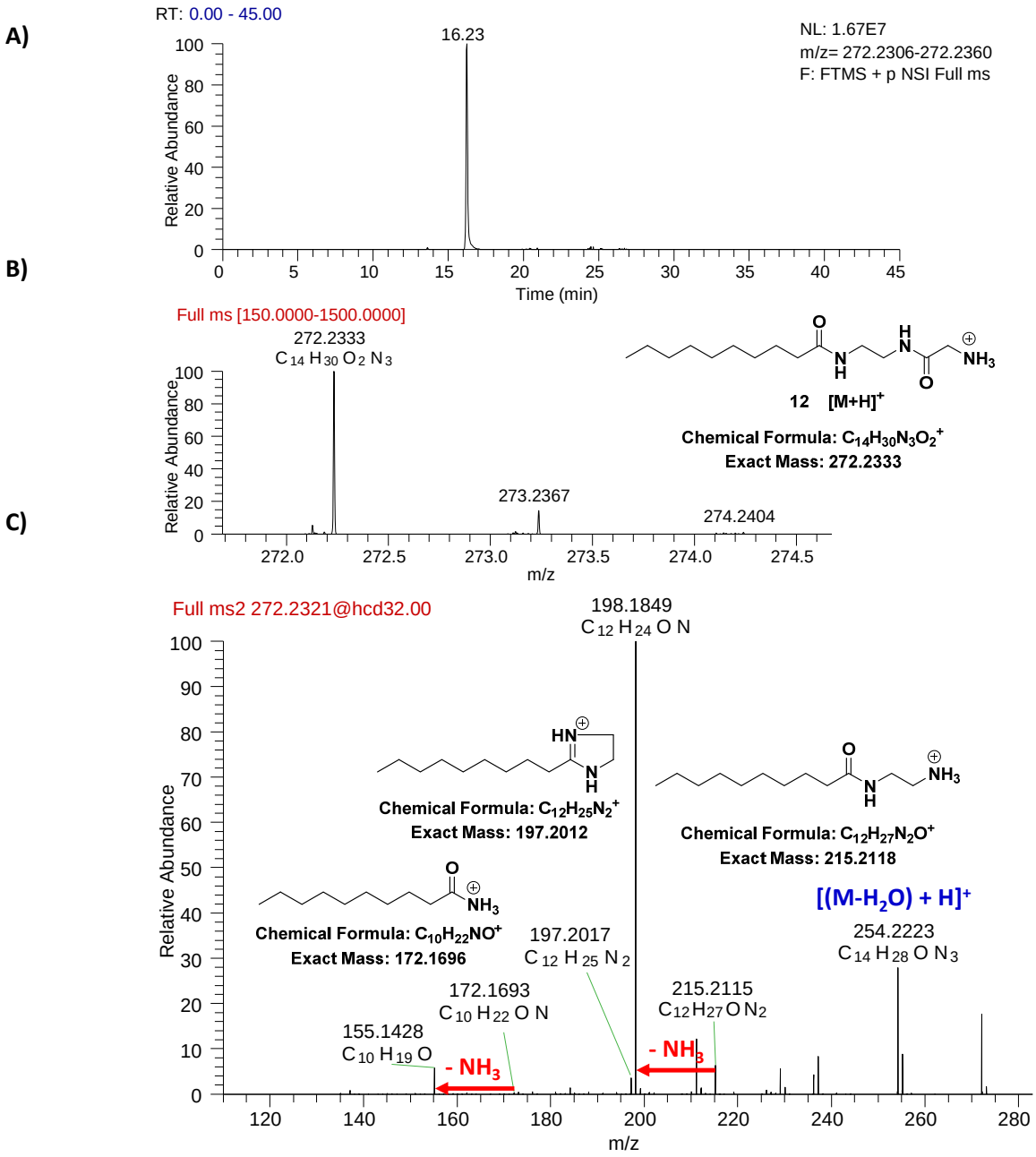
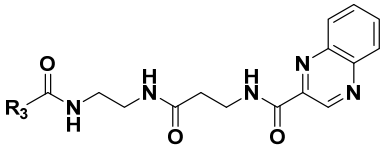
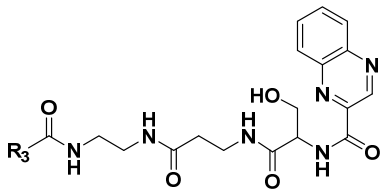
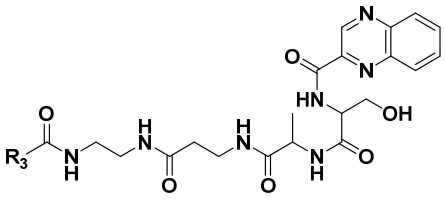
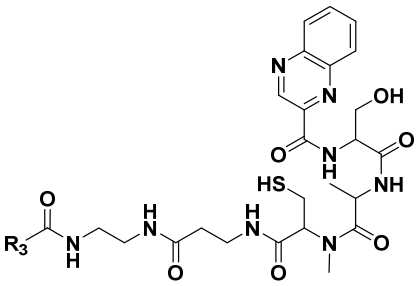


Figure 28S: Detection and characterisation of probe **12**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **12** (0.4 mM on MYM agar): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **12**.

No peptide intermediates were found/characterised from experiments with **12**.

3.7 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A)* via *β-alanine-based chain termination probes 13-15*

Table 6S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) *via* β-alanine-based probes **13-15** (analysed on a MaXis Impact instrument[a] and on an Orbitrap Fusion instrument[b]).

Putative intermediate structures	**Probe 13** [a],[b] $R_3 = (CH_2)_2CH_3$	**Probe 14** [a], [b] $R_3 = (CH_2)_5CH_3$	**Probe 15** [a], [b] $R_3 = (CH_2)_8CH_3$
	✓ High abundance	✓ High abundance	n.d.
	✓ Low abundance	✓ Low abundance	n.d.
	✓ Low abundance	n.d.	n.d.
	n.d.	n.d.	n.d.

3.7.1 Putative echinomycin intermediate capture *via* *N*-(2-acetamidoethyl)-3-aminopropanamide probe (13)

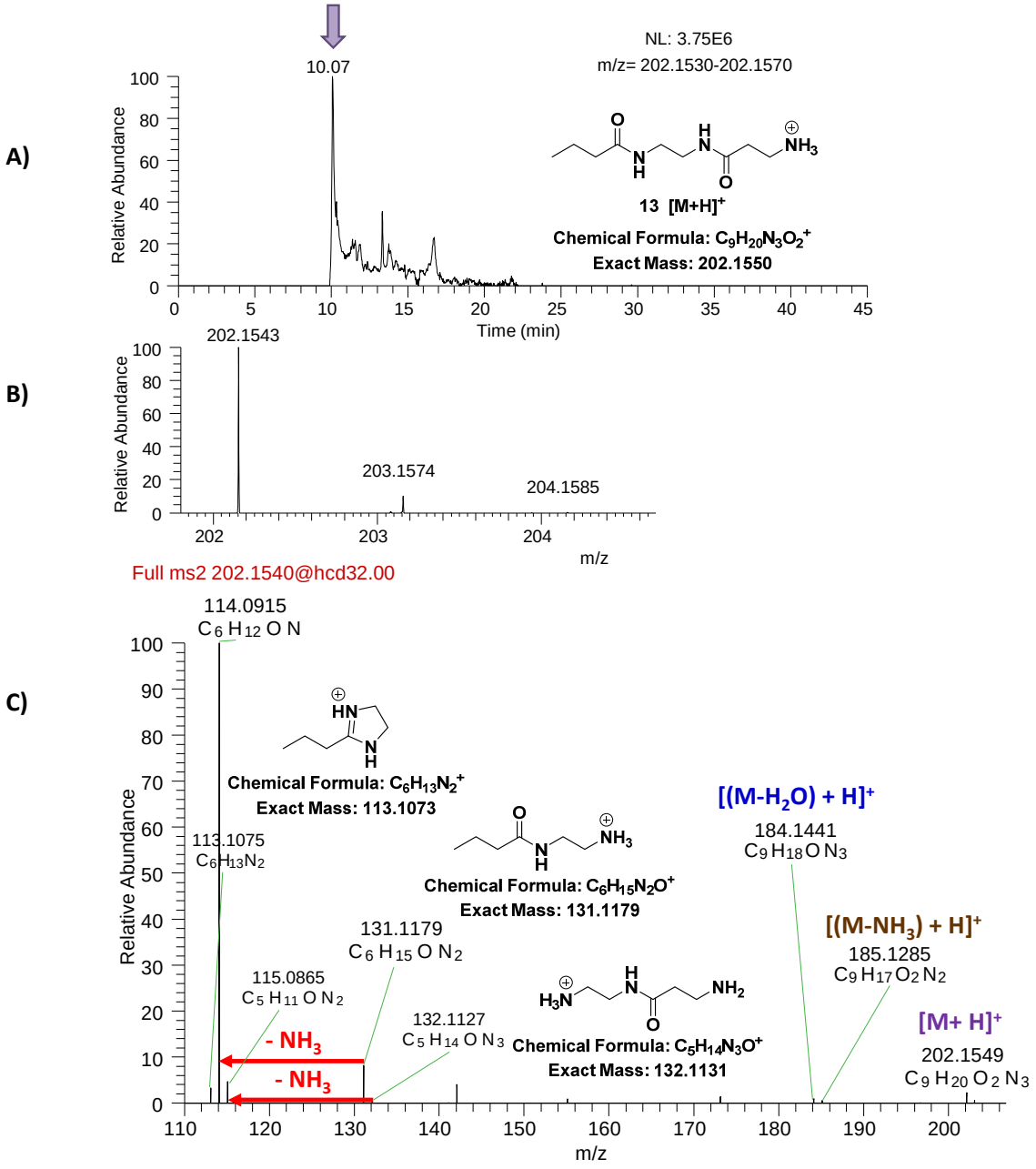


Figure 29S: Detection and characterisation of probe **13**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **13** (2.0 mM on MYM agar): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **13**.

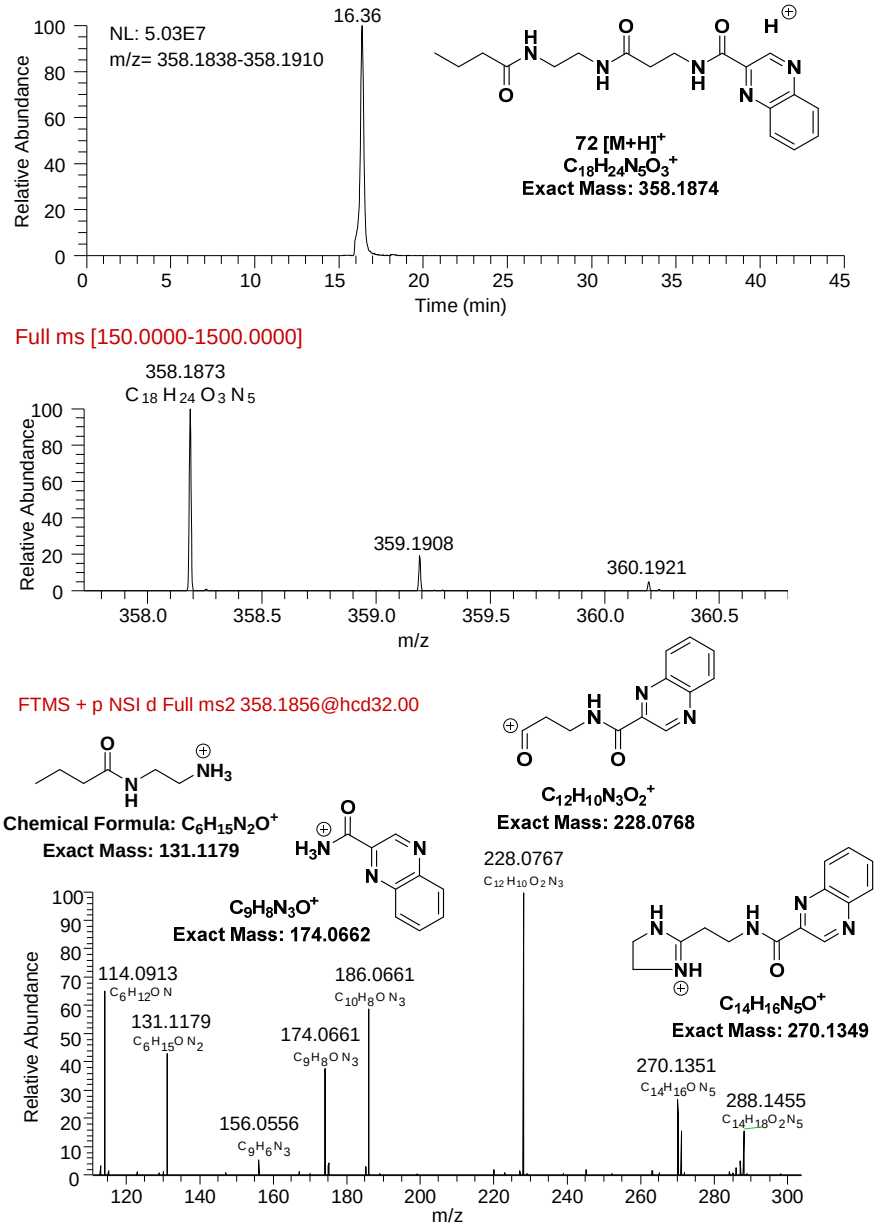


Figure 30S: Detection and characterisation of putative dipeptide intermediate **72**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **13** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top), accurate mass isotopic distribution (middle), and MS^2 fragmentation for **72** (bottom). This species was not found in control samples (data not shown).

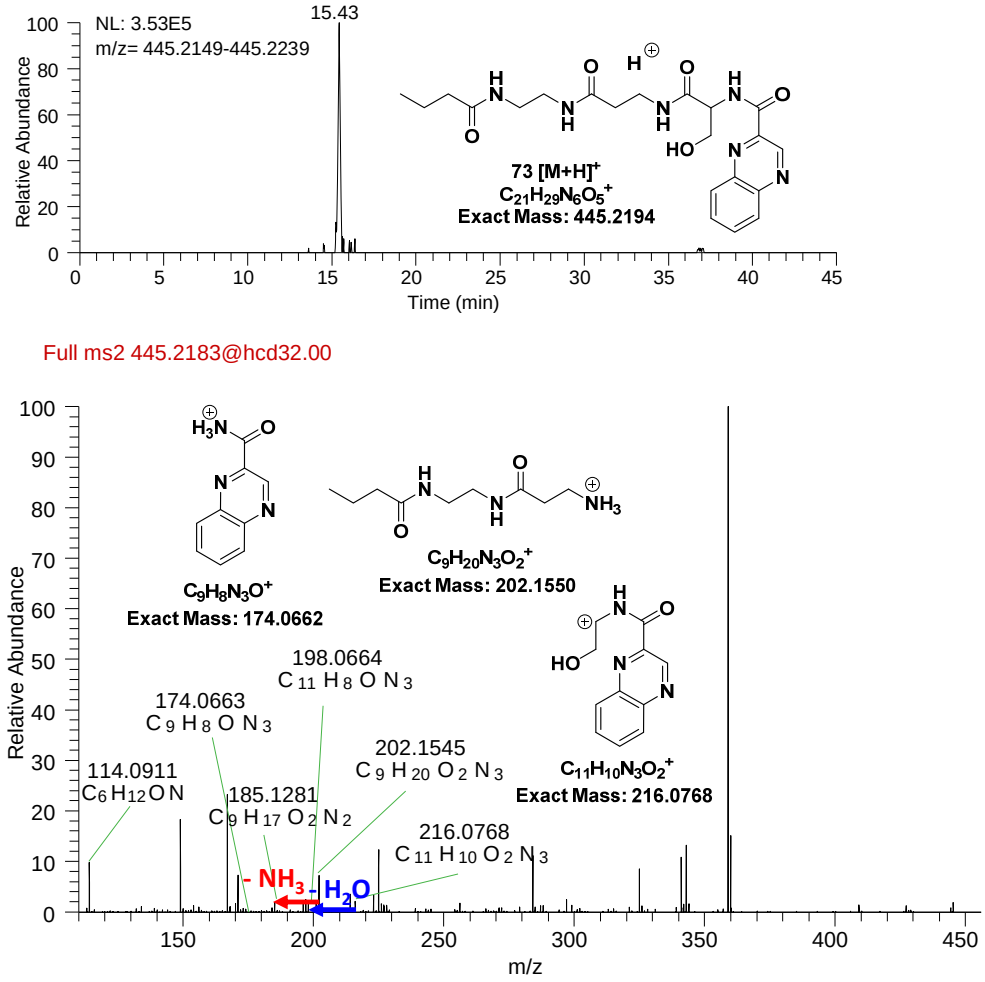


Figure 31S: Detection and characterisation of putative tripeptide intermediate **73**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **13** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **73** (bottom). This species was not found in control samples (data not shown).

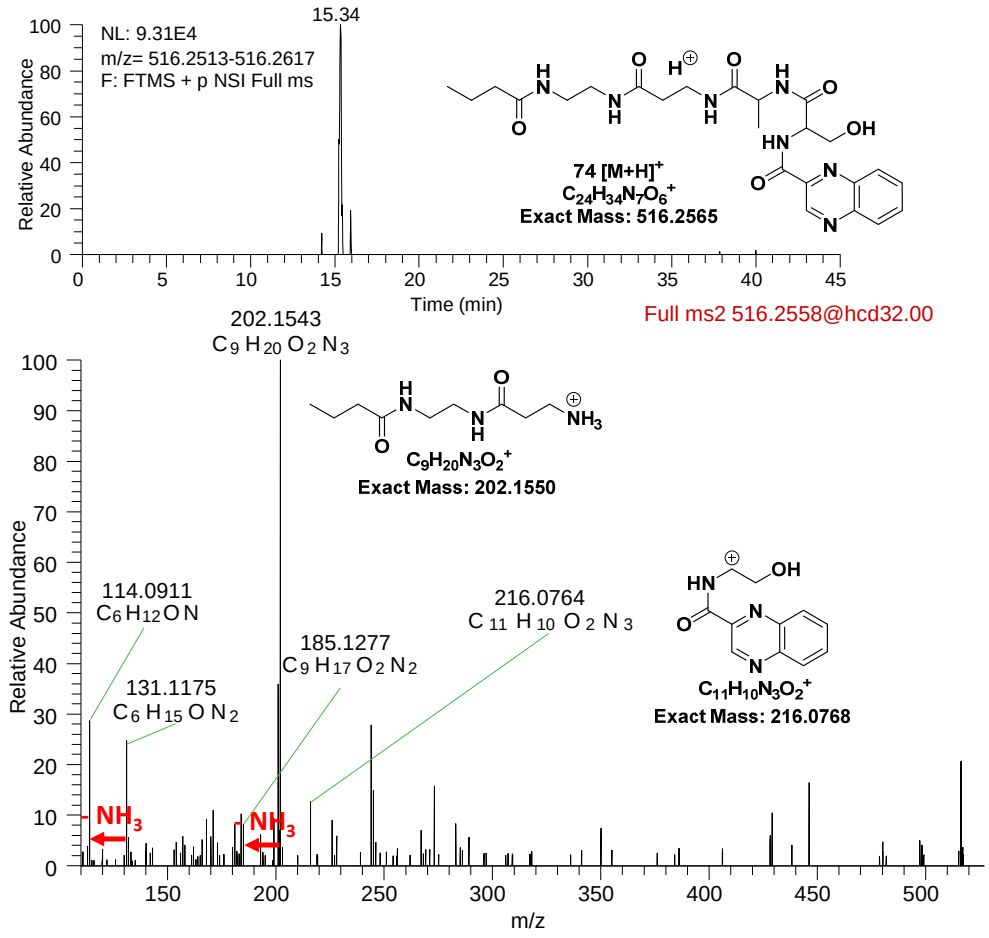


Figure 32S: Detection and characterisation of putative tetrapeptide intermediate **74**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **13** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **74** (bottom). This species was not found in control samples (data not shown).

3.7.2 Putative echinomycin intermediate capture *via* *N*-(2-(3-aminopropanamido)ethyl) heptanamide probe (14)

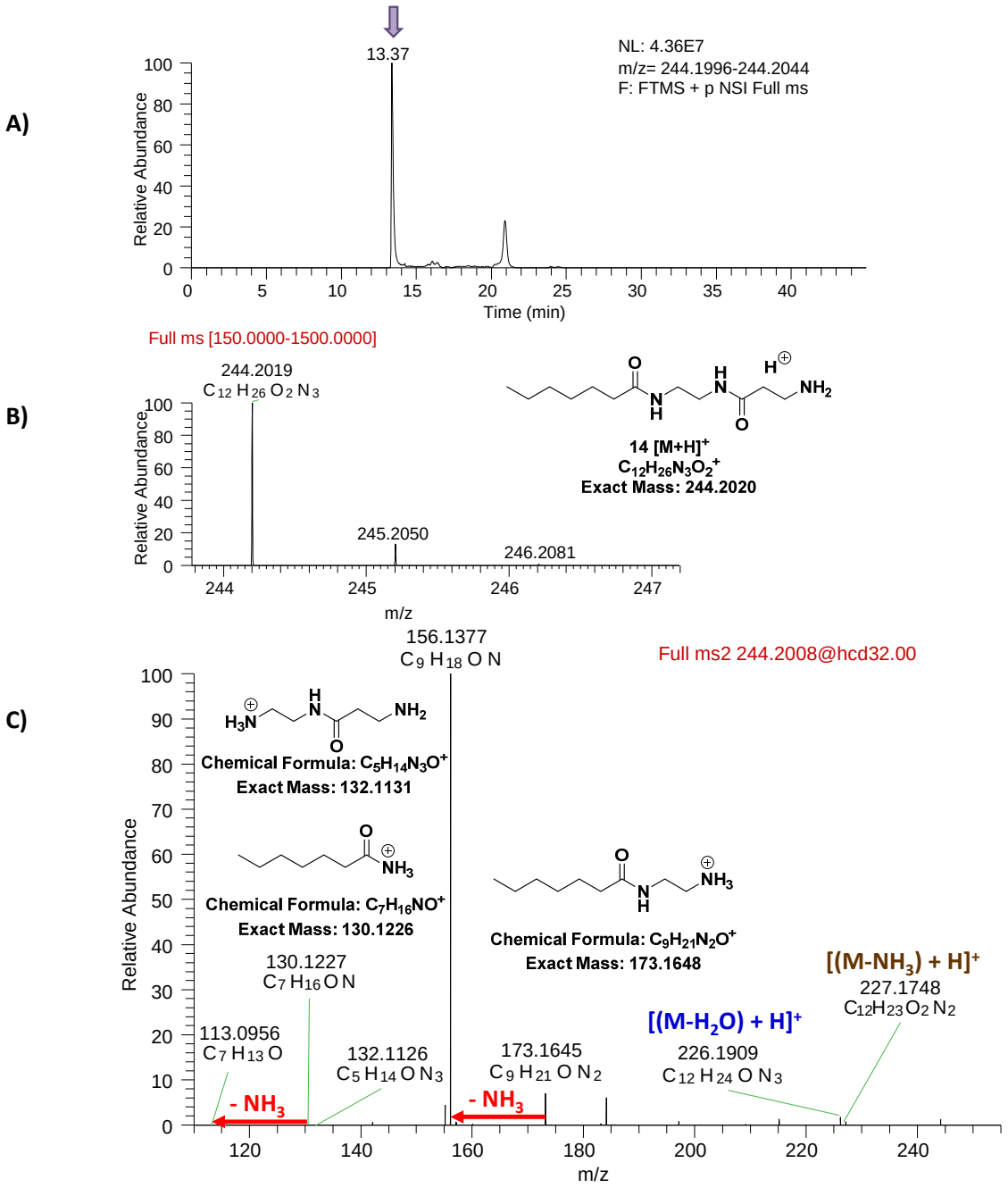


Figure 33S: Detection and characterisation of probe **14**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **14** (2.0 mM on MYM agar): (A) extracted ion chromatogram ($[M+H]^+$), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for **14**.

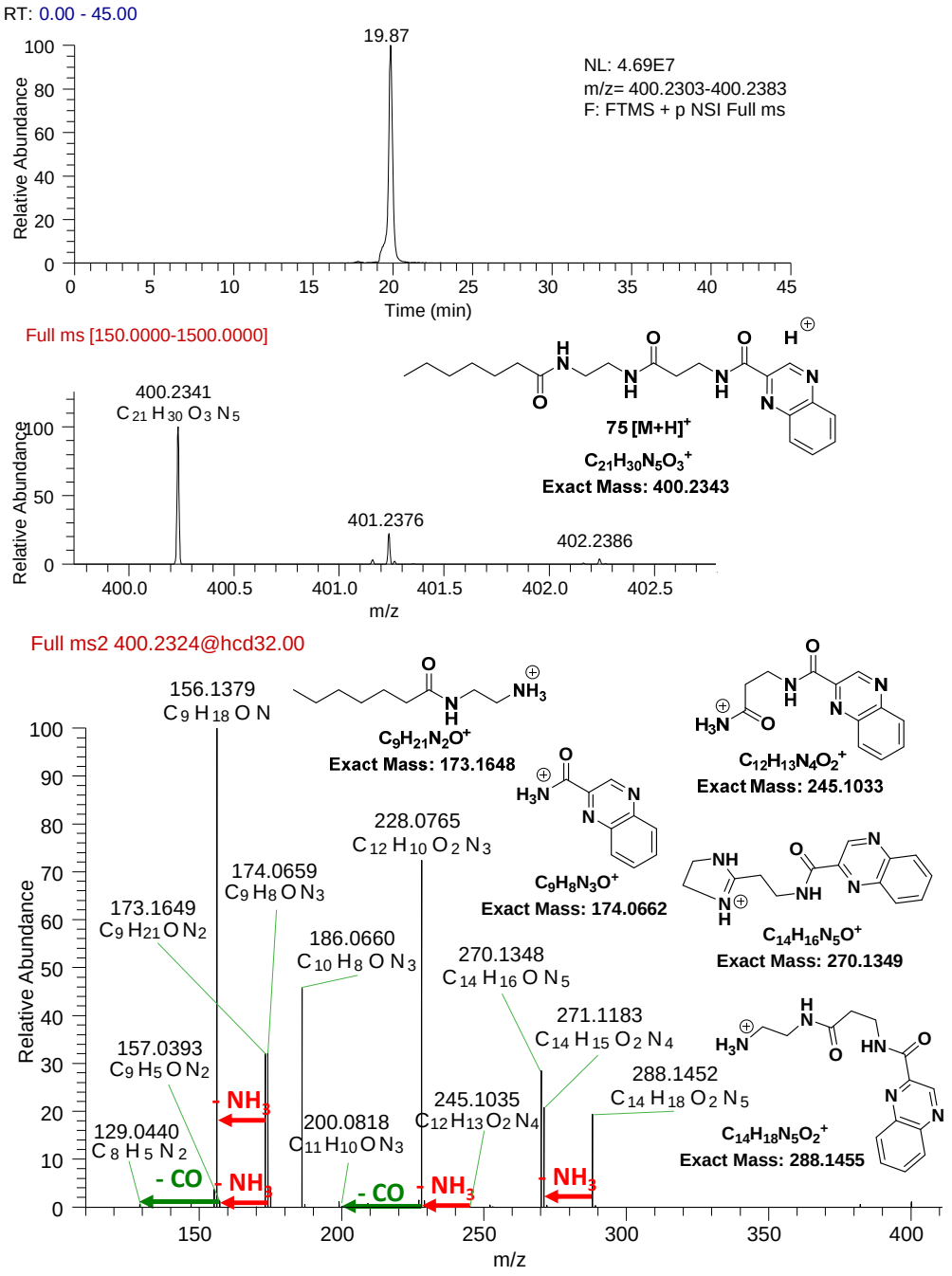


Figure 34S: Detection and characterisation of putative dipeptide intermediate **75**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **14** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **75** (bottom). This species was not found in control samples (data not shown).

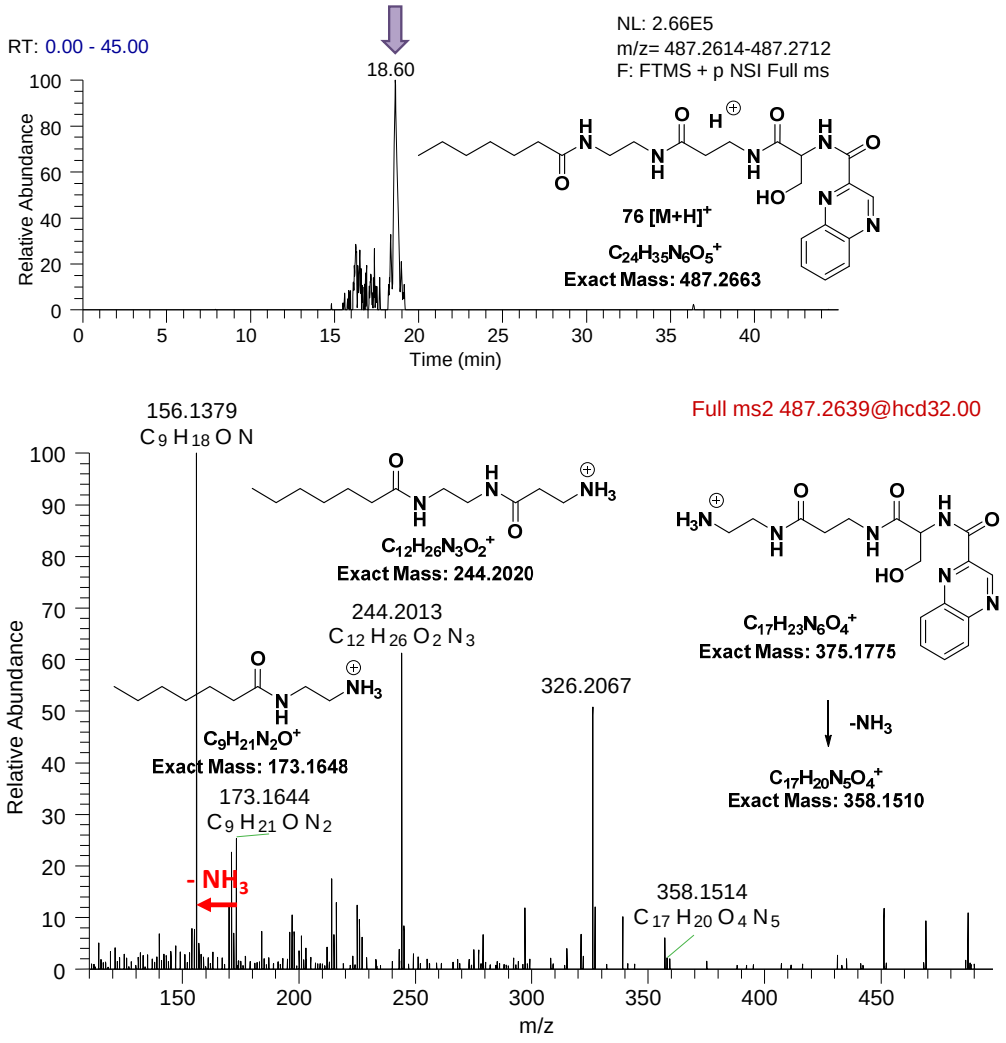


Figure 35S: Detection and characterisation of putative tripeptide intermediate **76**. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **14** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **76** (bottom). **76** was not detected in control samples (data not shown).

3.7.3 Putative echinomycin intermediate capture *via N*-(2-(3-aminopropanamido)ethyl) decanamide probe (15)

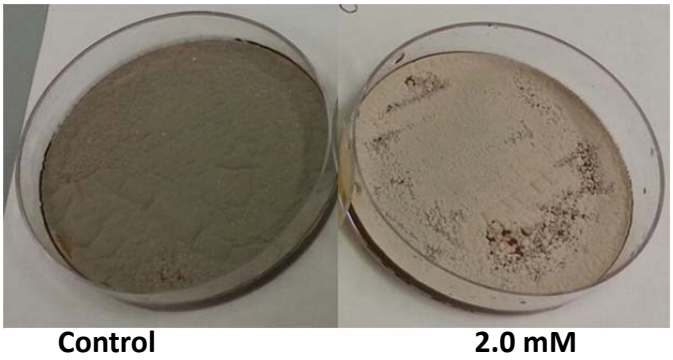


Figure 36S: Growth of *S. lasaliensis* ACP12(S970A) in the absence (control) and in the presence of 2.0 mM probe **15** on MYM agar plates. Oppositely to other *N*-decanoyl probes, **15** is not cytotoxic at concentrations above 1.0 mM.

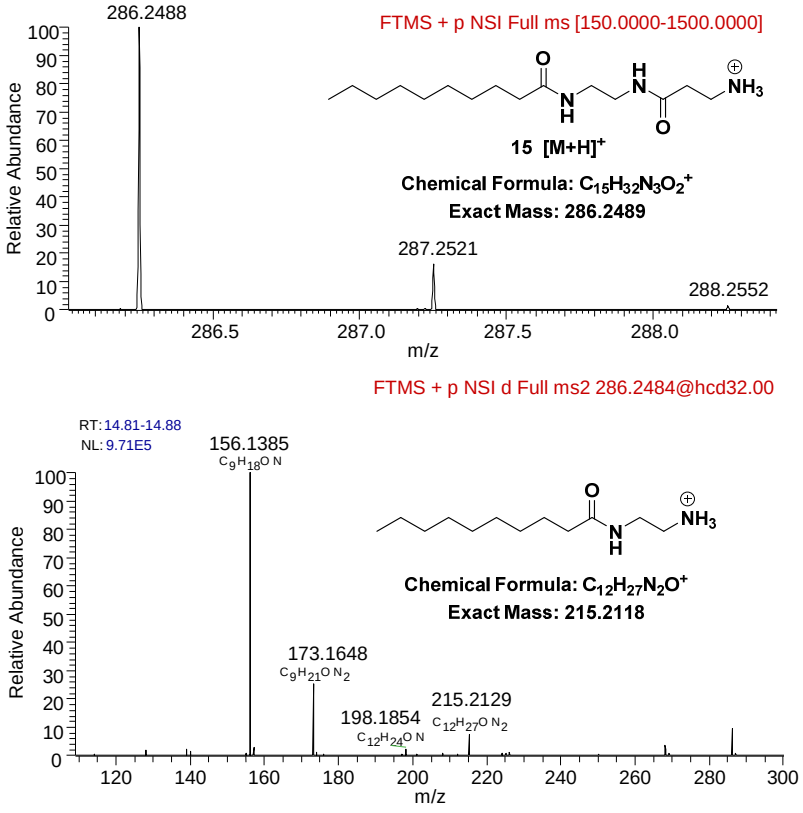
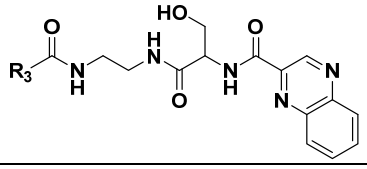
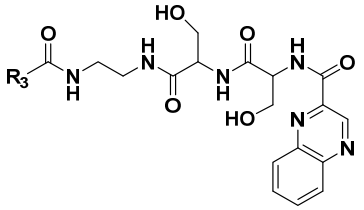
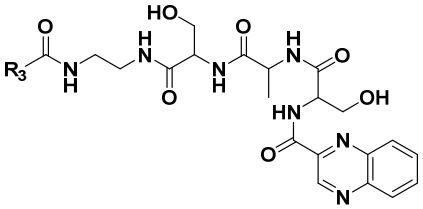
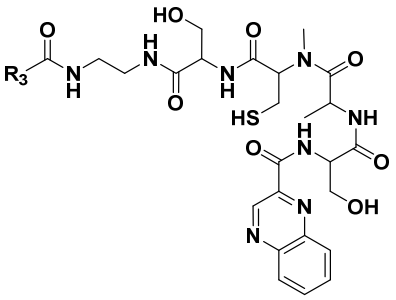


Figure 37S: Characterisation of probe **15** from ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **15** (MYM agar, 2.0 mM concentration): accurate mass isotopic (for $[M+H]^+$, top) and HR-MS^2 fragmentation (bottom).

No peptide intermediates were found/characterised from experiments with **15**.

3.8 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A)* via *D-Serine-based chain termination probe 16*

Table 7S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) *via* D-Ser-based probe **16** (analysed on an Orbitrap Fusion instrument).

Putative intermediate structures	**Probe 16** $R_3 = (CH_2)_5CH_3$
	✓ low abundance
	n.d.
	n.d.
	n.d.

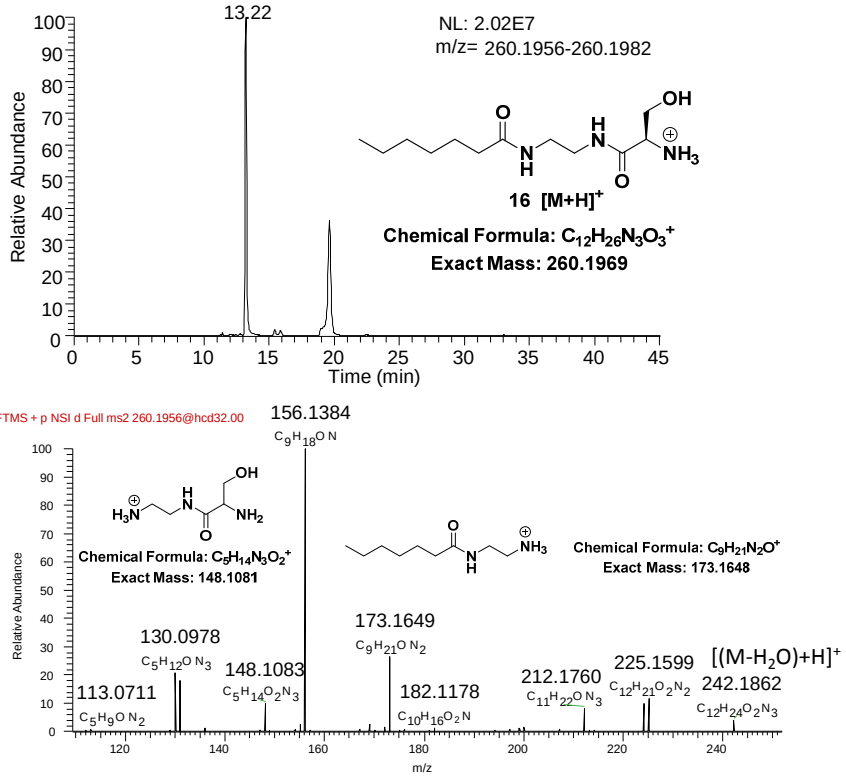


Figure 38S: Detection and characterisation of probe **16**. LC-HRMS analysis (Orbitrap Fusion) of the organic extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **16** (2.0 mM on MYM agar): extracted ion chromatogram ($[M+H]^+$, top), and accurate MS^2 fragmentation (bottom).

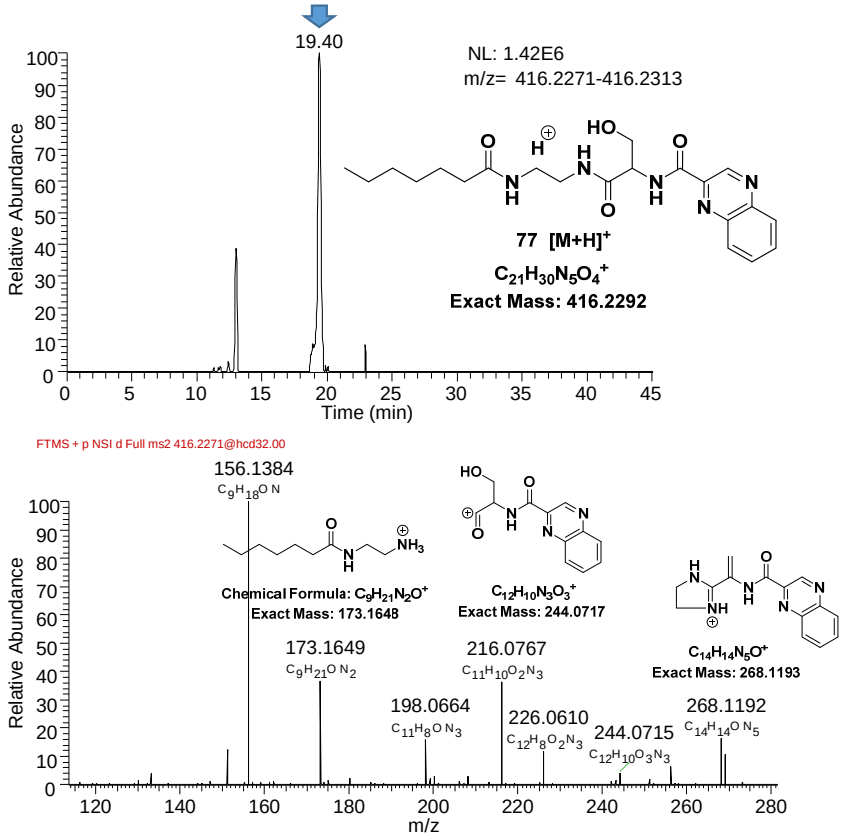


Figure 39S: Detection and characterisation of putative dipeptide intermediate **77**. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **16** (MYM agar, 2.0 mM concentration): extracted ion chromatogram ($[M+H]^+$, top) and MS^2 fragmentation for **77** (bottom). This species was not found in control samples (data not shown). The stereochemistry of **77** is currently under investigation.

3.9 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A)* via *L-phenylalanine-based chain termination probe 17*

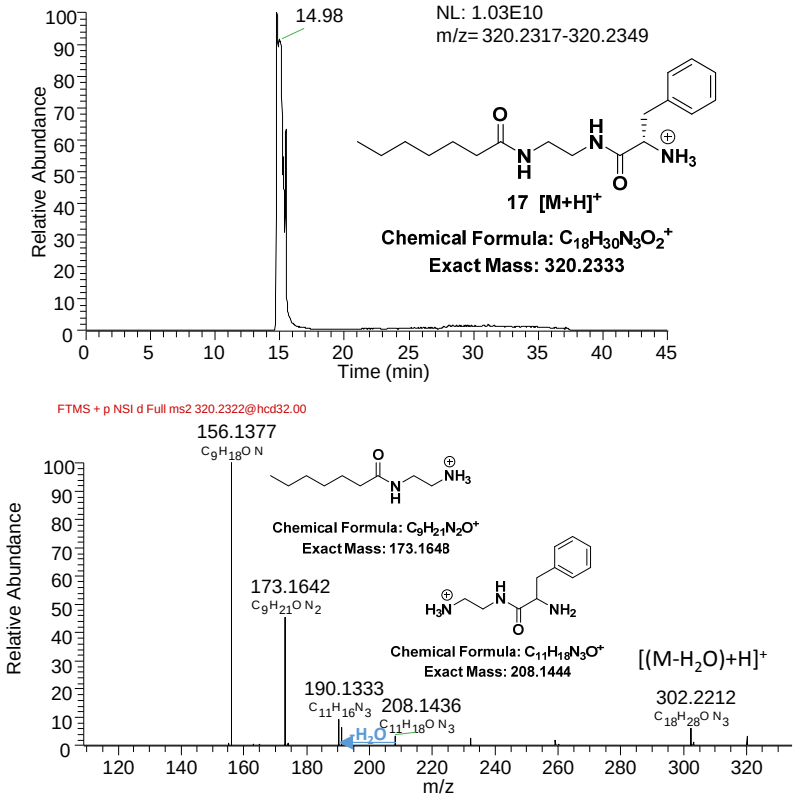


Figure 40S: Characterisation of probe **17** from organic extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe **18** (MYM agar, 2.0 mM concentration): extracted ion chromatogram (for $[M+H]^+$, top) and HR-MS^2 fragmentation (bottom).

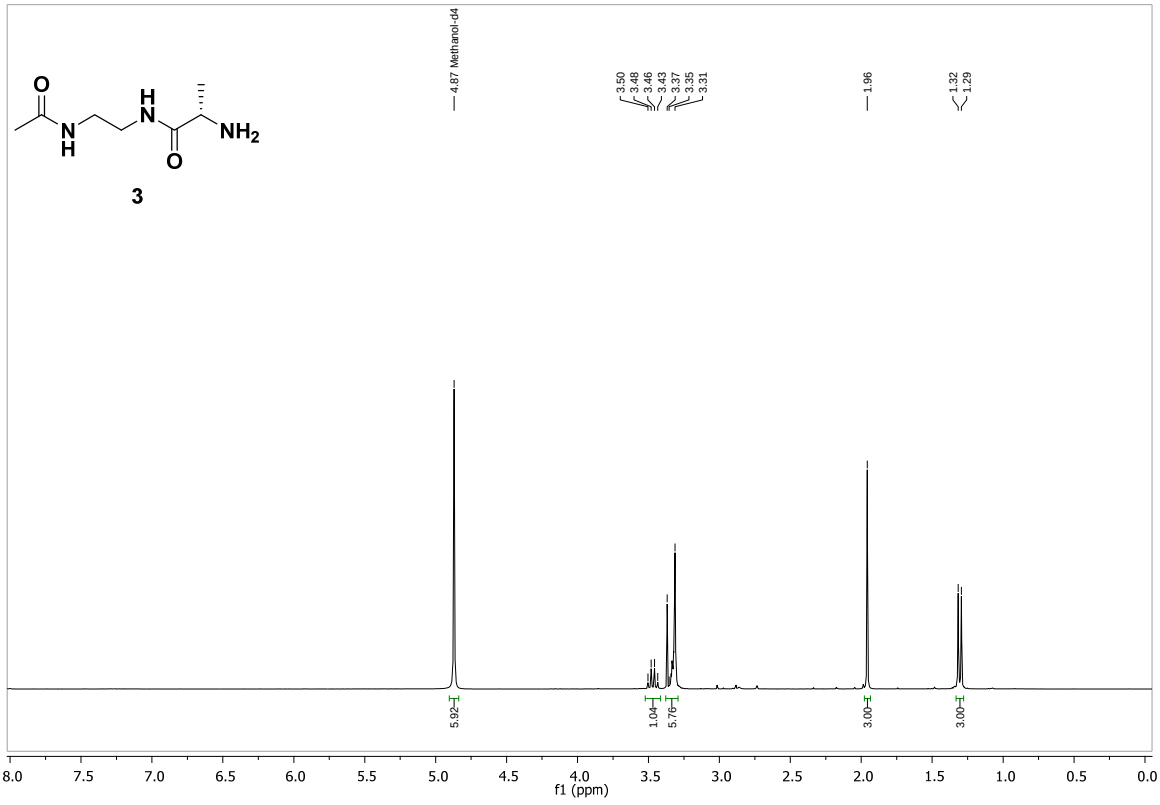
No peptide intermediates were consistently found/characterised from experiments with **17**.

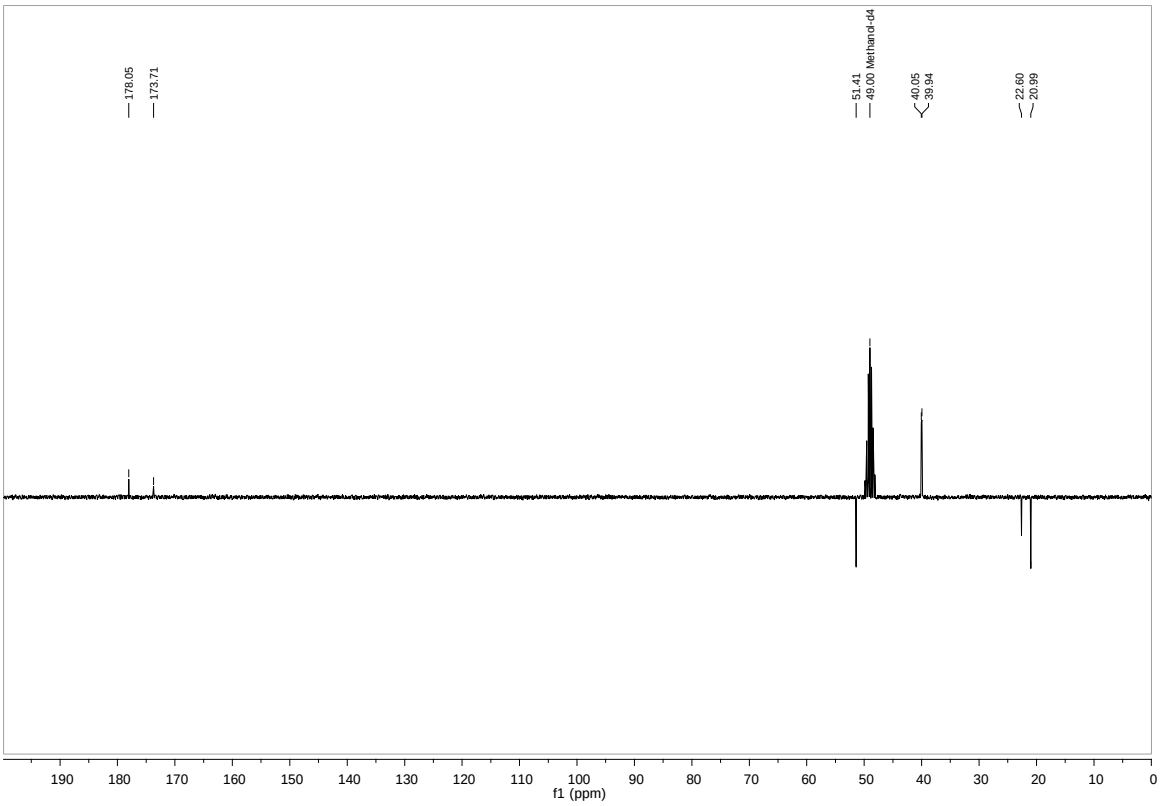
4 Literature

1. Krivickas, S. J.; Tamanini, E.; Todd, M. H.; Watkinson, M., *J. Org. Chem.* **2007,** *72*, 8280-8289.
2. a) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A., *Practical Streptomyces Genetics*, **2000**; b) Forget, M.S.; Robertson, A. W.; Overy, D. P.; Kerr, R. G.; Jakeman, D. L., *J. Nat. Prod.*, **2017**, Article ASAP, DOI: 10.1021/acs.jnatprod.7b00152.
3. a) Riva, E.; Wilkening, I.; Gazzola, S.; Li, A.; Smith, L.; Leadlay, P. F. L.; Tosin, M., *Angew. Chem. Int. Ed.* **2014**, *53*, 11944-11949; b) Wilkening, I.; Gazzola, S.; Riva, E.; Parascandolo, J. S.; Song, L.; Tosin, M., *ChemComm* **2016**, *52*, 10392-10395.

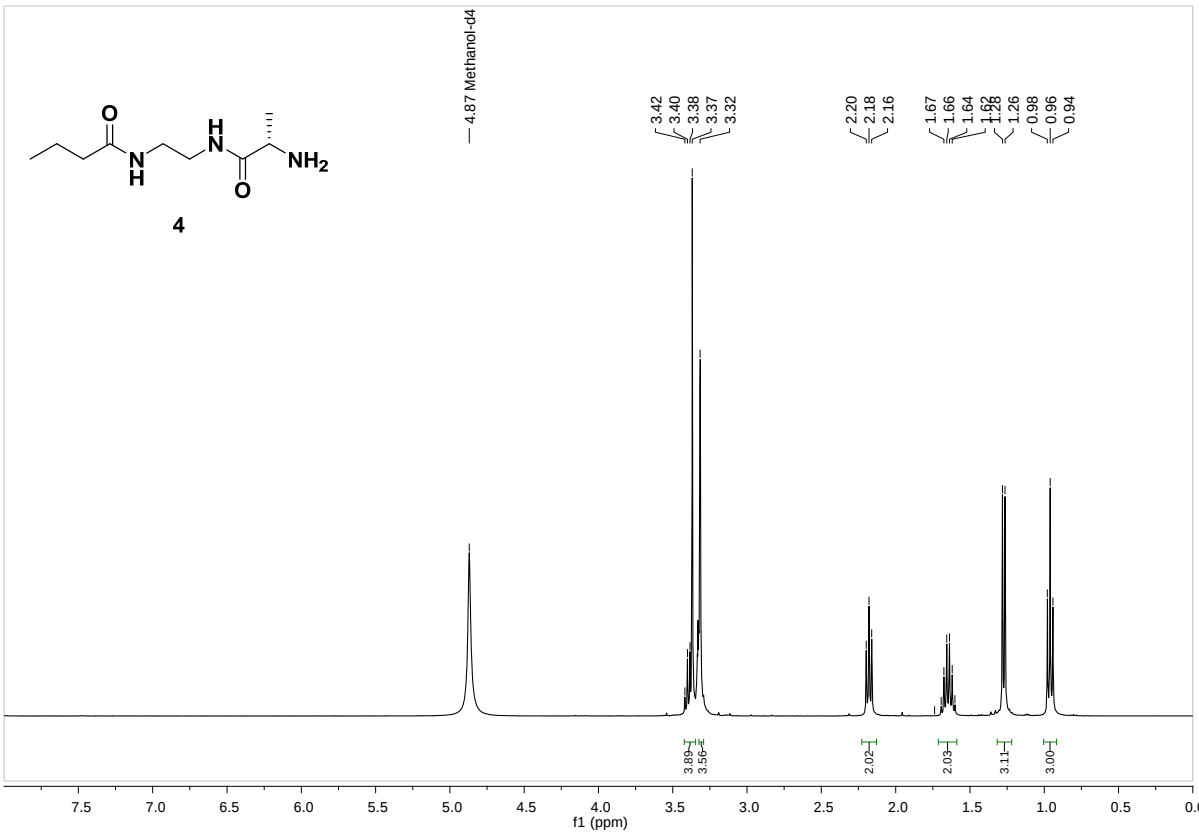
5 NMR spectra of probes 3-17

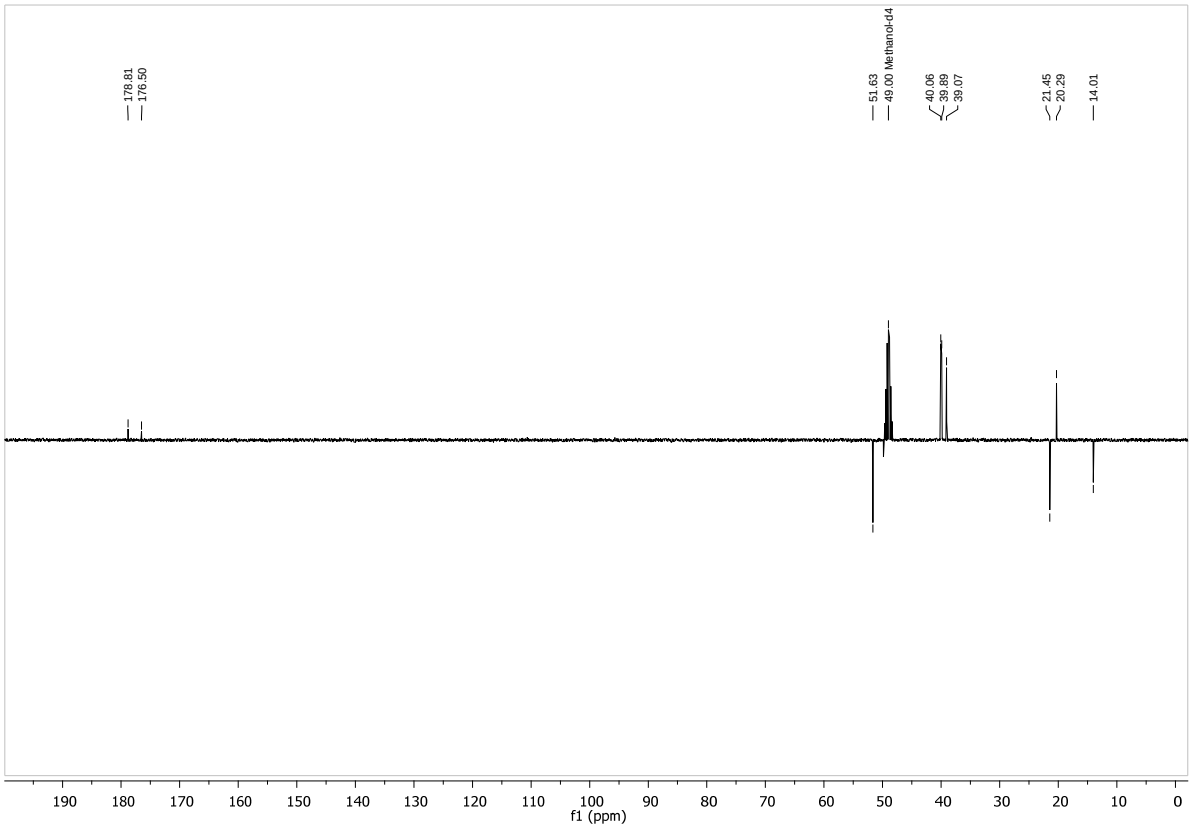
5.1 *^{1}H- and ^{13}C-NMR of probe 3*



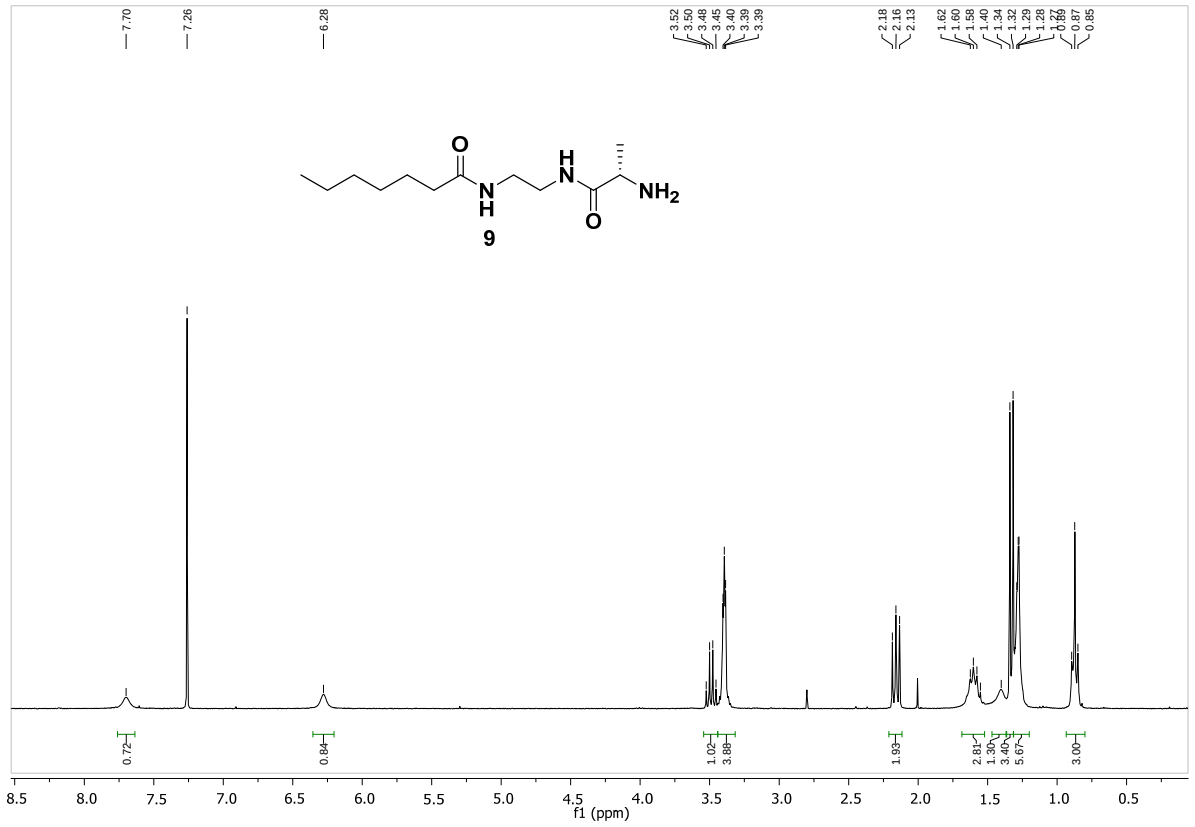


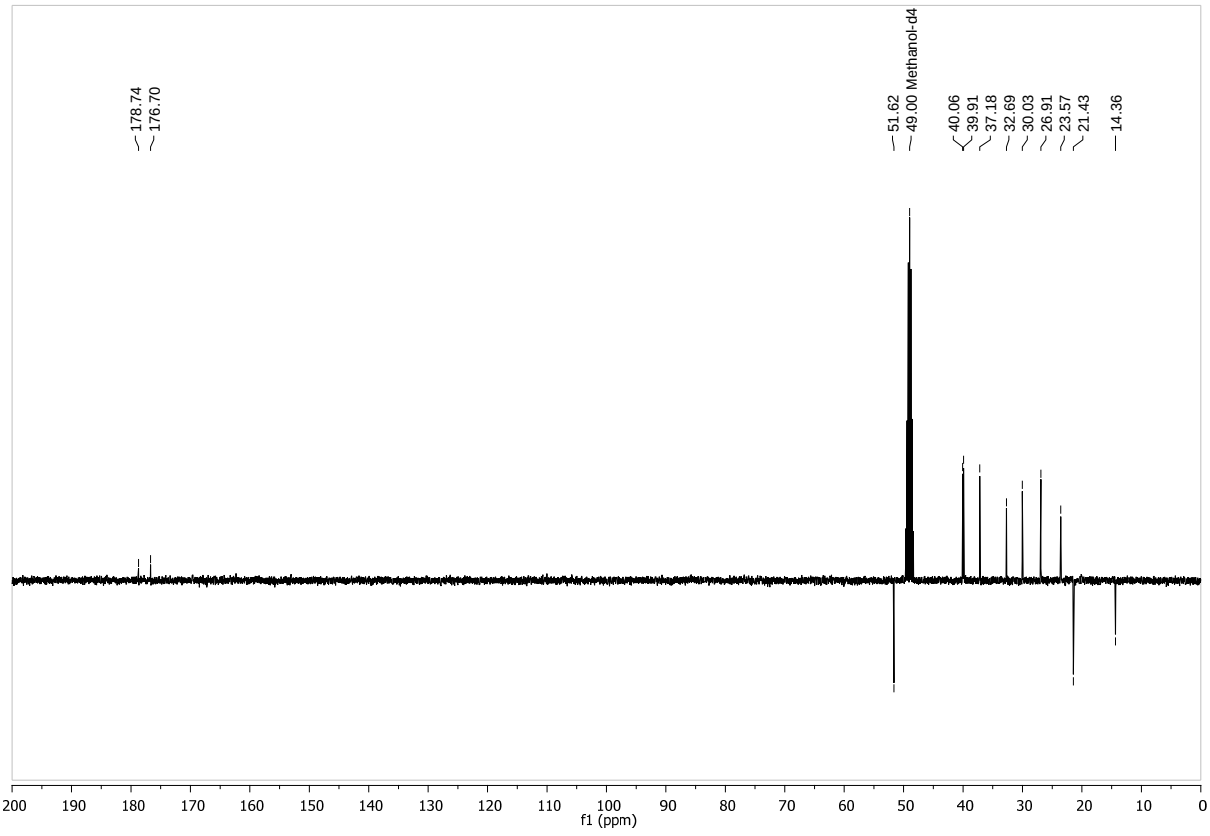
5.2 *^{1}H- and ^{13}C-NMR of probe 4*



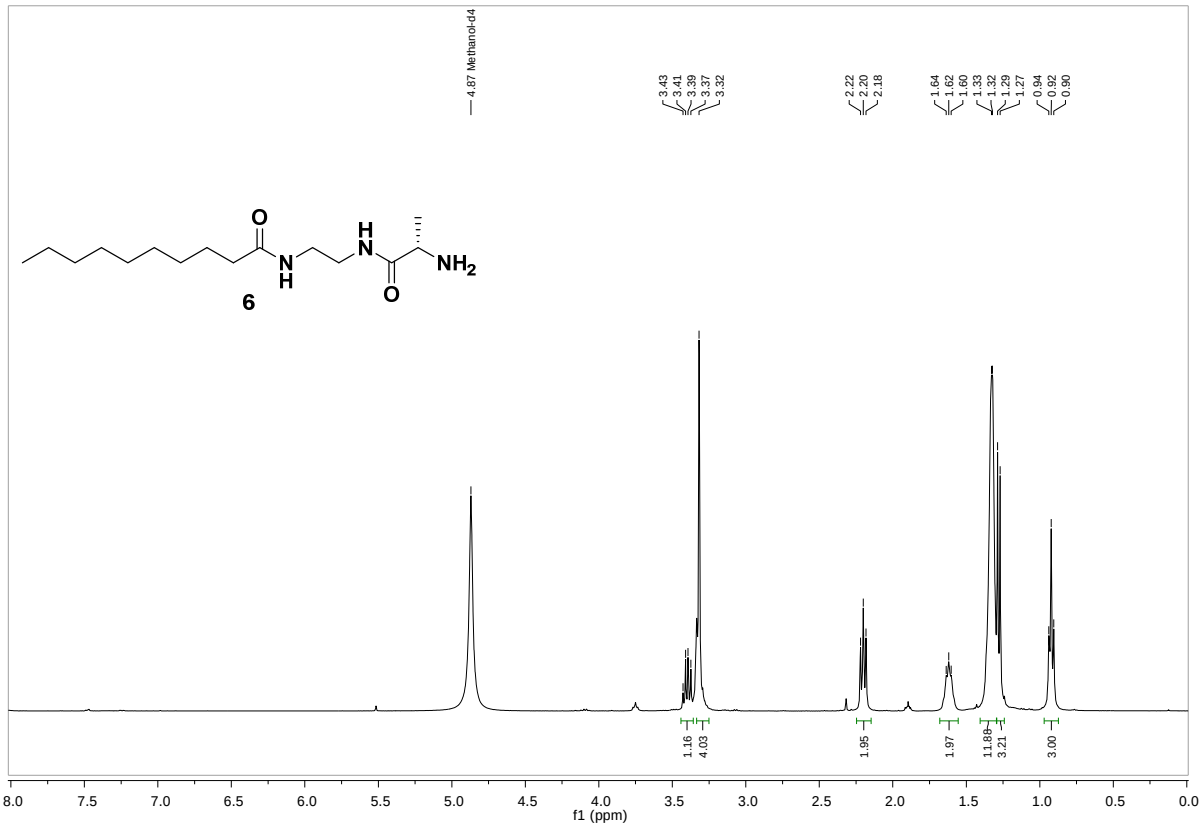


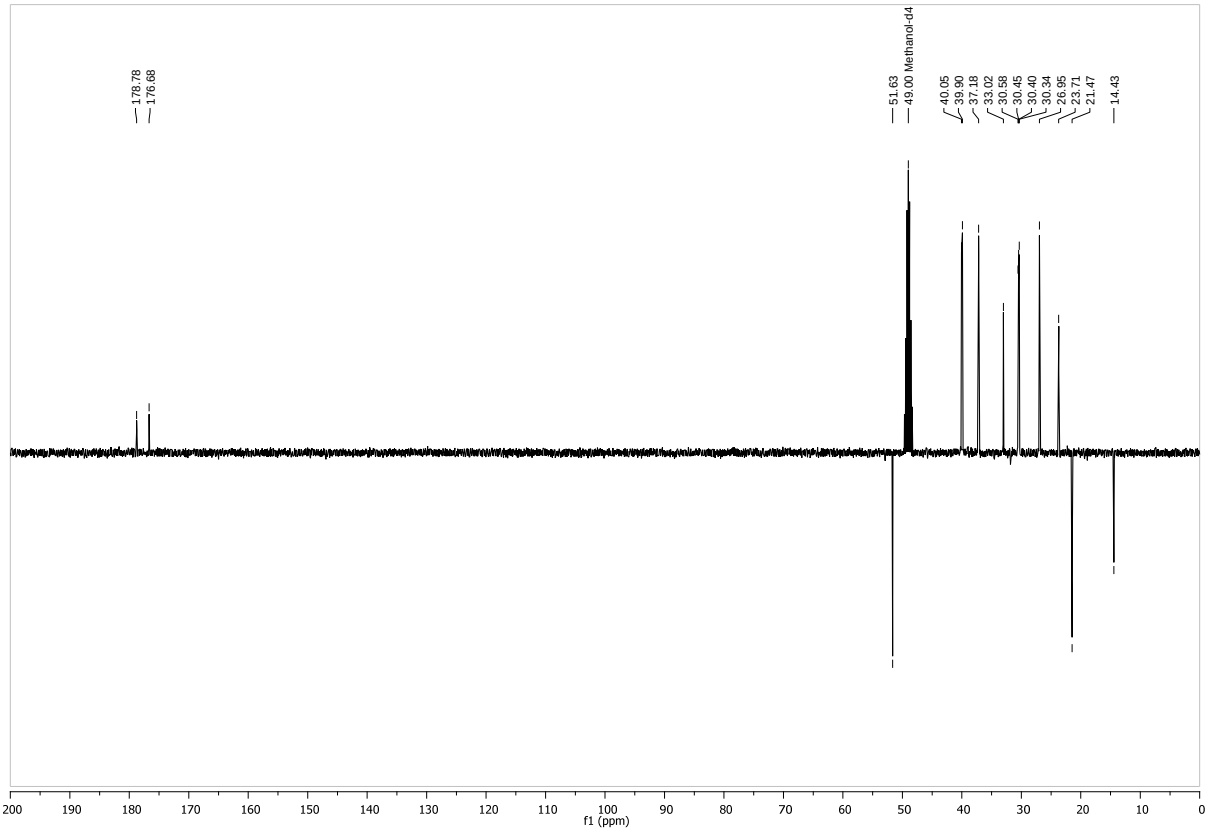
5.3 *^{1}H- and ^{13}C-NMR of probe 5*



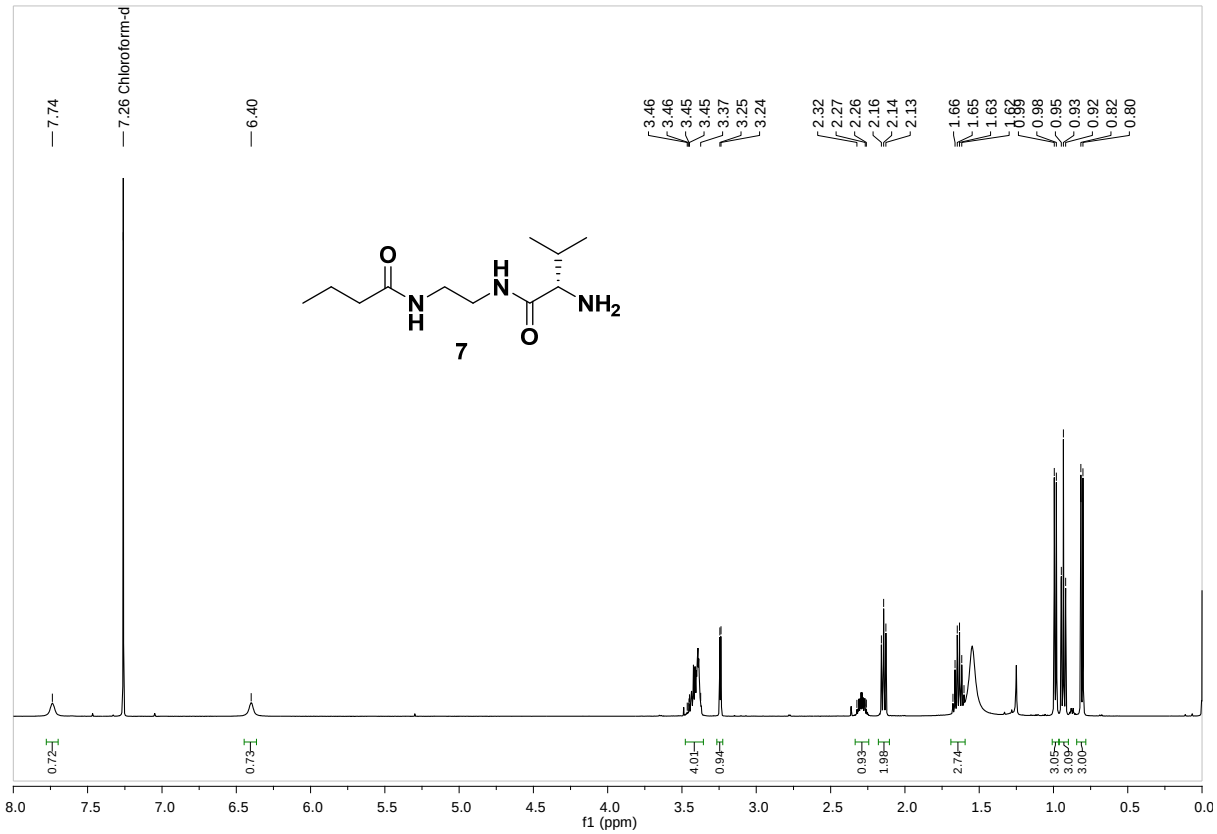


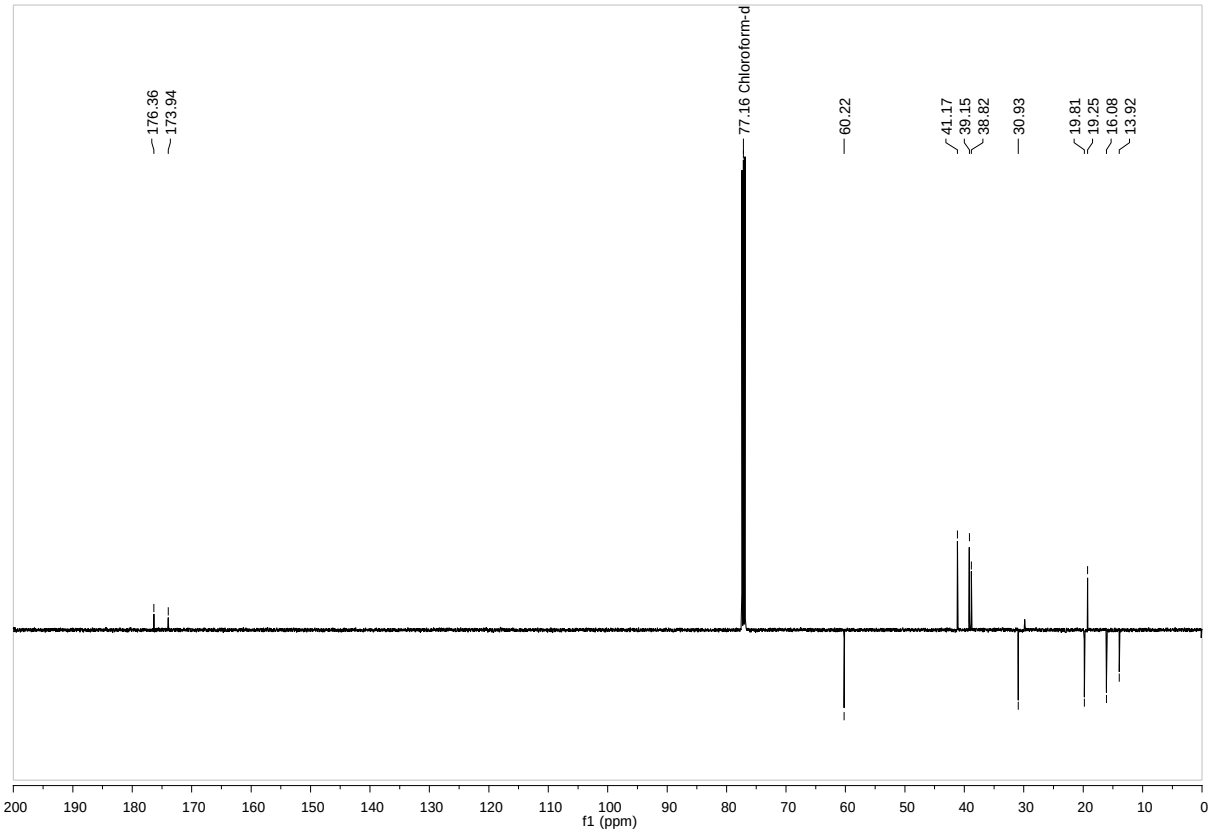
5.4 *^{1}H- and ^{13}C-NMR of probe 6*



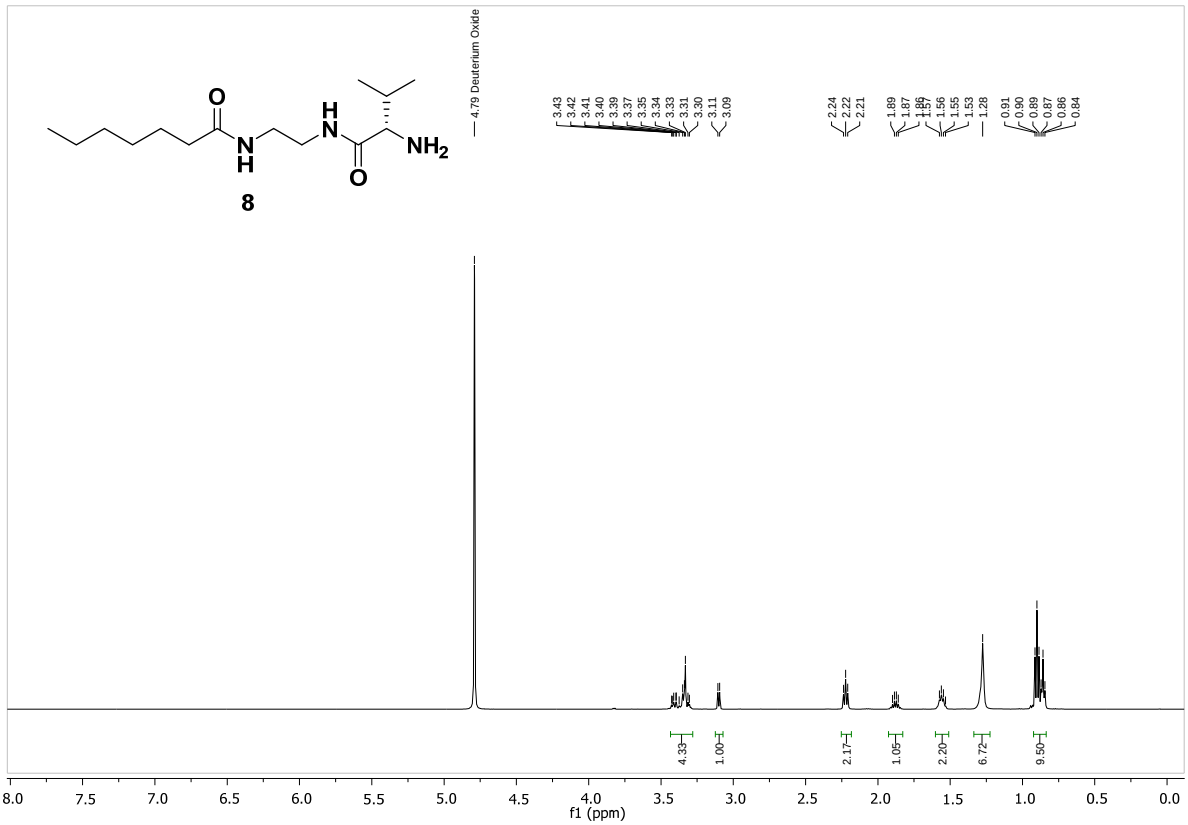


5.5 *^{1}H- and ^{13}C-NMR of probe 7*

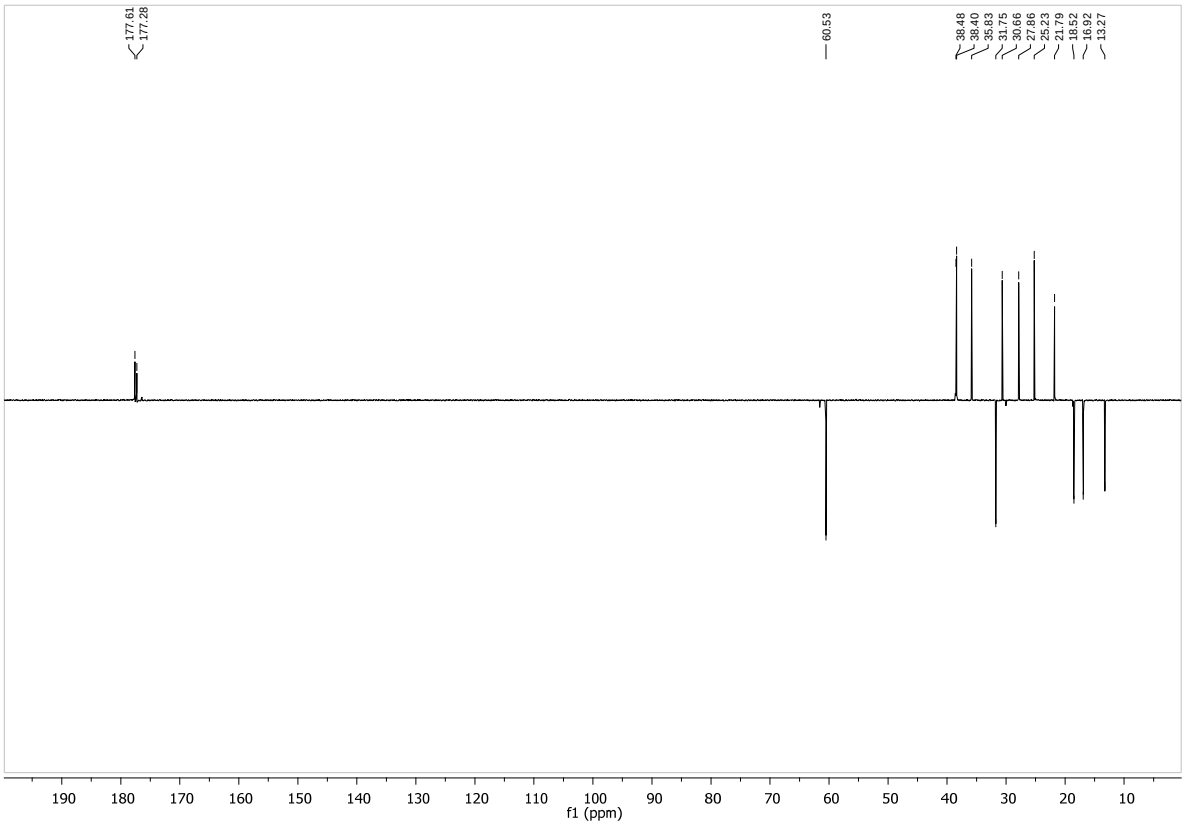




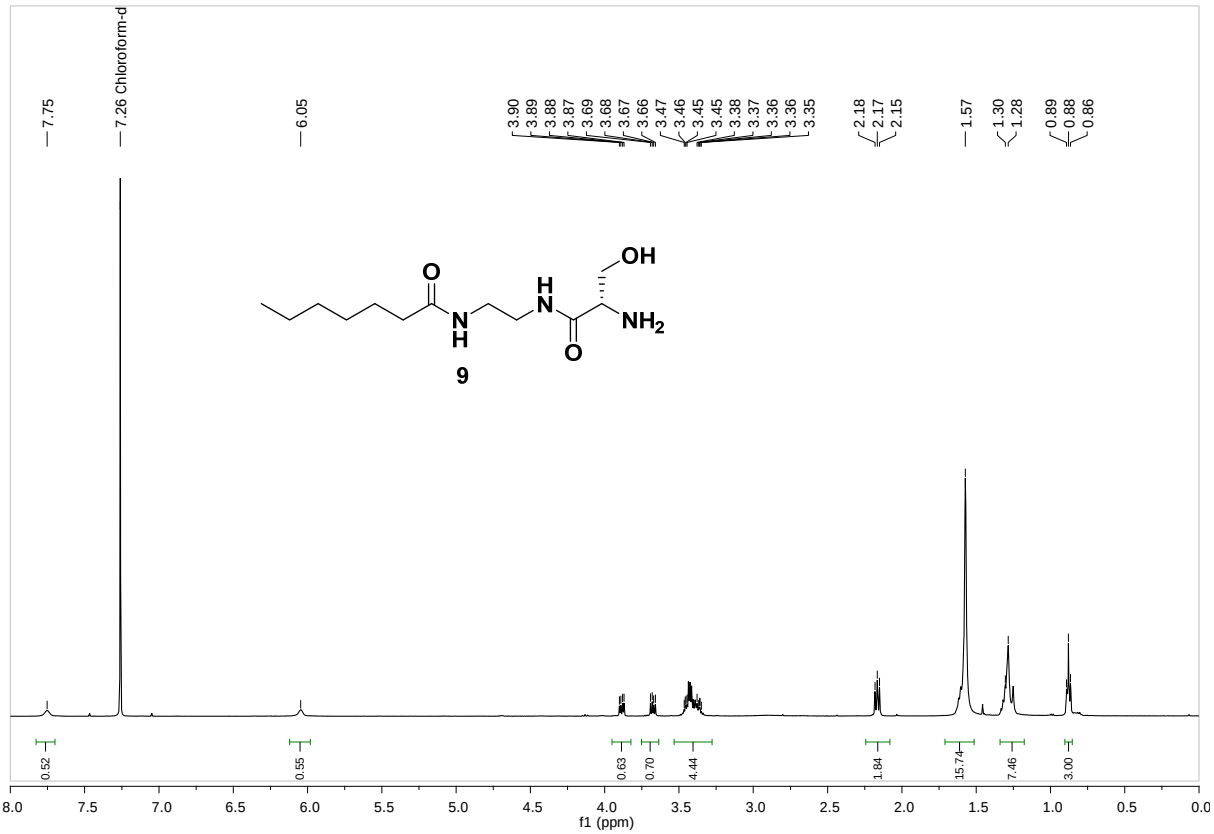
5.6 *^{1}H- and ^{13}C-NMR of probe 8*

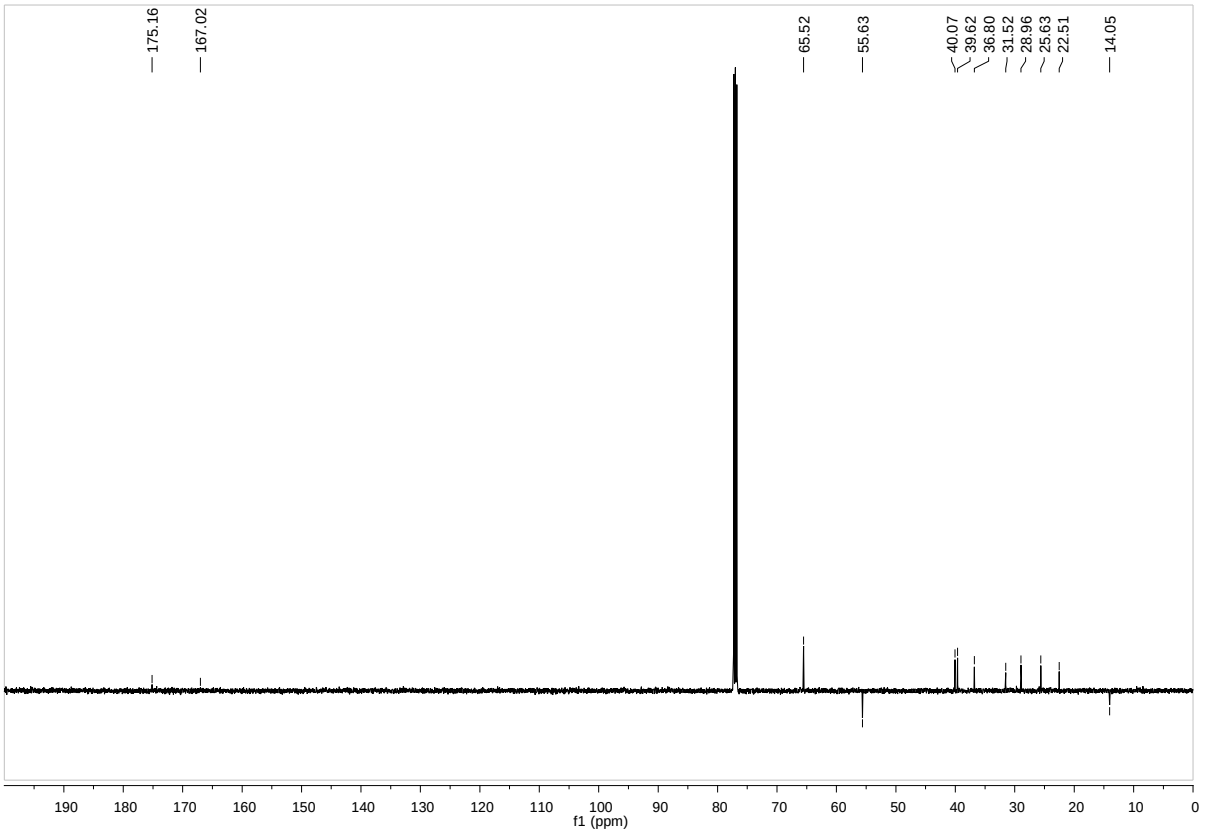


8

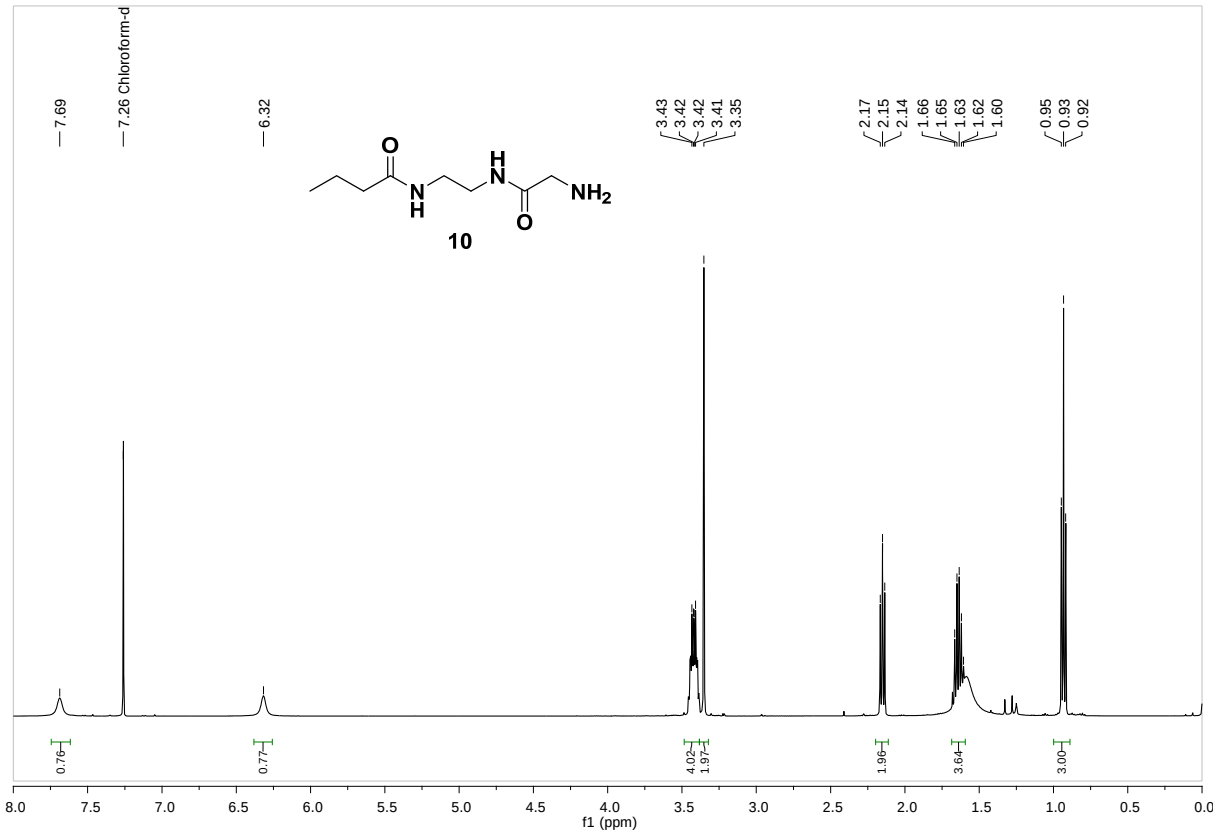


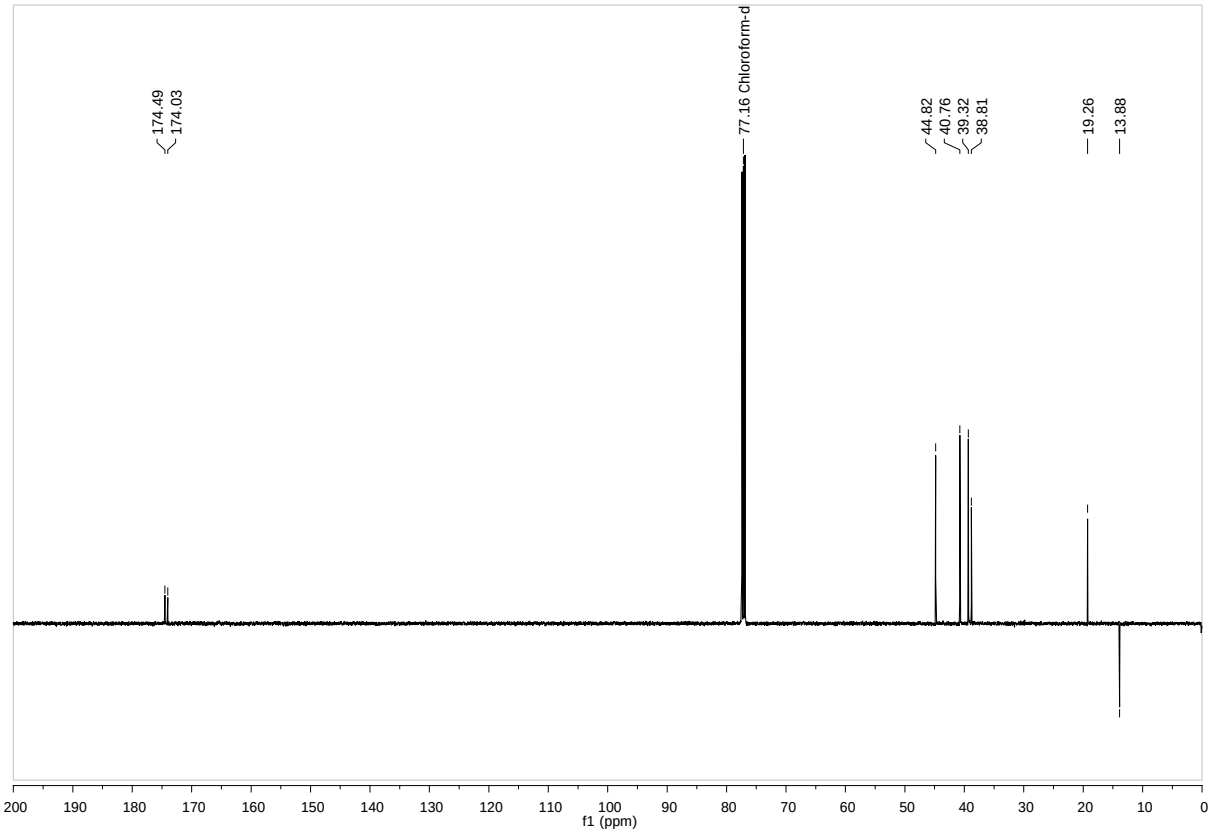
5.7 *^{1}H- and ^{13}C-NMR of probe 9*



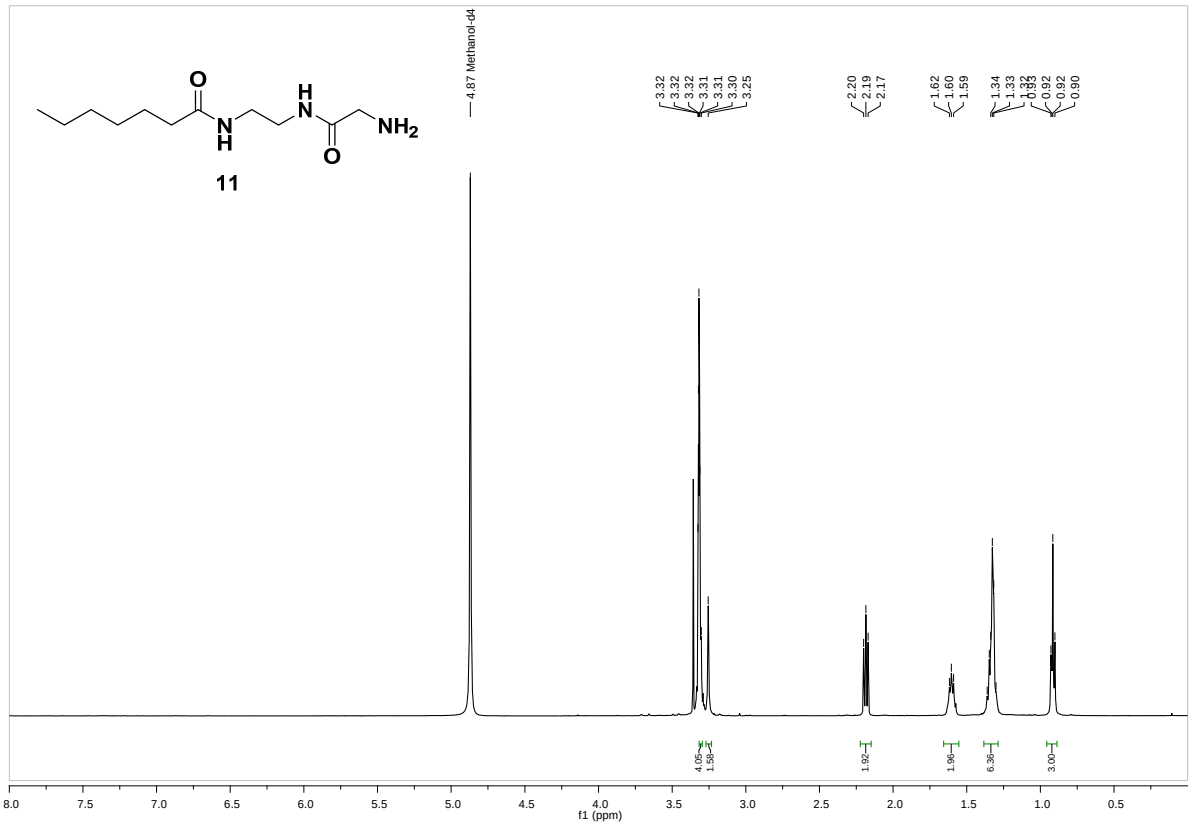


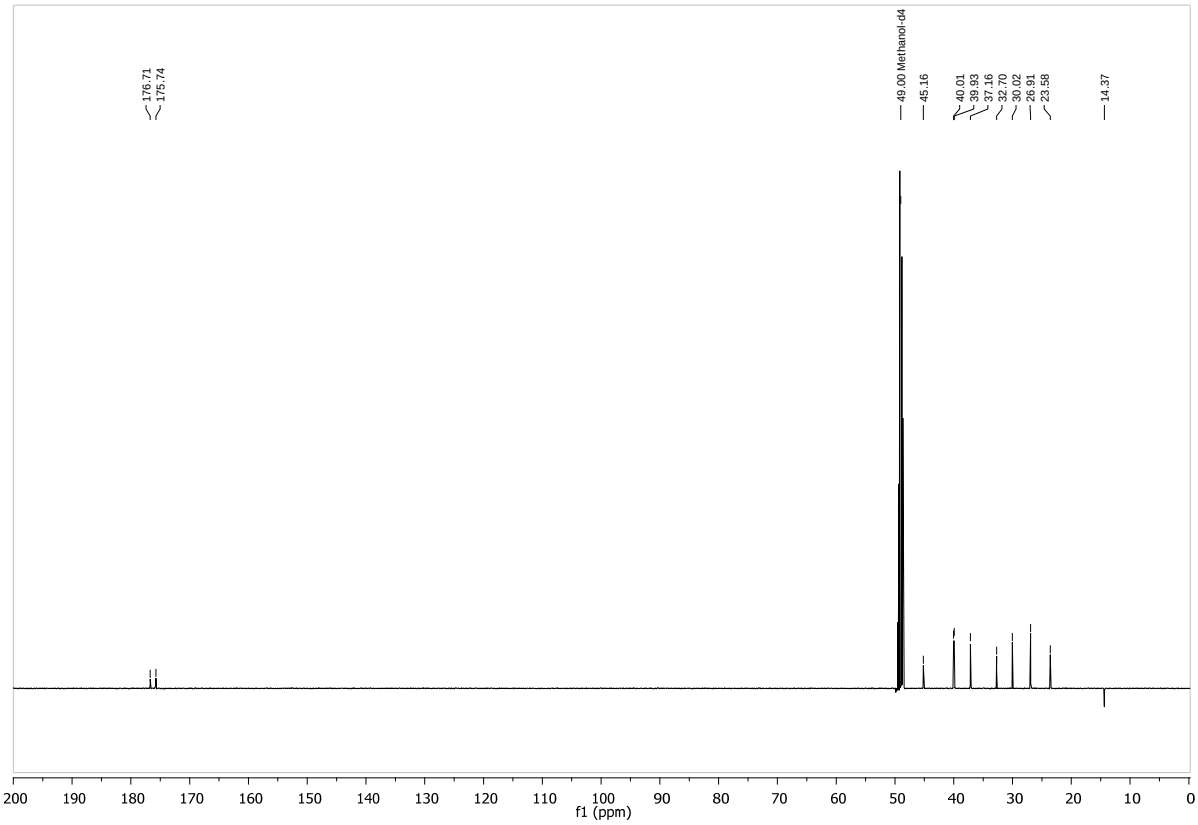
5.8 *^{1}H- and ^{13}C-NMR of probe 10*



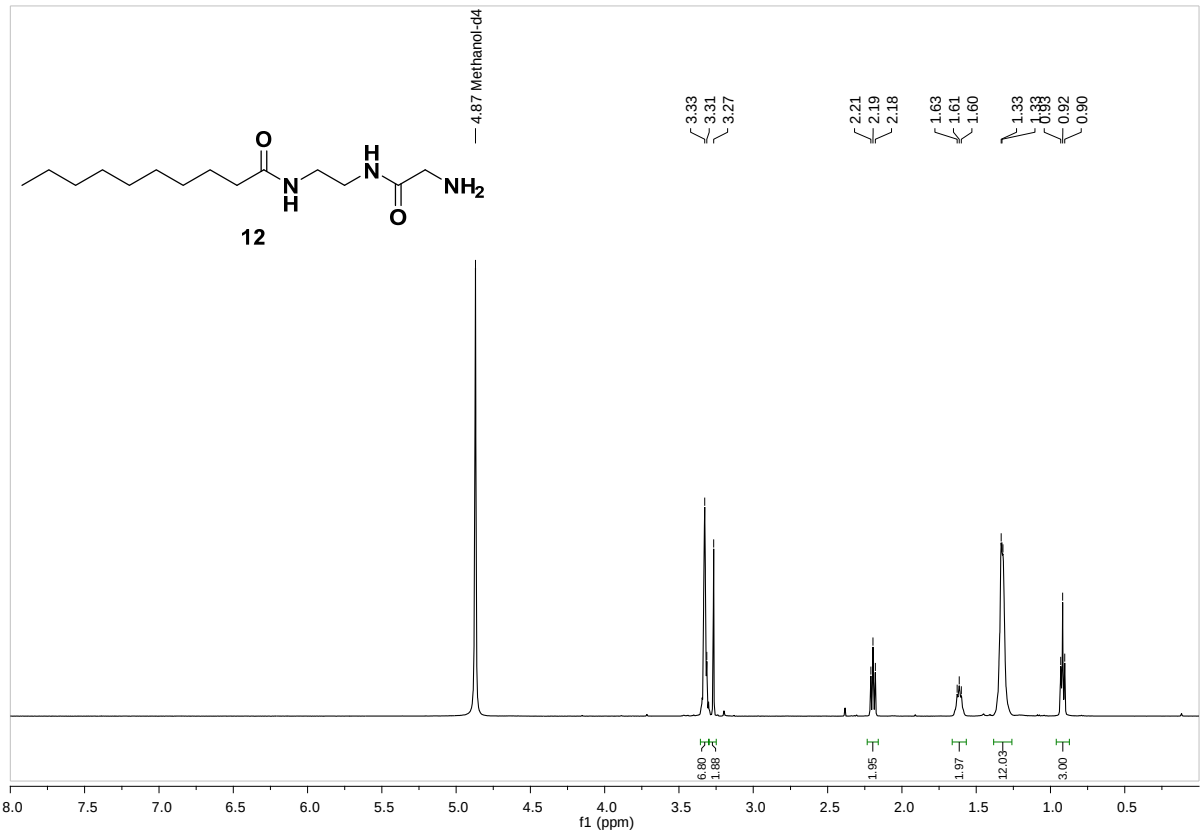


5.9 *^{1}H- and ^{13}C-NMR of probe 11*

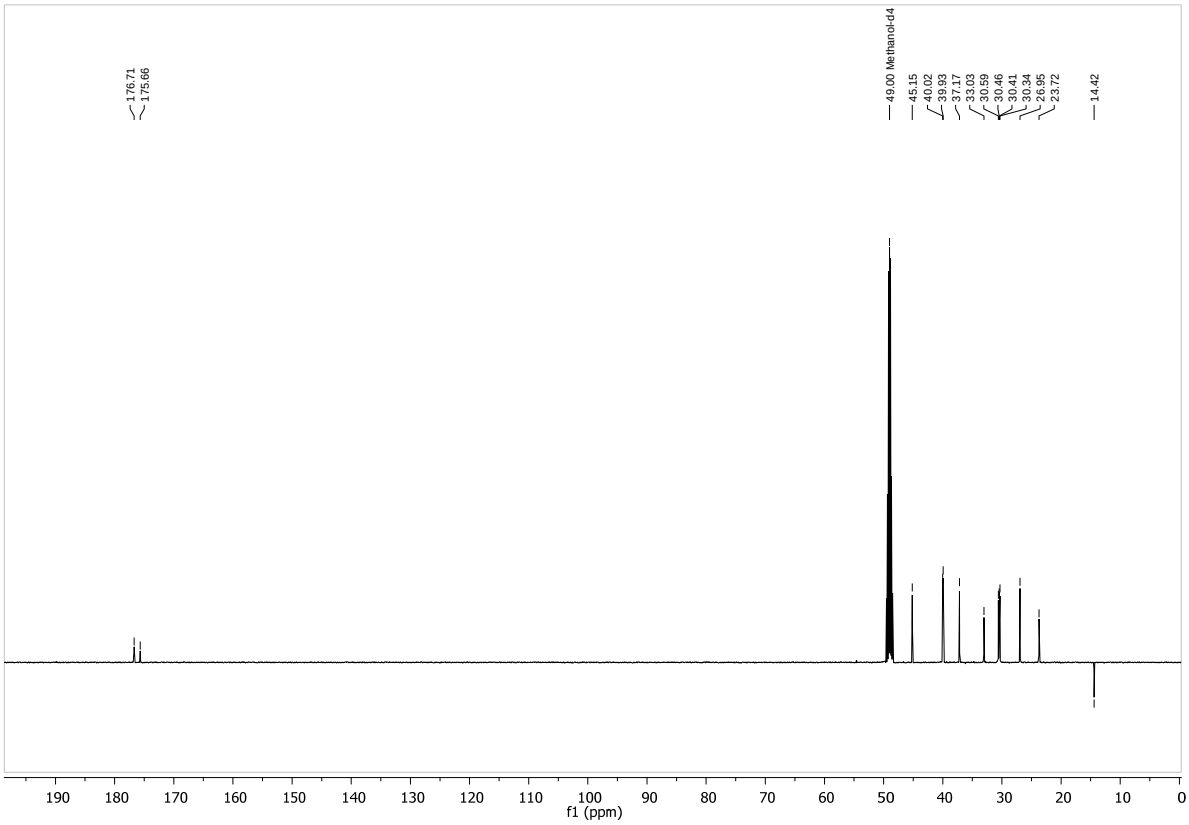




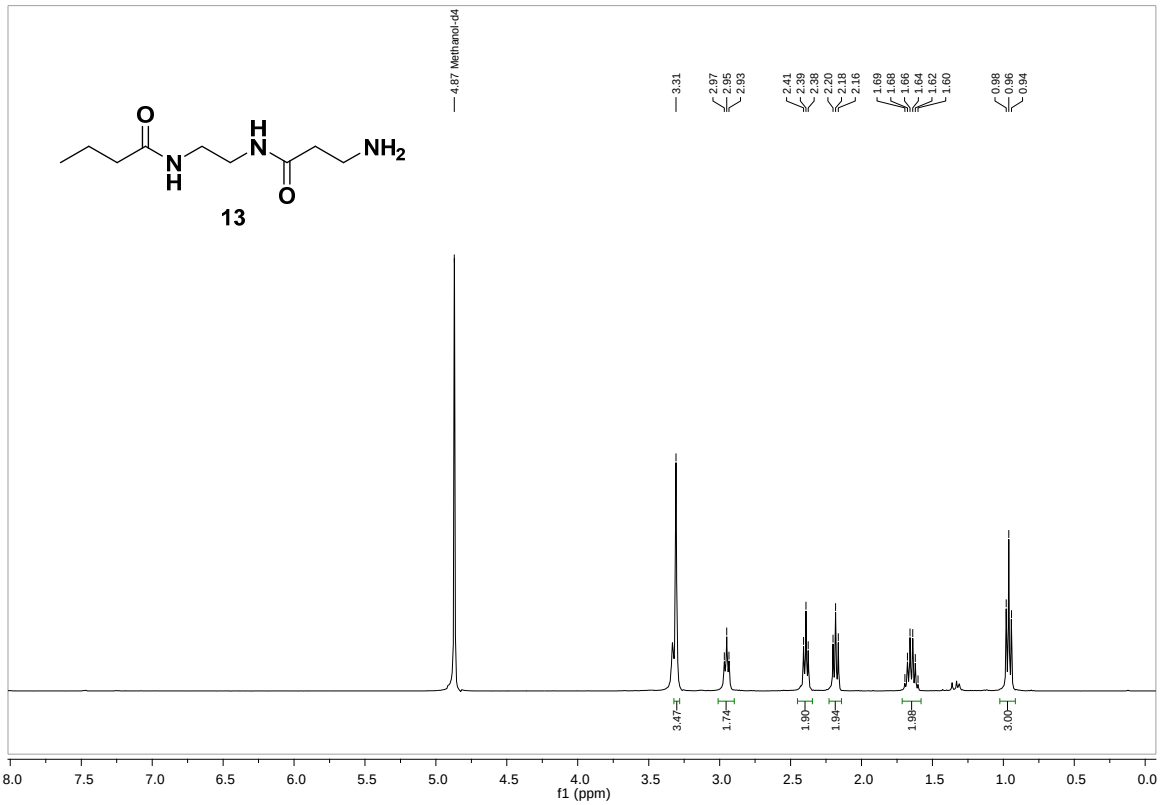
5.10 *^{1}H- and ^{13}C-NMR of probe 12*

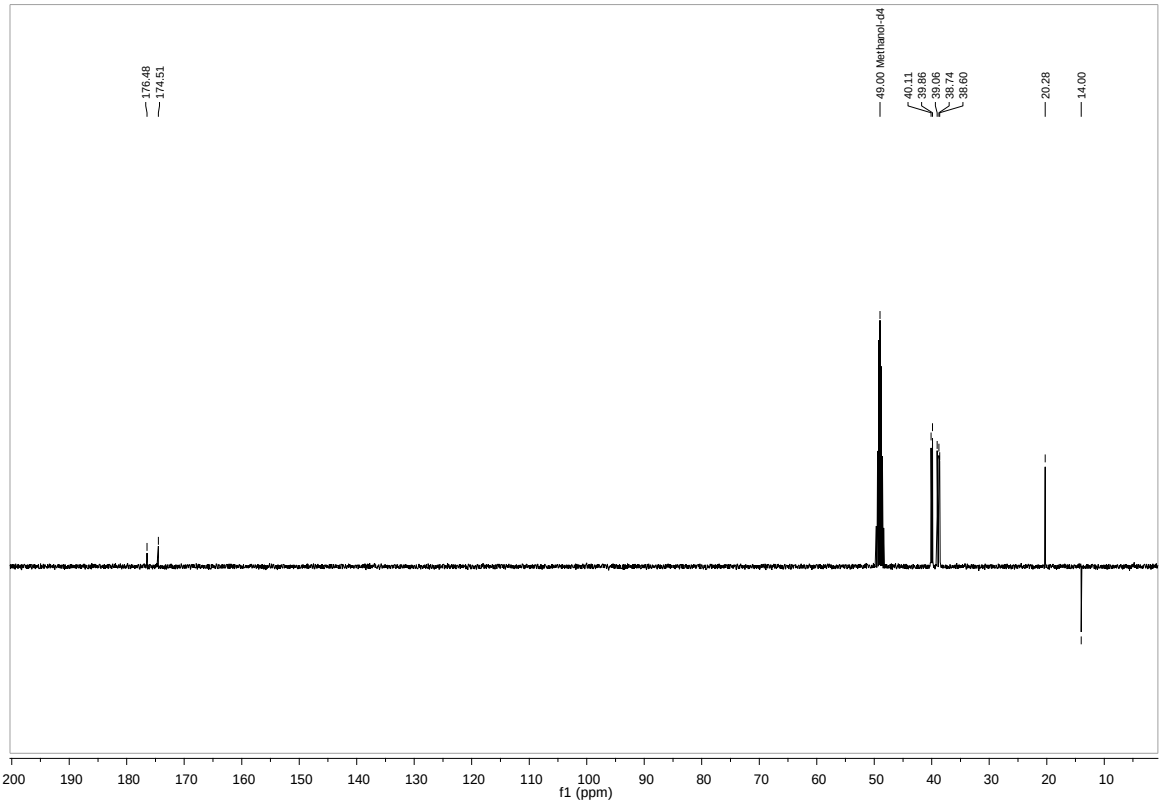


12

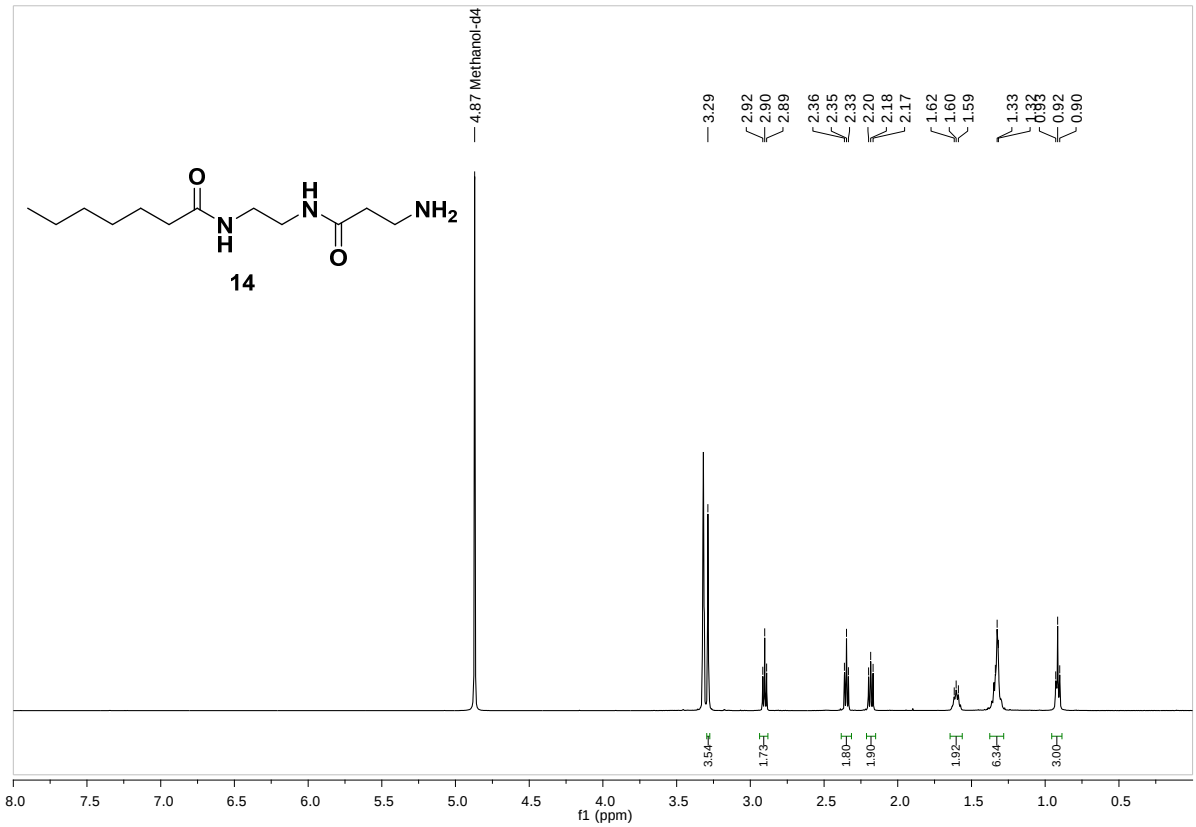


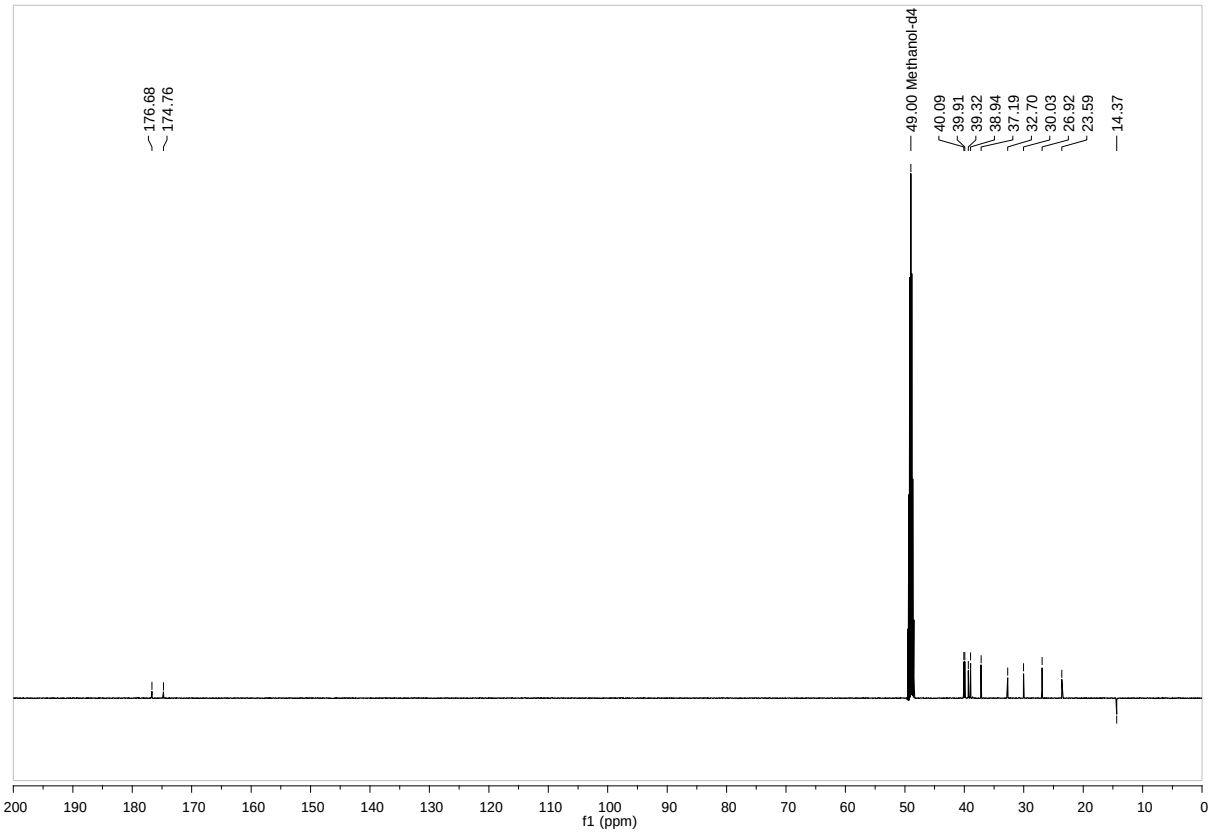
5.11 *^{1}H- and ^{13}C-NMR of probe 13*



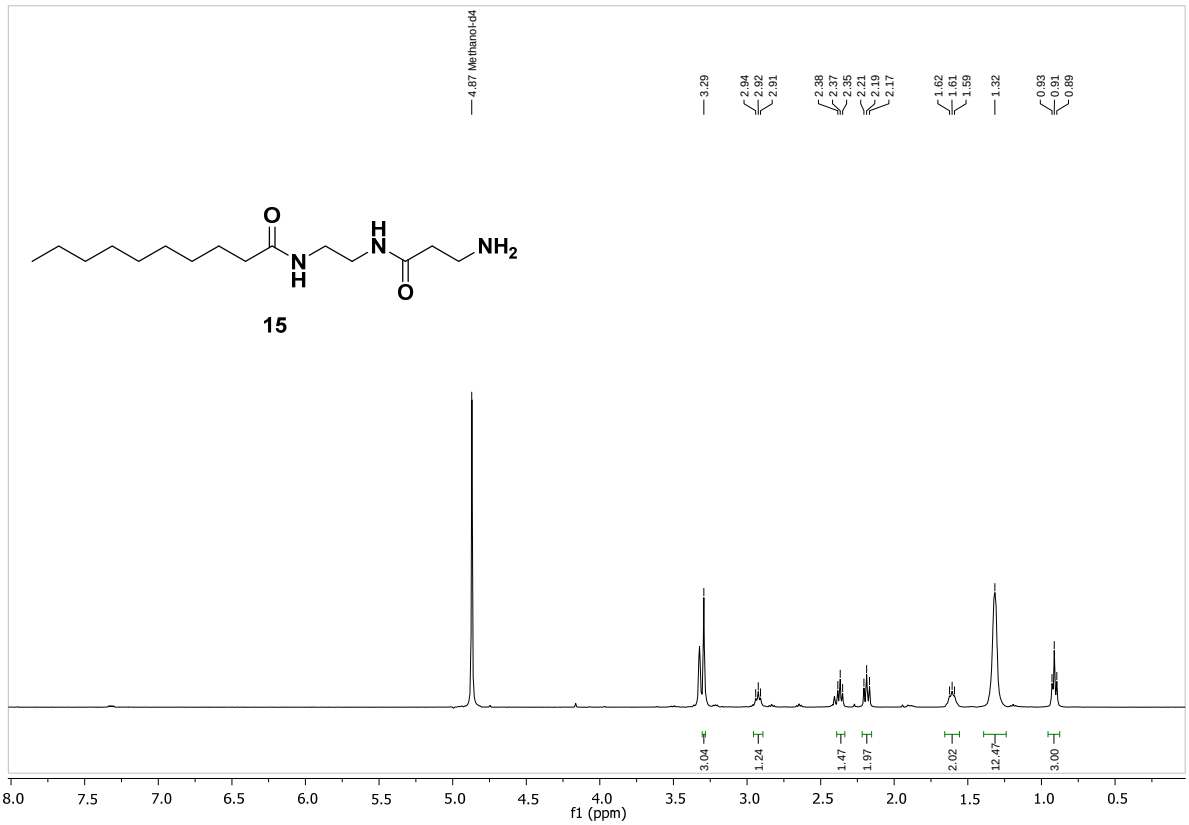


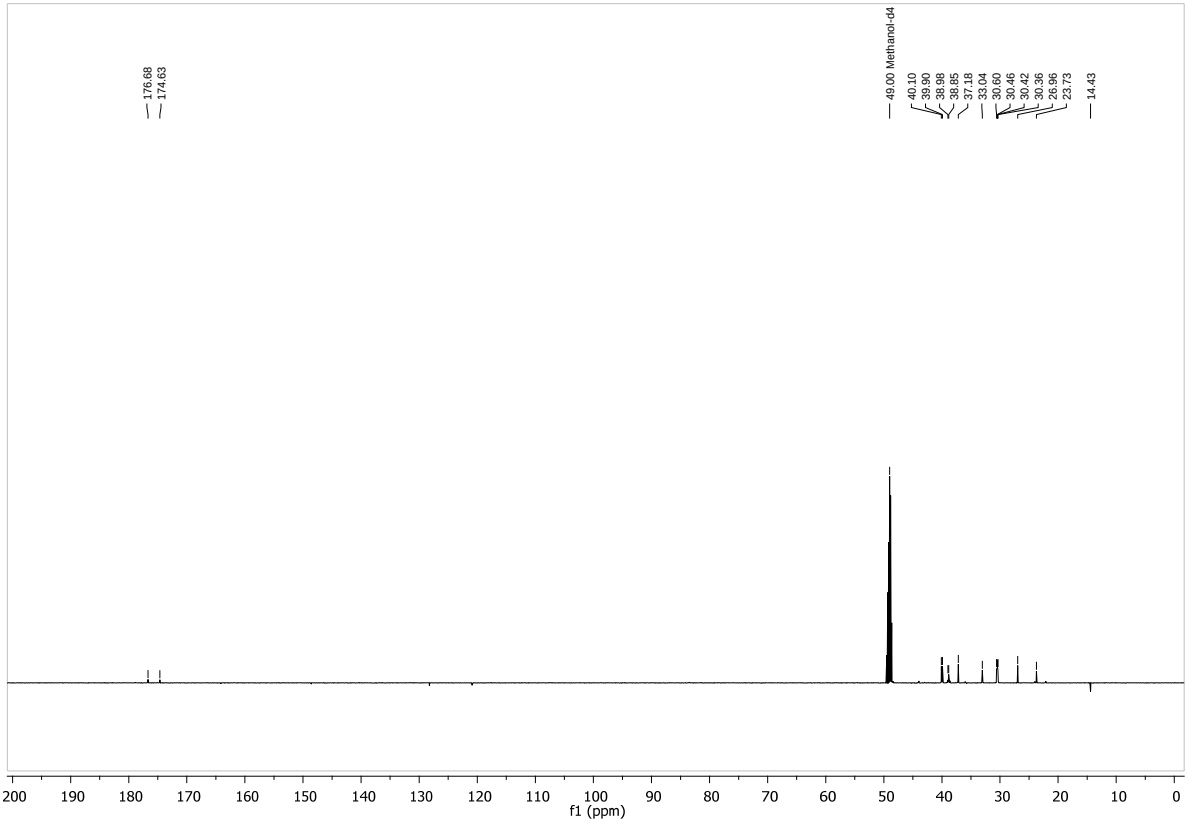
5.12 *^{1}H- and ^{13}C-NMR of probe 14*



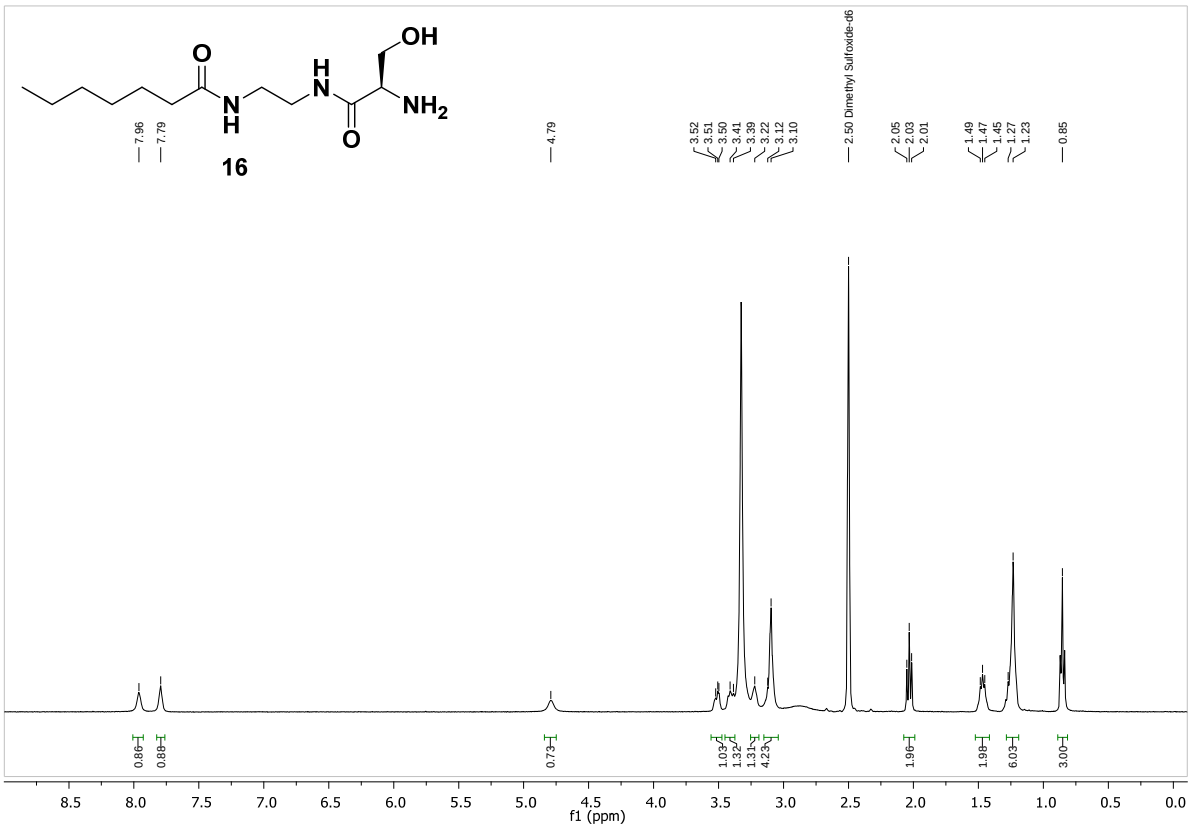


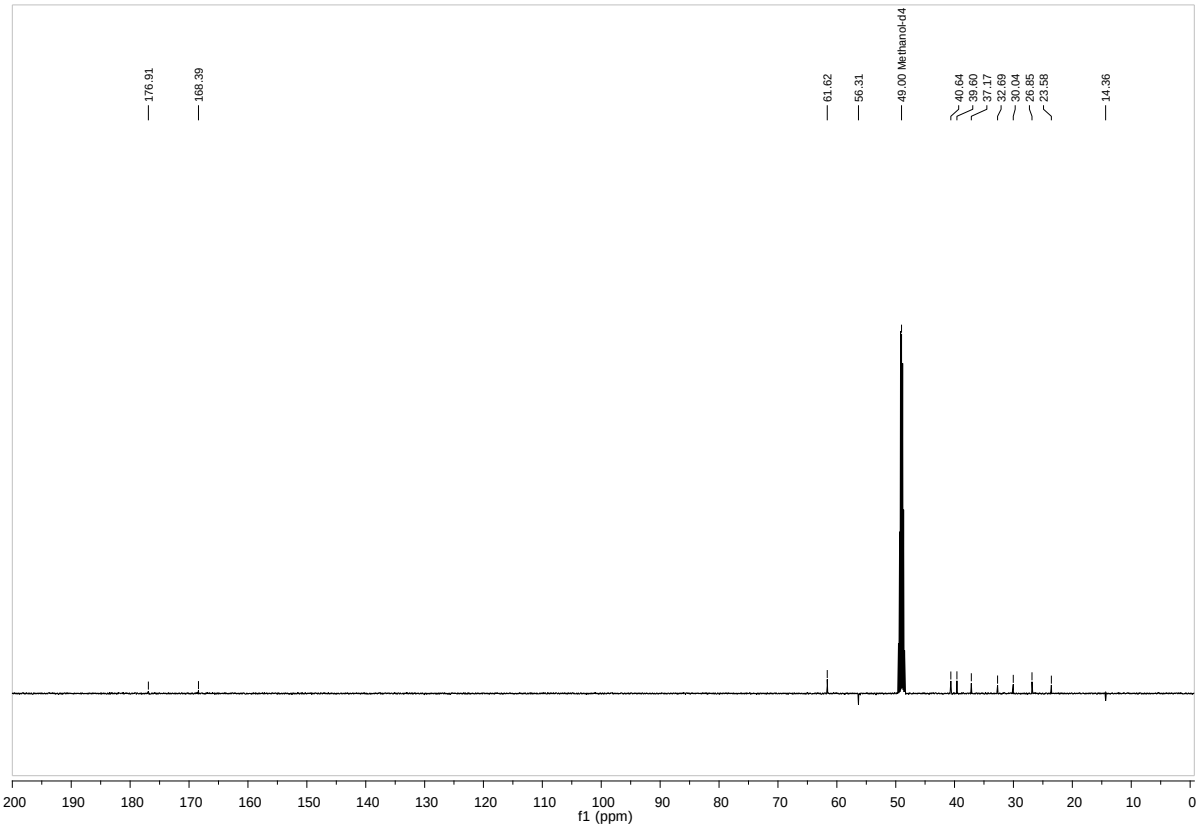
5.13 *^{1}H- and ^{13}C-NMR of probe 15*





5.14 *^{1}H- and ^{13}C-NMR of probe 16*





5.15 *^{1}H- and ^{13}C-NMR of probe 17*