Electronic Supplementary Material (ESI) for ChemComm.

Chemical probes reveal the timing of early chlorination in vancomycin biosynthesis

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Supplementary Information

Contents

Synthetic General Methods

All chemicals were purchased from Sigma-Aldrich, VWR International, Fisher Scientific, Fluorochem or Carbosynth and used without further purification. Dry solvents were purchased from Fisher Scientific or dried using solvent towers. Reagent grade solvents were purchased from Fisher Scientific. Analytical TLC was performed on aluminium sheets precoated with silica gel 60 (F_{254}, Merck) and visualised under ultra-violet light (short wave) and using potassium permanganate or ninhydrin stains and heating with a heat gun. Silica gel for column chromatography was purchased from Sigma-Aldrich (Tech grade, pore size 60 Å, 230-400 mesh).

Infra-red spectra were recorded on a Bruker Alpha-T FTOR spectrometer using 16 scans. Absorption maxima (v_{max}) are quoted in wavenumbers (cm^{-1}). ^{1}H and ^{13}C were recorded in d_4-MeOD or $CDCl_3$ unless stated otherwise on the following Bruker Avance instruments: DPX-300 MHz, DPX-400 MHz, DRX-500MHz or AV-600 MHz. Chemical shifts are reported in parts per million (ppm) and coupling constants (*J*) are reported in Hertz (Hz). High-resolution mass spectra (HRMS) of synthesized compounds were acquired using electrospray ionisation (ESI) on a MaXis UHR-TOF (Bruker Daltonics) or on a Bruker MaXis (ESI-HR-MS). Low-resolution mass spectra were recorded on an Agilent 6130B ESI-MS. Optical rotations were obtained using an AA-1000 Polarimeter from Optical Activity Ltd.

General Method I – Cbz group deprotection

To a Cbz-protected compound (0.90 mmol) and Pd/C (0.72 mmol, 0.8 eqv.) under argon, anhydrous MeOH (18 mL) was added and the solution was degassed by bubbling argon through the solution. $H_{2\,(g)}$ was then bubbled through the solution. After 4 hours, the mixture was filtered through Celite and concentrated *in vacuo* to afford the deprotected product.

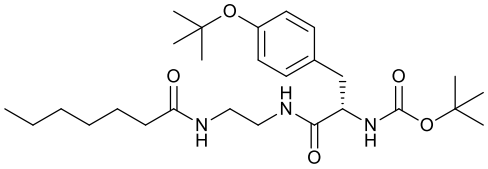
General Method II – Boc group deprotection

The Boc protected compound (0.056 mmol) was dissolved in Et_2O (2 mL) and 2M HCl in Et_2O (0.28 mL, 0.56 mmol, 10 eqv.) was added. The reaction was stirred at room temperature for 3 hours, and the solvent was removed *in vacuo* and the solid triturated with Et_2O, to afford the deprotected product.

Synthesis and Characterisation of Chemical Probes

N-(2-aminoethyl)heptanamide was synthesised according to the method previously reported by our group.[1] The hydrochloride salt 2-heptanamdioethan-1-aminium chloride, **4**, has been prepared previously.[2]

Synthesis of *tert*-butyl (*S*)-(3-(4-(tert-butoxy)phenyl)-1-((2-heptanamidoethyl)amino)-1-oxopropan-2-yl)carbamate (5)

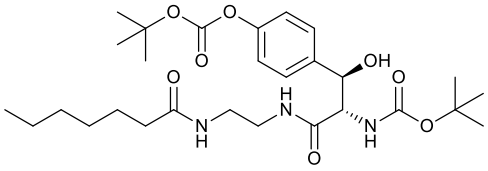


2-Heptanamidoethan-1-aminium chloride (**4,** 504 mg, 2.41 mmol) and Boc-L-Tyr(^{t}Bu)-OH (1.06 g, 3.14 mmol) under an argon atmosphere were dissolved in anhydrous THF (20 mL), then cooled to 0 °C and DIPEA (1.26 mL, 7.23 mmol) was added. After 15 minutes, HATU (1.19 g, 3.14 mmol) was added and the reaction allowed to return to room temperature. After 16 hours, the solvent was removed *in vacuo* and the residue redissolved in EtOAc (30 mL). The organic phase was washed with 1M HCl (40 mL), sat. $NaHCO_{3\,(aq)}$ (40 mL) and sat. $NaCl_{\,(aq)}$ (40 mL). The organics were dried with $MgSO_{4\,(s)}$ and filtered, then concentrated *in vacuo*. The crude product was purified by column chromatography with a stepwise gradient, from 3:1 petroleum ether: EtOAc to pure EtOAc, to afford *tert*-butyl (*S*)-(3-(4-(*ter*t-butoxy)phenyl)-1-((2-heptanamidoethyl)amino)-1-oxopropan-2-yl)carbamate, **5**, as a white solid (832 mg, 70%). $\mathbf{R_f}$= 0.37 in EtOAc; **IR** (thin film) υ_{max}: 2929 (C-H stretch), 1649 (C=O stretch), 1162 (C-O stretch), 845 (C-H bend);

^{1}H NMR (500 MHz, MeOD): δ 7.15 (2H, t, 7.0 Hz, CC**H**CH), 6.91 (2H, t, 8.4 Hz, CCHC**H**), 4.23 – 4.18 (1H, m, COC**H**NH), 3.28 – 3.21 (4H, m, NHC$\mathbf{H_2}$C$\mathbf{H_2}$NH), 3.04 (1H, dd, 5.9 Hz, 13.7 Hz, CHC$\mathbf{H_2}$C), 2.79 (1H, dd, 9.1 Hz, 13.6 Hz, CHC$\mathbf{H_2}$C). 2.17 (2H, t, 7.6 Hz, C$\mathbf{H_2}$CO), 1.59 (2H, quin, 7.0 Hz, C$\mathbf{H_2}$$CH_2$CO), 1.39 (9H, s, COC(C$\mathbf{H_3}$)$_3$), 1.36 – 1.30 (15H, m, COOC(C$\mathbf{H_3}$)$_3$, C$\mathbf{H_2}$C$\mathbf{H_2}$C$\mathbf{H_2}$), 0.91 (3H, t, 6.6 Hz, C$\mathbf{H_3}$); **^{13}C NMR** (125 MHz, MeOD): δ 176.6 (CH_2**C**ONH), 174.8 (NH**C**OCH), 157.7 (NH**C**OO), 155.3 (**C**OC$(CH_3)_3$), 133.8 (CHCH_2**C**), 130.9 (C**C**HCH), 125.2 (CCH**C**H), 80.6 (CHCO**C**$(CH_3)_3$), 79.5 (COO**C**$(CH_3)_3$), 57.87 (CO**C**HNH), 40.1, 39.8 (NH**C**H_2**C**H_2NH), 38.7 (CH**C**H_2C), 37.2 (**C**H_2CO), 32.7 (**C**H_2), 30.1 (COOC(**C**H_3)$_3$), 29.2 (**C**H_2), 28.7 (CHCOC(**C**H_3)$_3$), 26.9

(**C**H_2CH_2CO), 23.6 (**C**H_2), 14.4 (**C**H_3CH_2); **HRMS(ESI):** calculated for $C_{27}H_{45}N_3O_5Na$ $[M+Na]^+$: 514.3251, found: 514.3250; $[\alpha]_D^{28}$: -2.9 (0.068, MeOH).

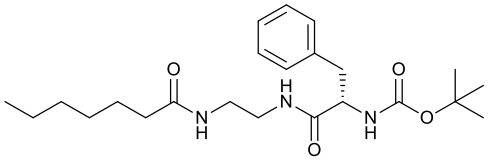
Synthesis of *tert*-butyl ((1*R*,2*S*)-1-(4-((*tert*-butoxycarbonyl)oxy)phenyl)-3-((2-heptanamidoethyl)amino)-1-hydroxy-3-oxopropan-2-yl)carbamate (6)



2-Heptanamidoethan-1-aminium chloride (**4,** 14 mg, 0.066 mmol) and (*2S,3R*)-2-((*ter*t-butoxycarbonyl)amino)-3-(4-((*tert*-butoxycarbonyl)oxy)phenyl)-3-hydroxypropanoic acid (29 mg, 0.073 mmol) were dissolved in anhydrous DMF (1 mL), cooled to 0 °C, and DIPEA (35 µL, 0.20 mmol) added. After 10 minutes, HATU (28 mg, 0.073 mmol) was added, and the reaction stirred at room temperature for 45 minutes. The solvent was removed *in vacuo* by azeotroping with heptane. The crude product was purified by column chromatography using a stepwise gradient, from 1:1 petroleum ether: EtOAc to pure EtOAc, to afford **6** as a white solid (31 mg, 86%). $\mathbf{R_f}$= 0.26 (EtOAc).

^{1}H NMR (500 MHz, $CDCl_3$): 7.40 (2H, d, 8.4 Hz, OCCHC**H**), 7.15 (2H, d, 8.6 Hz, OCC**H**), 6.85 (CON**H**), 6.04 (CON**H**), 5.38 (1H, d, 5.4 Hz, CHO**H**), 5.31 (1H, d, 2.3 Hz, CCHOHC**H**), 4.31 (1H, d, 5.8 Hz, COC**H**NH), 3.48 (2H, q, 7.0 Hz, C$\mathbf{H_2}CH_2$NHCOCH), 3.40 – 3.34 (1H, m, $CH_2C\mathbf{H_2}$NHCOCH), 3.28 – 3.22 (1H, m, $CH_2C\mathbf{H_2}$NHCOCH), 2.15 (2H, t, 7.5 Hz, C$\mathbf{H_2}$CO), 1.63 – 1.56 (2H, m, C$\mathbf{H_2}CH_2$CO), 1.55 (18H, s, C(C$\mathbf{H_3}$)$_3$), 1.36 – 1.31 (6H, m, C$\mathbf{H_2}$), 0.87 (3H, t, 6.8 Hz, C$\mathbf{H_3}CH_2$); **^{13}C NMR** (125 MHz, $CDCl_3$): 174.6 (CH_2**C**ONH), 171.8 (NH**C**OCH), 156.1 (NH**C**OO), 150.6 (O**C**OO), 137.3 (CHOH**C**CH), 127.0 (CHOHC**C**H), 121.2 (OCOC**C**H), 83.6 (OCOO**C**$(CH_3)_3$), 80.7 (NHCOO**C**$(CH_3)_3$), 72.2 (COCH**C**HOH), 59.9 (CO**C**HNH), 40.0 (NH**C**H_2**C**H_2NH), 39.6 (NH**C**H_2**C**H_2NH), 36.7 (**C**H_2CO), 31.5 (**C**H_2), 29.0 (**C**H_2) 27.7 (C(**C**$H_3)_3$), 25.5 (**C**H_2CH_2CO), 22.5 (**C**H_2), 14.0 (**C**H_3CH_2); **HRMS (ESI)**: calculated for $C_{28}H_{45}N_3O_8Na$ $[M+Na]^+$: 574.3099, found: 574.3091.

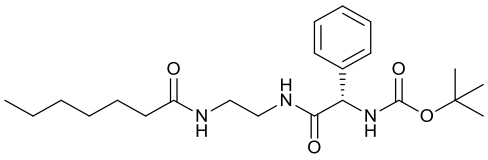
Synthesis of *tert*-butyl (*S*)-(1-((2-heptanamidoethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (7)



2-Heptanamidoethan-1-aminium chloride (**4**, 300 mg, 1.44 mmol), Boc-L-Phe-OH (458 mg, 1.72 mmol), and HATU (654 mg, 1.72 mmol) were dissolved in anhydrous DMF (7 mL) and stirred at room temperature for 10 minutes. DIPEA (0.75 mL, 4.32 mmol) was added, and the reaction mixture stirred at room temperature for 2 hours. The solvent was removed *in vacuo*, and the residue redissolved in EtOAc. The organic phase was washed with 1M HCl (2 x 30 mL), sat. $NaHCO_{3\,(aq)}$ (2 x 25 mL) and sat. $NaCl_{\,(aq)}$ (2 x 25 mL), then dried with $MgSO_{4\,(s)}$, filtered, and concentrated. The crude product was purified using column chromatography using an isocratic elution with EtOAc, to afford *tert*-butyl (*S*)-(1-((2-heptanamidoethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate, **7**, as a white solid (388 mg, 64%). $\mathbf{R_f}$ = 0.39 (EtOAc).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.32 – 7.06 (10H, m, Ar**H**), 6.33 (1H, s, CON**H**), 5.87 (1H, s, CON**H**), 5.34 – 5.31 (1H, m, COCHN**H**), 5.11 – 5.06 (2H, m, COOC**H$_2$**Ar), 4.34 (1H, dd, 6.7 Hz, 6.6 Hz,, COC**H**NH), 3.31 – 3.23 (4H, m, NHC**H$_2$**C**H$_2$**NH), 3.10 – 3.02 (2H, m, CHC**H$_2$**Ar), 2.10 (2H, t, 7.7 Hz, C**H$_2$**CO), 1.61 – 1.53 (2H, m, C**H$_2$**CH_2CO), 1.30 – 1.24 (6H, m, C**H$_2$**), 0.87 (3H, t, 6.5 Hz, C**H$_3$**CH_2); **^{13}C NMR** (125 MHz, $CDCl_3$): δ 174.3 (CH_2**C**ONH), 171.8 (**C**OCH), 156.1 (NH**C**OO), 136.5 (CH**C**CH_2), 136.2 (CH**C**CH_2), 129.4 (**C**H), 128.9 (**C**H), 128.7 (**C**H), 128.4 (**C**H), 128.2 (**C**H), 127.3 (**C**H), 67.3 (COO**C**H_2Ar), 56.6 (CO**C**HNH), 40.2 (NH**C**H_2**C**H_2NH), 39.7 (NH**C**H_2**C**H_2NH), 38.7 (CH**C**H_2Ar), 31.7 (**C**H_2), 29.1 (**C**H_2), 25.8 (**C**H_2CH_2CO), 22.7 (**C**H_2), 14.2 (**C**H_3CH_2); **HRMS (ESI)**: calculated for $C_{26}H_{36}N_3O_4Na$ $[M+Na]^+$: 476.2607, found: 476.2609.

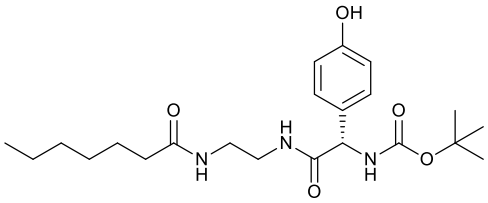
Synthesis of *tert*-butyl (*S*)-(2-((2-heptanamidoethyl)amino)-2-oxo-1-phenylethyl)carbamate (8)



2-Heptanamidoethan-1-aminium chloride (**4,** 250 mg, 1.20 mmol) and Boc-L-Phg-OH (332 mg, 1.32 mmol) under an argon atmosphere were dissolved in anhydrous DMF (3 mL), cooled to 0 °C and DIPEA (627 µL, 3.60 mmol) added. After 15 minutes, HATU (502 mg, 1.32 mmol) was added and the reaction mixture stirred at room temperature for 4 hours. The solvent was removed *in vacuo* and the residue redissolved in EtOAc. The organic phase was washed with 5% LiCl $_{(aq)}$ (5 x 10 mL), 1M HCl (10 mL), sat. $NaHCO_{3\,(aq)}$ (10 mL) and sat. NaCl $_{(aq)}$ (2 x 20 mL), then dried with $MgSO_{4\,(s)}$, filtered and concentrated. The crude product was purified by column chromatography with a stepwise gradient, from 1:1 EtOAc: petroleum ether to pure EtOAc, to afford *tert*-butyl (*S*)-(2-((2-heptanamidoethyl)amino)-2-oxo-1-phenylethyl)carbamate as a white solid (399 mg, 82%). R_f = 0.17 1:1 EtOAc: petroleum ether.

^{1}H NMR (500 MHz, MeOD): δ 7.43 – 7.30 (5H, m, Ar**H**), 5.10 (1H, s, COC**H**NH), 3.33 – 3.25 (4H, m, NHC**H$_2$**C**H$_2$**NH), 2.14 (2H, t, 7.4 Hz, C**H$_2$**CO), 1.61 – 1.54 (2H, m, C**H$_2$**CH_2CO), 1.45 (9H, s, C(C**H$_3$**)$_3$), 1.37 – 1.29 (6H, m, C**H$_2$**), 0.93 (3H, t, 6.9 Hz, C**H$_3$**CH_2); **^{13}C NMR** (125 MHz, MeOD): δ 176.7 (CH_2**C**ONH), 173.6 (NH**C**OCH) ,157.5 (NH**C**OO), 139.2 (COCH**C**CH), 129.8 (**C**H), 129.3 (**C**H), 128.5 (**C**H), 80.9 (**C**$(CH_3)_3$), 60.4 (CO**C**HNH), 40.1 (NH**C**H_2**C**H_2NH), 39.8 (NH**C**H_2**C**H_2NH), 37.2 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 28.7 (C(**C**H_3)$_3$), 26.8 (**C**H_2CH_2CO), 23.6 (**C**H_2), 14.4 (**C**H_3CH_2); **HRMS (ESI)**: calculated for $C_{22}H_{35}N_3O_4Na$ $[M+Na]^+$: 428.2520, found: 428.2522.

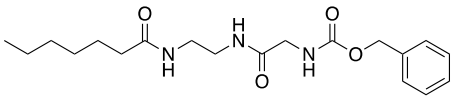
Synthesis of *tert*-butyl (*S*)-(2-((2-heptanamidoethyl)amino)-1-(4-hydroxyphenyl)-2-oxoethyl)carbamate (9)



2-Heptanamidoethan-1-aminium chloride (**4,** 400 mg, 1.92 mmol) and Boc-L-Hpg-OH (564 mg, 2.11 mmol) under an argon atmosphere were dissolved in DMF (5 mL), cooled to 0 °C, and DIPEA (1.00 mL, 5.75 mmol) were added. After 15 minutes, HATU (802 mg, 2.11 mmol) was added and stirred for 4 hours. DMF was removed *in vacuo* and the residue redissolved in EtOAc. The organic phase was washed with 5% LiCl $_{(aq)}$ (5 x 10 mL), 1M HCl (2 x 10 mL), sat. $NaHCO_{3\ (aq)}$ (2 x 10 mL) and sat. NaCl $_{(aq)}$ (2 x 10 mL), then dried with $MgSO_{4\ (s)}$, filtered and concentrated. The crude product was purified by column chromatography with a stepwise gradient, from 1:1 petroleum ether: EtOAc to pure EtOAc, affording *tert*-butyl (*S*)-(2-((2-heptanamidoethyl)amino)-1-(4-hydroxyphenyl)-2-oxoethyl)carbamate, **9**, as a white solid (275 mg, 34%). **R_f =** 0.32 (EtOAc); **IR** (thin film) υ_{max}: 2929 (C-H stretch), 1641 (C=O stretch), 1161 (C-O stretch);

^{1}H NMR (500 MHz, MeOD): δ 7.20 (2H, d, 8.5 Hz, COCHCC**H**), 6.75 (2H, d, 8.5 Hz, COCHCCHC**H**), 4.96 (1H, s, COC**H**NH), 3.35 – 3.22 (4H, m, NHC**H_2**C**H_2**NH), 2.12 (2H, t, 7.4 Hz, C**H_2**CO), 1.58 – 1.52 (2H, m, C**H_2**CH_2CO), 1.44 (9H, s, C(C**H_3**)$_3$), 1.34 – 1.28 (6H, m, C**H_2**), 0.91 (3H, t, 6.8 Hz, C**H_3**CH_2); **^{13}C NMR** (125 MHz, MeOD): δ 176.7 (**C**H_2CONH), 174.2 (NH**C**OCH), 158.7 (CH**C**OH), 157.4 (NH**C**OO), 129.7 (COCH**C**), 116.5 (**C**HCOH), 80.8 (**C**(CH_3)$_3$), 59.9 (CO**C**HNH), 40.1 (NH**C**H_2**C**H_2NH), 39.9 (NH**C**H_2**C**H_2NH), 37.2 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 28.7 (C(**C**H_3)$_3$), 26.8 (**C**$H_2$$CH_2$CO), 23.6 (**C**$H_2$), 14.4 (**C**$H_3$$CH_2$); **HRMS (ESI):** calculated for $C_{22}H_{35}N_3O_5Na$ $[M+Na]^+$: 444.2469, found: 444.2472; **$[\alpha]_D^{28}$**: -57.4 (0.070, MeOH).

Synthesis of *tert*-butyl (2-((2-heptanamidoethyl)amino)-2-oxoethyl)carbamate (10)

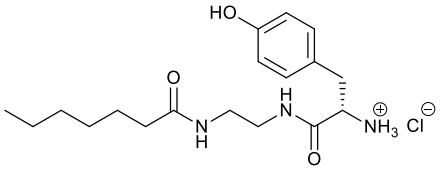


2-Heptanamidoethan-1-aminium chloride (**4**, 347 mg, 1.66 mmol) and Boc-Gly-OH (378 mg, 2.16 mmol) under an argon atmosphere were dissolved in anhydrous DMF (4 mL), cooled to 0 °C and DIPEA (0.86 mL, 4.98 mmol) was added. After stirring for 10 minutes, HATU (821 mg, 2.16 mmol) was added and the reaction allowed to return to room temperature. DMF was largely removed *in vacuo*, and residue dissolved in DCM (25 mL) and washed with 1M HCl (2 x 30 mL), sat. $NaHCO_{3\,(aq)}$ (2 x 30 mL) and sat. NaCl (2 x 30 mL). The organic phase was dried with $MgSO_{4(s)}$, filtered and concentrated to afford *tert*-butyl (2-((2-heptanamidoethyl)amino)-2-oxoethyl)carbamate, **10**, as a white solid (399 mg, 73%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36 (5H, s, Ar**H**), 6.74 (1H, s, CON**H**), 6.02 (1H, s, CON**H**), 5.39 (1H, s, CON**H**), 5.14 (2H, s, C**H$_2$**Ar), 3.85 (2H, d, 5.5 Hz COC**H$_2$**NH), 3.43 – 3.36 (4H, m, NHC**H$_2$**C**H$_2$**NH), 2.16 (2H, t, 4.5 Hz, C**H$_2$**CO), 1.65 – 1.57 (2H, m, C**H$_2$**CH_2CO), 1.32 – 1.26 (6H, m, C**H$_2$**), 0.86 (3H, t, 6.0 Hz, C**H$_3$**CH_2), **LRMS (ESI)**: calculated for $C_{19}H_{30}N_3O_4$ $[M+H]^+$: 363.2, found: 364.0.

The data is in accordance with those reported in the literature.[1]

Synthesis of (*S*)-1-((2-heptanamidoethyl)amino)-3-(4-hydroxyphenyl)-1-oxopropan-2-aminium chloride (11)

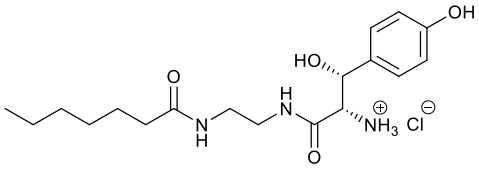


tert-Butyl (*S*)-(3-(4-(*tert*-butoxy)phenyl)-1-((2-heptanamidoethyl)amino)-1-oxopropan-2-yl)carbamate (**5**, 638 mg, 1.30 mmol) was dissolved in 5:3 Et_2O: MeOH (8 mL), and 2M HCl in Et_2O (3.24 mL, 6.49 mmol) added dropwise. The reaction mixture was stirred at room temperature for 5 hours and the solvent removed *in vacuo*. The residue was washed with Et_2O (3 x 5 mL) to afford (*S*)-1-((2-heptanamidoethyl)amino)-3-(4-hydroxyphenyl)-1-oxopropan-2-aminium chloride, **11**, as a foamy brown solid (475 mg, 98%). **R_f**: 0.86 (1:1 DCM:

MeOH); **IR** (thin film) υ_{max}: 3244 (N-H stretch), 2926 (C-H stretch), 1613 (C=O stretch), 826 (C-H bend);

^{1}H NMR (500 MHz, MeOD): δ 7.09 (2H, d, 8.4 Hz, CH_2CC**H**), 6.78 (2H, d, 8.4 Hz, CH_2CCHC**H**), 3.92 (1H, t, 7.3 Hz, COC**H**NH), 3.30 – 3.15 (4H, m, NHC**H$_2$**C**H$_2$**NH), 3.08 (1H, dd, 6.8 Hz, 14.1 Hz, CHC**H$_2$**C), 2.94 (1H, dd, 7.9 Hz, 14.1 Hz, CHC**H$_2$**C), 2.18 (2H, t, 7.6 Hz, C**H$_2$**CO), 1.63 – 1.53 (2H, m, C**H$_2$**CH_2CO), 1.37 – 1.25 (6H, m, C**H$_2$**), 0.89 (3H, t, 6.6 Hz, C**H$_3$**CH_2); **^{13}C NMR** (125 MHz, MeOD): δ 176.8 (CH_2**C**ONH), 169.9 (**C**OCH), 158.3 (**C**OH), 131.6 (CH_2C**C**H), 126.1 (CH_2**C**CH), 116.8 (CH_2CCHC**H**), 56.1 (CO**C**HNH), 40.3 (NH**C**H_2**C**H_2NH), 39.6 (NH**C**H_2**C**H_2NH), 37.9 (CH**C**H_2C), 37.2 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 26.9 (**C**H_2CH_2CO), 23.6 (**C**H_2), 14.4 (**C**H_3CH_2); **HRMS (ESI):** calculated for $C_{18}H_{30}N_3O_3$ $[M+H]^+$: 336.2282, found: 336.2279; **[α]$_D^{28}$**: +32.0 (0.077, MeOH).

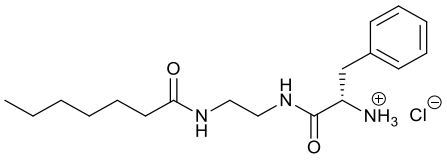
Synthesis of (2*S*,3*R*)-1-((2-heptanamidoethyl)amino)-3-hydroxy-3-(4-hydroxyphenyl)-1-oxopropan-2-aminium chloride (12)



Compound **12** was prepared from **6** (31 mg, 0.056 mmol) according to General Procedure II to afford (2*S*, 3*R*)-1-((2-heptanamidoethyl)amino)-3-hydroxy-3-(4-hydroxyphenyl)-1-oxopropan-2-aminium chloride, **12**, as a white solid (16 mg, 86%). **R$_f$**: 0.72 (1:1 MeOH: DCM); **IR** (thin film) υ_{max}: 3251 (N-H stretch), 2954 (C-H stretch), 2852 (O-H stretch), 1613 (C=O stretch), 726 (C-H bend);

^{1}H NMR (500 MHz, MeOD): δ 7.24 (2H, d, 8.5 Hz, CH_2CC**H**), 6.82 (2H, d, 8.4 Hz, CH_2CCHC**H**), 4.83 (1H, CHC**H**OH), 3.77 (1H, d, 7.7 Hz, COC**H**NH_2), 3.15-3.05 (4H, m, NHC**H$_2$**C**H$_2$**NH), 2.14 (2H, t, 7.8 Hz, C**H$_2$**CONH), 1.59 – 1.52 (2H, m, C**H$_2$**CH_2CO), 1.32 – 1.26 (6H, m, C**H$_2$**), 0.89 (3H, t, 6.8 Hz, C**H$_3$**); **^{13}C NMR** (125 MHz, MeOD): δ 176.8 (CH_2**C**ONH), 168.3 (**C**OCHNH_2), 159.1 (CH**C**OH), 131.3 (**C**HCHCOH), 129.1 (CH_2**C**CH), 116.5 (CH**C**HCOH), 74.6 (CH**C**HOH), 61.2 (CO**C**HNH_2), 40.2 (NH**C**H_2**C**H_2NH), 39.5 (NH**C**H_2**C**H_2NH), 37.1 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 26.8 (**C**H_2CH_2CO). 23.6 (**C**H_2), 14.4 (**C**H_3); **HRMS (ESI)**: calculated for $C_{18}H_{29}N_3O_4Na$ $[M+Na]^+$: 374.2050, found: 374.2048; **[α]$_D^{28}$**: +16.7 (0.042, MeOH).

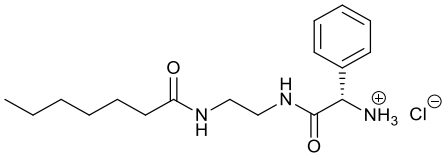
Synthesis of (*S*)-1-((2-heptanamidoethyl)amino)-1-oxo-3-phenylpropan-2-aminium chloride (13)



Compound **13** was prepared from **7** (588 mg, 1.40 mmol) according to General Procedure II. The residue obtained was triturated with Et_2O (3 x 5 mL) to afford (*S*)-1-((2-heptanamidoethyl)amino)-1-oxo-3-phenylpropan-2-aminium chloride, **13**, as a white solid (416 mg, 93%). $\mathbf{R_f}$ = 0.97 (1:1 DCM: MeOH); **IR** (thin film) υ_{max}: 3222 (N-H stretch), 2927 (C-H stretch), 1635 (C=O stretch), 748 (C-H bend);

^{1}H NMR (500 MHz, MeOD): δ 7.32 – 7.19 (5H, m, Ar**H**), 3.50 (1H, t, 7.0 Hz, COC**H**NH_2), 3.25 – 3.15 (4H, m, NHC$\mathbf{H_2}$C$\mathbf{H_2}$NH), 2.99 (1H, dd, 13.5 Hz, 6.5 Hz, CHC$\mathbf{H_2}$), 2.80 (1H, dd, 13.5 Hz, 7.5 Hz, CHC$\mathbf{H_2}$), 2.15 (2H, t, 7.5 Hz, C$\mathbf{H_2}$CO), 1.61 – 1.55 (2H, m, C$\mathbf{H_2}$$CH_2$CO), 1.34 – 1.28 (6H, m, C$\mathbf{H_2}$), 0.92 – 0.88 (3H, m, C$\mathbf{H_3}$$CH_2$); **^{13}C NMR** (125 MHz, MeOD): δ 177.1 (**C**OCH), 176.6 (CH_2**C**ONH), 138.9 (CH_2**C**), 130.4 (CCH**C**HCH), 129.6 (C**C**HCH), 127.8 (CCHCH**C**H), 57.9 (CO**C**HNH), 42.5 (CH**C**H_2), 39.9 (NH**C**H_2**C**H_2NH), 39.8 (NH**C**H_2**C**H_2NH), 37.2 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 26.9 (**C**H_2CH_2CO), 23.6 (**C**H_2), 14.4 (**C**H_3CH_2); **HRMS (ESI)**: calculated for $C_{18}H_{30}N_3O_2$ $[M+H]^+$: 319.2020, found: 319.2019; $\mathbf{[\alpha]_D}^{28}$: +33.4 (0.061, MeOH).

Synthesis of (*S*)-2-((2-heptanamidoethyl)amino)-2-oxo-1-phenylethan-1-aminium chloride (14)

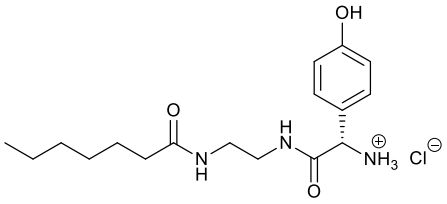


Compound **14** was prepared from **8** (391 mg, 096 mmol) according to General Procedure II to afford (*S*)-2-((2-heptanamidoethyl)amino)-2-oxo-1-phenylethan-1-aminium chloride, **14**, as an off-white solid (300 mg, 91%). $\mathbf{R_f}$ = 0.86 (1:1 DCM: MeOH); **IR** (thin film) υ_{max}: 3294 (N-H stretch), 2910 (C-H stretch), 1682 (C=O stretch), 1641 (C=O stretch), 733 (C-H bend);

^{1}H NMR (500 MHz, MeOD): δ 7.55 – 7.47 (5H, m, Ar**H**), 4.93 (1H, s, COC**H**NH), 3.32 – 3.21 (4H, m, NHC$\mathbf{H_2}$C$\mathbf{H_2}$NH), 2.11 (2H, m, C$\mathbf{H_2}$CO), 1.58 – 1.52 (2H, m, C$\mathbf{H_2}$$CH_2$CO), 1.36 – 1.29 (6H, m, C$\mathbf{H_2}$), 0.93 (3H, t, 6.9 Hz, C$\mathbf{H_3}$$CH_2$); **^{13}C NMR** (125 MHz, MeOD): δ 176.7 (CH_2**C**ONH), 169.2 (**C**OCHNH), 134.5 (COCH**C**CH), 131.1 (**C**H), 130.6 (**C**H), 129.3 (**C**H), 58.0 (CO**C**HNH), 40.5 (NH**C**H_2**C**H_2NH), 39.5 (NH**C**H_2**C**H_2NH), 37.1 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 26.8 (**C**H_2CH_2CO),

23.6 (**C**H_2), 14.4 (**C**H_3CH_2); **HRMS (ESI)**: calculated for $C_{17}H_{28}N_3O_2$: 306.2176, found: 306.2177; **[α]$_D^{26}$**: +64.2 (0.083, MeOH).

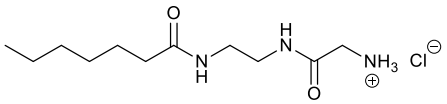
Synthesis of (*S*)-2-((2-heptanamidoethyl)amino)-1-(4-hydroxyphenyl)-2-oxoethan-1-aminium chloride (15)



Compound **15** was prepared from **9** (235 mg, 0.56 mmol) according to General Procedure II to afford (*S*)-2-((2-heptanamidoethyl)amino)-1-(4-hydroxyphenyl)-2-oxoethan-1-aminium chloride, **15**, as a pale yellow solid (198 mg, 99%). **R_f** = 0.59 (1:1 MeOH: DCM); **IR** (thin film) υ_{max}: 3270 (N-H stretch), 2924 (C-H stretch), 1639 (C=O stretch), 835 (C-H bend);

^{1}H NMR (500 MHz, MeOD): δ 7.30 (2H, d, 8.5 Hz, CHCC**H**), 6.84 (2H, d, 8.5 Hz, CHCCHC**H**), 4.81 (1H, s, COC**H**NH_2), 3.32 – 3.18 (4H, m, NHC**H$_2$**C**H$_2$**NH), 2.10 (2H, t, 7.6 Hz, C**H$_2$**CO), 1.56 – 1.51 (2H, m, C**H$_2$**CH_2CO), 1.34 – 1.29 (6H, m, C**H$_2$**), 0.89 (3H, t, 6.8 Hz, C**H$_3$**); **^{13}C NMR** (125 MHz, MeOD): δ 176.8 (CH_2**C**O), 169.7 (**C**O$CHNH_2$), 160.3 (**C**OH), 130.8 (C**C**HCH), 124.9 (**C**CHCH), 117.1 (CCH**C**H), 57.6 (CO**C**HNH_2), 40.4, 39.6 (NH**C**H_2**C**H_2NH), 37.0 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 26.8 (**C**H_2CH_2CO), 23.6 (**C**H_2), 14.4 (**C**H_3); **HRMS (ESI)**: calculated for $C_{17}H_{27}N_3O_3Na$ $[M+Na]^+$: 344.1945, found: 344.1942; **[α]$_D^{26}$**: -21.3 (0.045, MeOH).

Synthesis of 2-((2-heptanamidoethyl)amino)-2-oxoethan-1-aminium chloride (16)

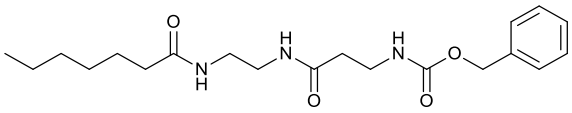


Compound **16** was prepared from **10** (326 mg, 0.90 mmol) according to General Procedure II to afford 2-((2-heptanamidoethyl)amino)-2-oxoethan-1-aminium chloride, **16**, as a white solid (315 mg, 99%).

^{1}H NMR (300 MHz, MeOD): δ 3.34 – 3.31 (4H, m, NHC**H$_2$**C**H$_2$**NH), 3.26 (2H, s, COC**H$_2$**NH), 2.19 (2H, t, 7.5 Hz, C**H$_2$**CO), 1.64 – 1.56 (2H, m, C**H$_2$**CH_2CO), 1.37 – 1.28 (6H, m, C**H$_2$**), 0.92 (3H, t, 6.0 Hz, C**H$_3$**CH_2); **LRMS (ESI):** calculated for $C_{11}H_{24}N_3O_2$ $[M+H]^+$: 229.2, found: 229.4.

The data is in accordance with those reported in the literature.[1]

Synthesis of benzyl (3-((2-heptanamidoethyl)amino)-3-oxopropyl)carbamate (17)

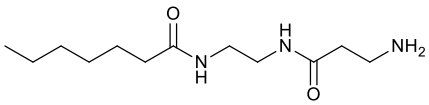


2-Heptanamidoethan-1-aminium chloride (**4**, 300 mg, 1.44 mmol) and Z-β-Ala-OH (353 mg, 1.58 mmol) under an argon atmosphere were dissolved in DMF (2 mL), cooled to 0 °C, and DIPEA (752 µL, 4.32 mmol) added. After 15 minutes, HATU (601 mg, 1.58 mmol) was added and stirred at room temperature for 16 hours. The solvent was removed *in vacuo* and the residue redissolved in EtOAc. The organic phase was washed with 5% $LiCl_{(aq)}$ (5 x 10 mL), 1M HCl (2 x 20 mL), sat. $NaHCO_{3\,(aq)}$ (2 x 20 mL) and sat. $NaCl_{(aq)}$ (20 mL). The organic phase was isolated and dried with $MgSO_{4\,(s)}$, filtered and concentrated to afford pure benzyl (3-((2-heptanamidoethyl)amino)-3-oxopropyl)carbamate (495 mg, 91%).

^{1}H NMR (300 MHz, MeOD): δ 7.35 (5H, s, Ar**H**), 5.08 (2H, s, C**H$_2$**Ar), 3.40 (2H, t, 6.0 Hz, $COCH_2C$**H$_2$**NH_2), 3.30 – 3.22 (4H, m, NHC**H$_2$**C**H$_2$**NH), 2.39 (2H, t, 6.4 Hz, COC**H$_2$**CH_2NH_2), 2.18 (2H, t, 7.20 Hz, C**H$_2$**CO), 1.61 – 1.54 (2H, m, C**H$_2$**CH_2CO), 1.35 – 1.27 (6H, m, C**H$_2$**), 0.91 (3H, t, 5.9 Hz, C**H$_3$**CH_2); **LRMS (ESI)**: calculated for $C_{20}H_{31}O_4Na$ $[M+Na]^+$: 400.2, found: 400.3.

The data are in accordance with those reported in the literature.[1]

Synthesis of *N*-(2-(3-aminopropanamido)ethyl)heptanamide (18)

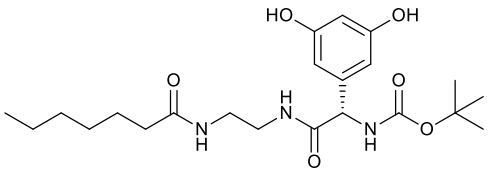


Compound **18** was prepared from **17** (495 mg, 1.31 mmol) according to General Procedure I to afford *N*-(2-(3-aminopropanamido)ethyl)heptanamide, **18**, as an off-white solid (302 mg, 95%).

^{1}H NMR (300 MHz, MeOD): δ 3.35 – 3.28 (4H, m, NHC**H$_2$**C**H$_2$**NH), 2.94 (2H, t, 6.3 Hz, $COCH_2C$**H$_2$**NH_2), 2.38 (2H, t, 6.2 Hz, COC**H$_2$**CH_2NH_2), 2.19 (2H, t, 7.4 Hz, C**H$_2$**CO), 1.66 – 1.57 (2H, m, C**H$_2$**CH_2CO), 1.38 – 1.30 (6H, m, C**H$_2$**), 0.92 (3H, t, 6.0 Hz, C**H$_3$**CH_2); **LRMS (ESI):** calculated for $C_{12}H_{26}N_3O_2$ $[M+H]^+$: 244.2, found: 244.4.

The data are in accordance with those reported in the literature.[1]

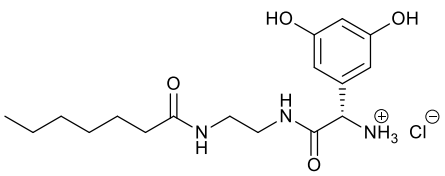
Synthesis of *tert*-butyl (*S*)-(1-(3,5-dihydroxyphenyl)-2-((2-heptanamidoethyl)amino)-2-oxoethyl)carbamate (19)



2-Heptanamidoethan-1-aminium chloride (**4**, 83 mg, 0.39 mmol) and Boc-L-Dhpg-OH (71 mg, 0.25 mmol) under an argon atmosphere were dissolved in anhydrous THF (5 mL) and cooled to 0 °C. $NaHCO_{3\,(s)}$ (84 mg, 1.0 mmol) was added, and after 5 minutes it was followed by 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT, 225 mg, 0.75 mmol). The reaction was stirred at room temperature overnight and diluted with H_2O (15 mL) and EtOAc (15 mL). The aqueous layer was isolated and extracted with EtOAc (3 x 10 mL). The combined organic phases were washed with 1M HCl (15 mL), sat. $NaHCO_{3\,(aq)}$ (3 x 10 mL) and sat. $NaCl_{\,(aq)}$ (15 mL), then dried with $MgSO_{4\,(s)}$, filtered and concentrated. The crude product was purified using column chromatography using an isocratic elution with EtOAc, to afford *tert*-butyl (*S*)-(1-(3,5-dihydroxyphenyl)-2-((2-heptanamidoethyl)amino)-2-oxoethyl)carbamate (29 mg, 27%). $\mathbf{R_f}$= 0.38 (EtOAc);

^{1}H NMR (500 MHz, MeOD): δ 6.32 (2H, d, 1.8 Hz, CHCOHC**H**C), 6.21 (1H, s, C**H**COHCHC), 4.91 (1H, s, COC**H**NH), 3.34 – 3.26 (4H, m, NHC$\mathbf{H_2}$C$\mathbf{H_2}$NH), 2.15 (2H, t, 7.6 Hz, C$\mathbf{H_2}$CO), 1.60 – 1.53 (2H, m, C$\mathbf{H_2}$$CH_2$CO), 1.46 (9H, s, C(C$\mathbf{H_3}$)$_3$), 1.35 – 1.29 (6H, m, C$\mathbf{H_2}$), 0.91 (3H, t, 6.8 Hz, C$\mathbf{H_3}$$CH_2$); **^{13}C NMR** (125 MHz, MeOD): δ 176.8 (CH_2**C**ONH), 173.7 (**C**OCHNH), 159.9 (**C**OH), 157.5 (NH**C**OO) 141.0 (COCH**C**CH), 106.9 (CHC**C**HCOH), 103.4 (COH**C**H), 80.9 (**C**$(CH_3)_3$), 60.2 (CO**C**HNH), 40.1 (NH**C**H_2**C**H_2NH), 39.8 (NH**C**H_2**C**H_2NH), 37.2 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 28.7 (C(**C**H_3)$_3$), 26.8 (**C**$H_2$$CH_2$CO), 23.6 (**C**$H_2$), 14.4 (**C**$H_3$$CH_2$); **LRMS (ESI)**: calculated for $C_{22}H_{35}N_3O_6Na$ $[M+Na]^+$: 460.2, found: 460.2.

Synthesis of (*S*)-1-(3,5-dihydroxyphenyl)-2-((2-heptanamidoethyl)amino)-2-oxoethan-1-aminium chloride (20)



tert-Butyl *(S)*-(1-(3,5-dihydroxyphenyl)-2-((2-heptanamidoethyl)amino)-2oxoethyl)carbamate (**19**, 29 mg, 0.066 mmol) was dissolved in EtOH (4 mL), and 2M HCl in Et_2O (2 mL, 4.0 mmol) and the reaction stirred overnight at room temperature. The solvent was removed *in vacuo* to afford *(S)*-1-(3,5-dihydroxyphenyl)-2-((2-heptanamidoethyl)amino)-2-oxoethan-1-aminium chloride as a pale brown solid (18 mg, 82%). $\mathbf{R_f}$ = 0.51 (1:1 MeOH: DCM); **IR** (thin film) υ_{max}: 2919 (C-H stretch), 1649 (C=O stretch), 1602 (C=O stretch), 1033 (C-O stretch); **^{1}H NMR** (500 MHz, MeOD): δ 6.42 (2H, d, 2.0 Hz, CHC**C**HCOH), 6.36 (1H, t, 2.0 Hz, CCHCOH**C**H), 4.70 (1H, s, COC**H**NH_2), 3.37 – 3.21 (4H, m, NHC$\mathbf{H_2}$C$\mathbf{H_2}$NH), 2.18 – 2.14 (2H, m, C$\mathbf{H_2}$CO), 1.59 – 1.54 (2H, m, C$\mathbf{H_2}$$CH_2$CO), 1.36 – 1.28 (6H, m, C$\mathbf{H_2}$), 0.92 (3H, t, 6.8 Hz, C$\mathbf{H_3}$$CH_2$); **^{13}C NMR** (125 MHz, MeOD): δ 176.9 (CH_2**C**ONH), 169.2 (**C**OCHNH), 160.7 (**C**OH), 136.2 (COCH**C**), 107.4 (CHC**C**HCOH), 104.9 (COH**C**HCOH), 57.9 (CO**C**HNH_2), 40.5 (NH**C**H_2**C**H_2NH), 39.5 (NH**C**H_2**C**H_2NH), 37.1 (**C**H_2CO), 32.7 (**C**H_2), 30.0 (**C**H_2), 26.8 (**C**$H_2$$CH_2$CO), 23.6 (**C**$H_2$), 14.4 (**C**$H_3$$CH_2$); **HRMS (ESI)**: calculated for $C_{17}H_{28}N_3O_4$ $[M+H]^+$: 338.2074, found: 338.2075; **[α]$_D^{28}$**: +48.4 (0.052, MeOH).

Supplementary References

1 Y. T. C. Ho, D. J. Leng, F. Ghiringhelli, I. Wilkening, D. P. Bushell, O. Kostner, E. Riva, J. Havemann, D. Passarella and M. Tosin, *Chem Commun.*, 2017, **53**, 7088–7091.

2 I.-H. Kim, C. Morisseau, T. Watanabe and B. D. Hammock, *J. Med. Chem.*, 2004, **47**, 2110–2122.

Feeding Experiments

General Microbiology Methods

For all feeding experiments on plate, the probes were dissolved in MeOH and added to the agar and media during plate preparation. All feeding experiments were conducted in duplicates. All glassware and media were sterilised by autoclaving (Astell). Liquid cultures were grown with shaking in an Innova 44 incubator/ shaker (New Brunswick Scientific); solid cultures were grown in a Heraeus incubator (Thermo).

Seed cultures of *A. orientalis* NRRL 2452 (10 mL, with springs) were grown in R5 media for 5 days at 30 °C with shaking at 180 rpm. For feedings on solid media, R5 agar plates containing the probes at 2.0 mM concentration were inoculated with 50 µL seed culture and grown for 5 days at 30 °C. For chemical supplementation of liquid cultures, R5 media (5 mL, with springs) was inoculated with 50 µL seed culture and grown overnight at 30 °C with shaking at 180 rpm. The culture was then supplemented once daily with probe (0.5 mM in 25 µL MeOH) for 4 days, to a final concentration of 2.0 mM and then lyophilized. The lyophilised solid was extracted with 10 mL MeOH, concentrated and redissolved in 1 mL HPLC-grade MeOH. The plates were extracted with MeOH (10 mL), concentrated and redissolved in 1 mL HPLC-grade MeOH for HPLC-MS^n analysis on a MaXis Impact and on an Orbitrap Fusion instrument.

R5 medium:
103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2.6H_2O$, 10 g glucose, 0.1 g casamino acids, 5 g yeast extract, 5.73 g TES buffer. dH_2O up to 893 mL. After autoclaving, add 2 mL trace elements solution (0.2 gL^{-1} $FeCl_3.6H_2O$, 0.01 gL^{-1} of $Na_2B_4O_7.10H_2O$, $(NH_4)_6Mo_7O_{24}.5H_2O$, $CuCl_2.2H_2O$, $MnCl_2.4H_2O$, 0.04 gL^{-1} $ZnCl_2$), and 80 mL of 0.33 M $CaCl_2$, 10 mL of 3.96 M KH_2PO_4, and 15 mL of 1.74 M L-proline.

R5 agar medium:
103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2.6H_2O$, 10 g glucose, 0.1 g casamino acids, 5 g yeast extract, 5.73 g TES buffer, 15 g agar. dH_2O up to 893 mL. After autoclaving, add 2 mL trace elements solution (0.2 gL^{-1} $FeCl_3.6H_2O$, 0.01 gL^{-1} of $Na_2B_4O_7.10H_2O$, $(NH_4)_6Mo_7O_{24}.5H_2O$, $CuCl_2.2H_2O$, $MnCl_2.4H_2O$, 0.04 gL^{-1} $ZnCl_2$), and 80 mL of 0.33 M $CaCl_2$, 10 mL of 3.96 M KH_2PO_4, and 15 mL of 1.74 M L-proline.

High resolution mass spectrometry analyses of extracts

Preliminary analysis of organic extracts using a MaXis Impact UHR-TOF (Bruker Daltonics) instrument. Samples (5 µL) were injected onto an Agilent Eclipse C18 column (1.8 µm, 100 mm x 2.1 mm). Mobile phase buffer A was composed of 0.1% aqueous formic acid and mobile phase buffer B was composed of 100% acetonitrile containing 0.1% formic acid. The following solvent gradient was applied: 5% B 0 - 5 min; 5 - 100% B 5 - 17.3 min; 100% B 17.3 - 22.3 min; 100 - 5% B 25.3 - 30 min, at a flow rate of 0.2 mL/min. Spectra were recorded in positive ionisation mode, scanning from m/z 0 to 2000 with the resolution set at 45K. Gathered data were internally calibrated once they have been acquired from the instrument using the Bruker Daltronics Data Analysis software. A calibration curve was fitted to the peaks and applied to the spectrum.

Detailed analysis of all the extracts deriving from chemical probe feeding experiments wasperformed on a Thermo Orbitrap Fusion (Q-OT-qIT) instrument. Reverse phase chromatography was used to separate the mixtures prior to MS analysis. Two columns were used: an Acclaim PepMap µ-precolumn cartridge 300 µm i.d. x 5 mm 5 µm 100 Å and an Acclaim PepMap RSLC 75 mm 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 buffer B was composed of 100% acetonitrile containing 0.1% formic acid. Samples (2 µL) were loaded onto the µ-precolumn equilibrated in 2% aqueous acetonitrile containing 0.1% trifluoroacetic acid for 8 mins at 10 µL min-1 after which compounds were eluted onto the analytical column following the gradient shown below. Eluting cations were converted to gas-phase ions by electrospray ionization and analysed. Scans of precursors from 150 to 1500 *m/z* were performed at 60K resolution (at 200 *m/z*) with a 4 x 10^5 ion count target. Tandem MS were performed by isolation at 1.6 Th with the quadrupole, HCD fragmentation with normalized collision energy of 32, and rapid scan MS analysis in the ion trap. The MS^2 ion count target with set to 2 x 10^4 and the maximum injection time was 50 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

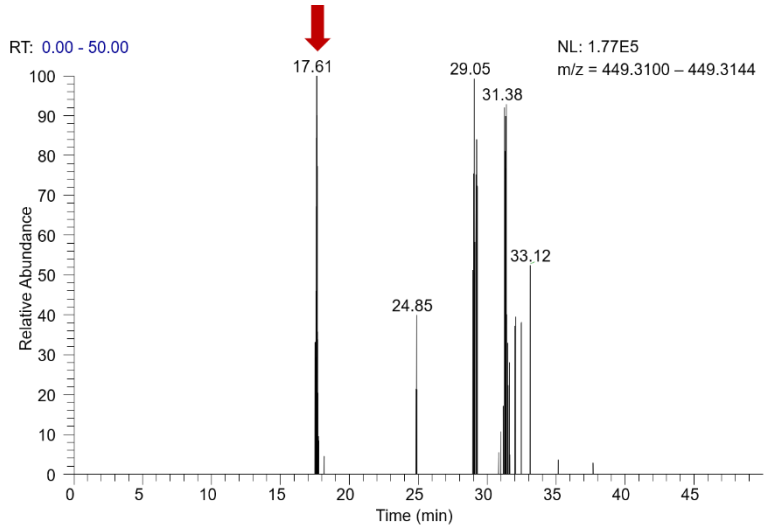
was turned on. The instrument was run in top speed mode with 5 s cycles, meaning the instrument would continuously perform MS^2 events until the list of non-excluded precursors diminishes to zero or 5 s, whichever is shorter. Fusion runs were performed with Survey scans of precursors from 150 to 1500 *m/z* 60K resolution.

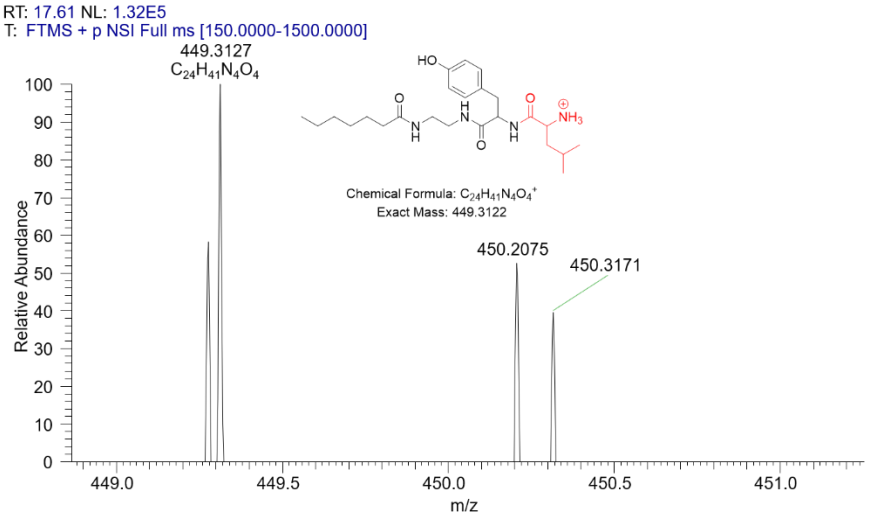
The gradient used to separate the mixtures is illustrated in the Table below. 2 µL of extract was injected, and a flow rate of 0.3 µL/min was used.

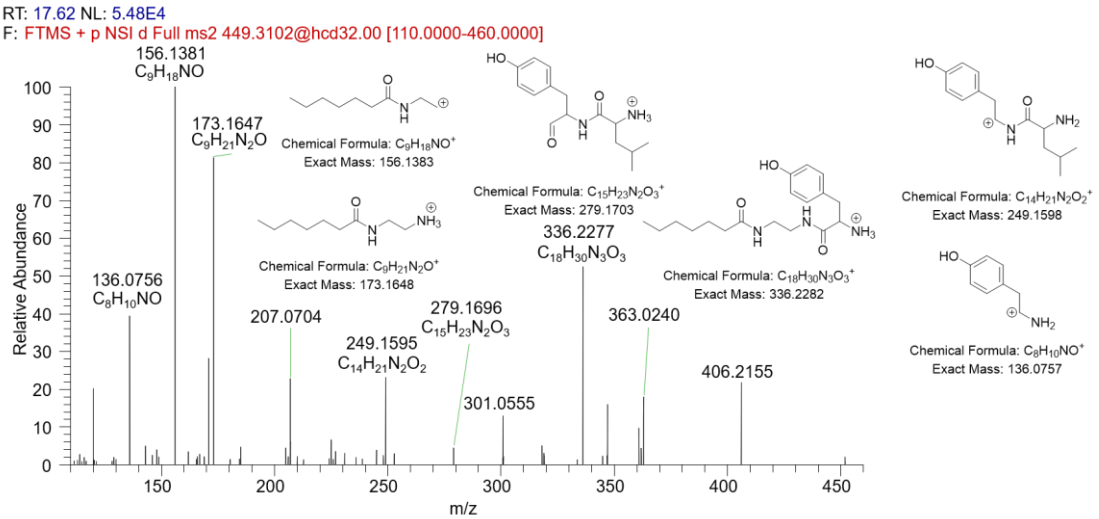
Time (mins)	**Solvent B (%)**
0	3
5	5
30	80
40	80
41	3
50	3

Capture of peptide intermediates from *A. orientalis* by tyrosine-based chain termination probe (11)

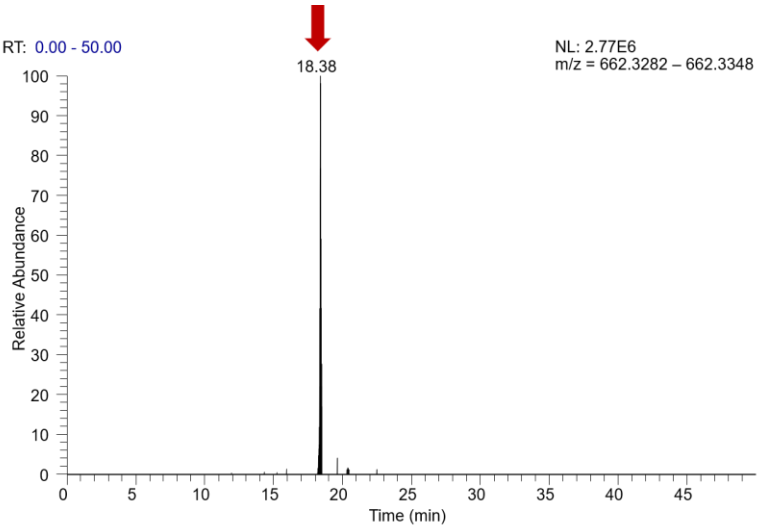
Dipeptide

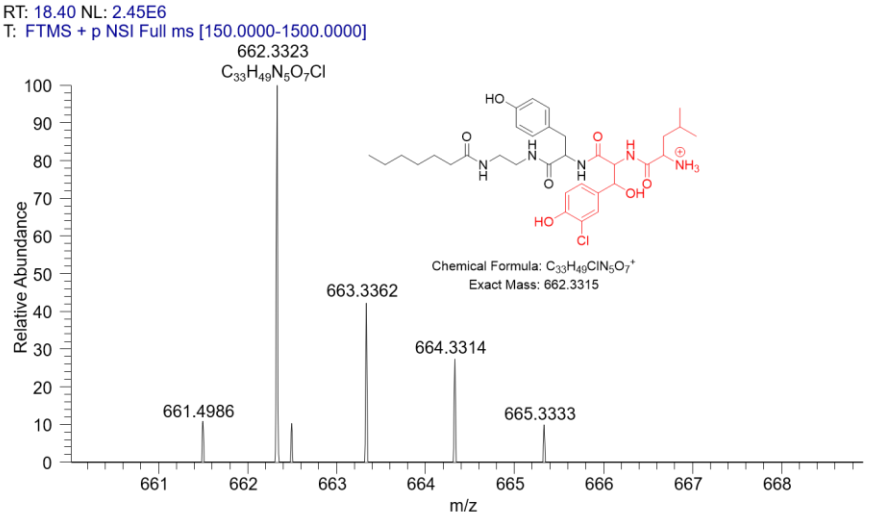


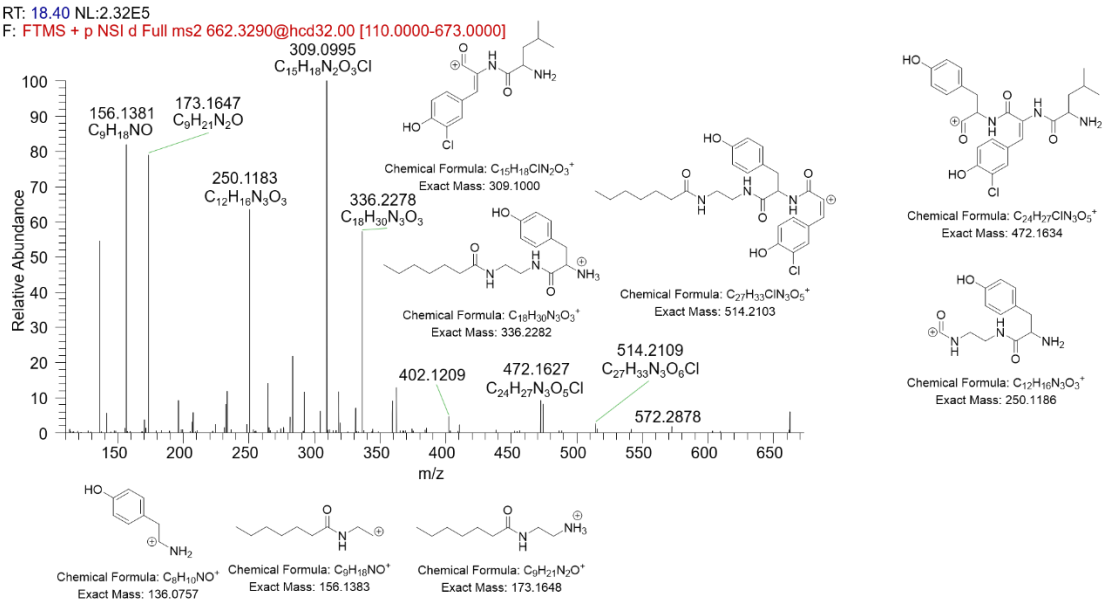




Tripeptide


RT: 0.00 - 50.00
18.38
NL: 2.77E6
m/z = 662.3282 – 662.3348
Relative Abundance
Time (min)


RT: 18.40 NL: 2.45E6
T: FTMS + p NSI Full ms [150.0000-1500.0000]
662.3323
C33H49N5O7Cl
663.3362
664.3314
661.4986
665.3333
Relative Abundance
m/z
Chemical Formula: C33H49ClN5O7+
Exact Mass: 662.3315

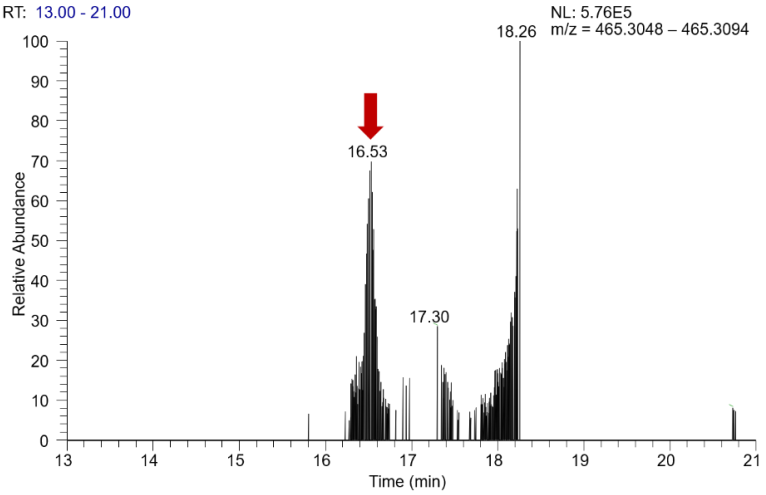

RT: 18.40 NL:2.32E5
F: FTMS + p NSI d Full ms2 662.3290@hcd32.00 [110.0000-673.0000]
309.0995
C15H18N2O3Cl
156.1381
C9H18NO
173.1647
C9H21N2O
250.1183
C12H16N3O3
336.2278
C18H30N3O3
402.1209
472.1627
C24H27N3O5Cl
514.2109
C27H33N3O6Cl
572.2878
Relative Abundance
m/z
Chemical Formula: C15H18ClN2O3+
Exact Mass: 309.1000
Chemical Formula: C18H30N3O3+
Exact Mass: 336.2282
Chemical Formula: C27H33ClN3O5+
Exact Mass: 514.2103
Chemical Formula: C8H10NO+
Exact Mass: 136.0757
Chemical Formula: C9H18NO+
Exact Mass: 156.1383
Chemical Formula: C9H21N2O+
Exact Mass: 173.1648

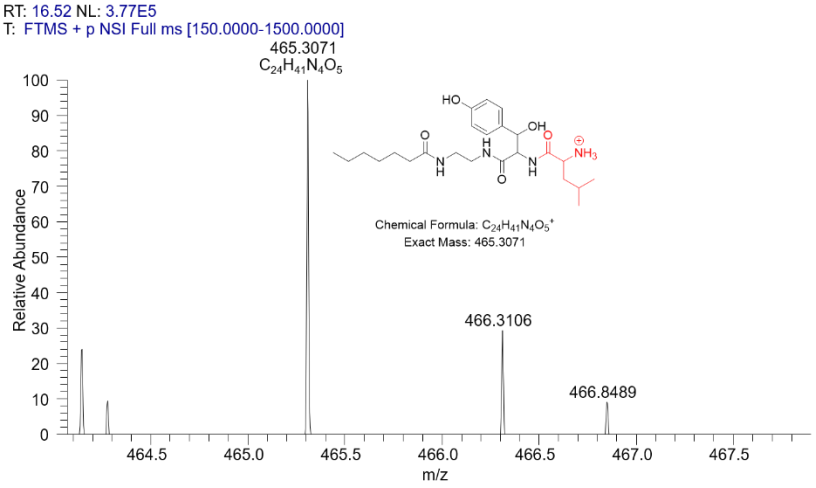
Chemical Formula: C24H27ClN3O5+
Exact Mass: 472.1634

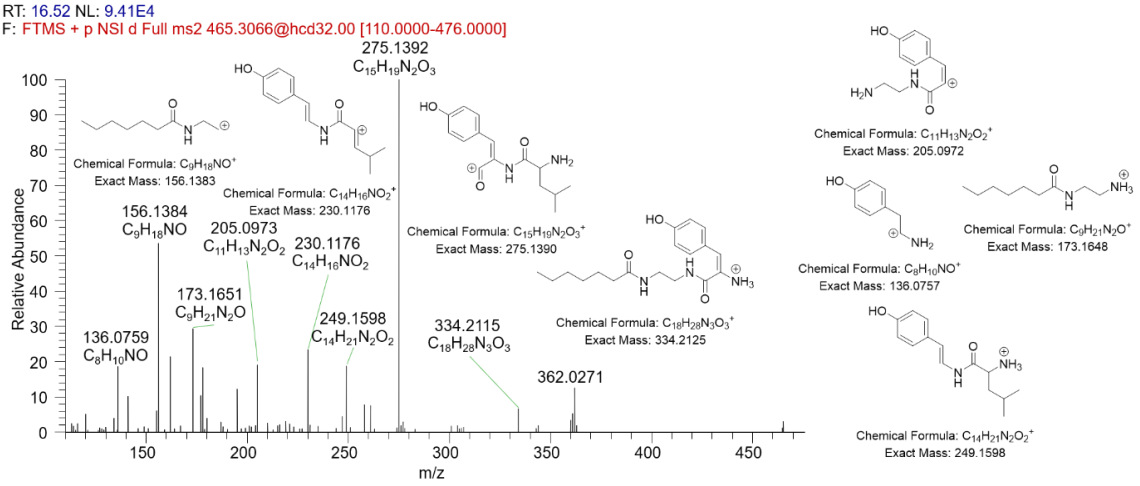
Chemical Formula: C12H16N3O3+
Exact Mass: 250.1186

Capture of peptide intermediates from *A. orientalis* by β-hydroxytyrosine-based chain termination probe (12)

Dipeptide

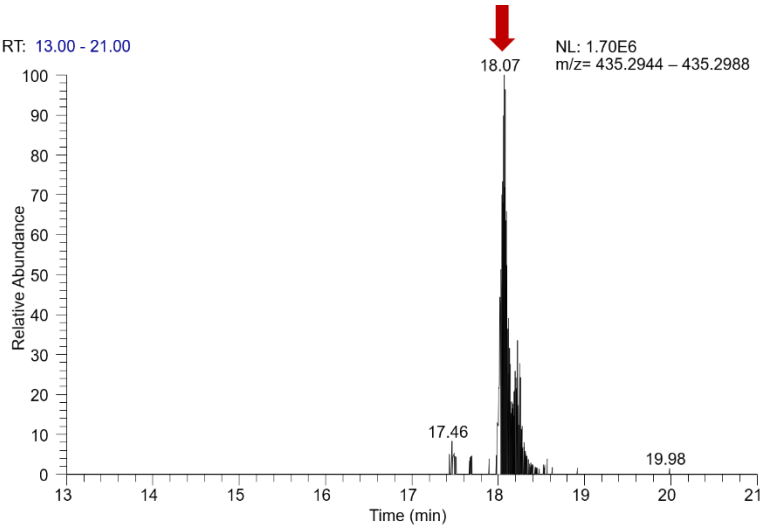


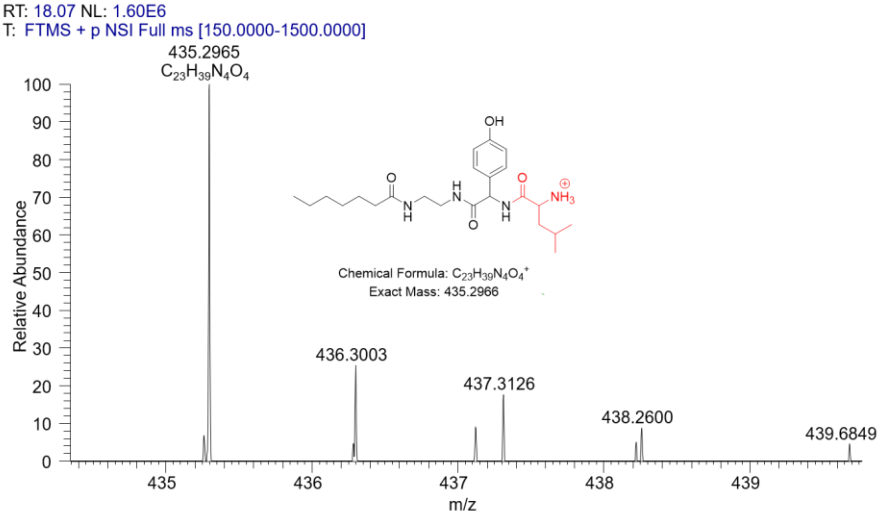


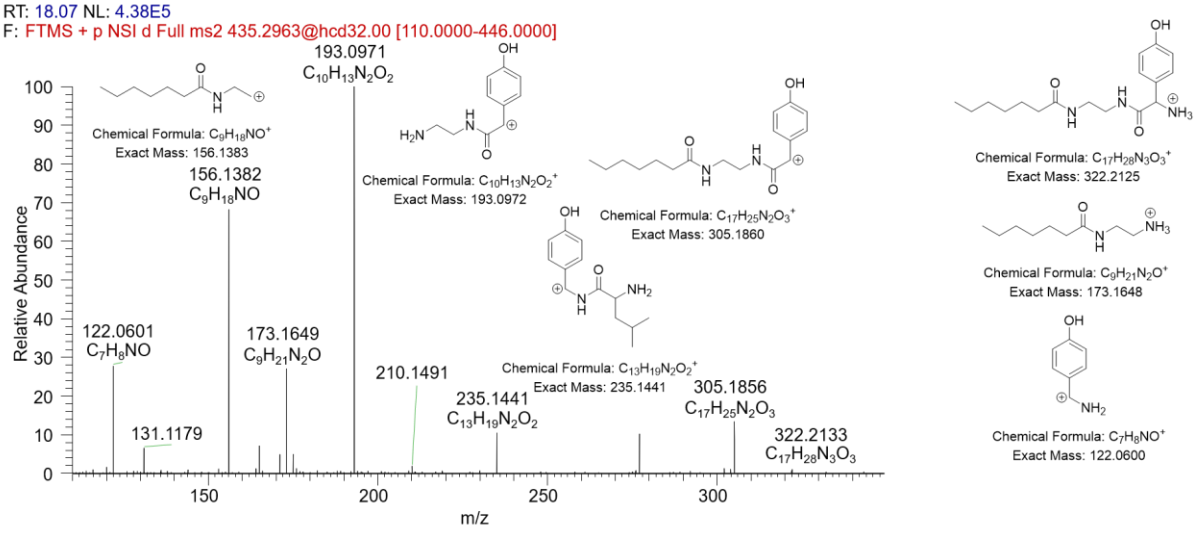


Capture of peptide intermediates from *A. orientalis* by 4-hydroxyphenylglycine-based chain termination probe (15)

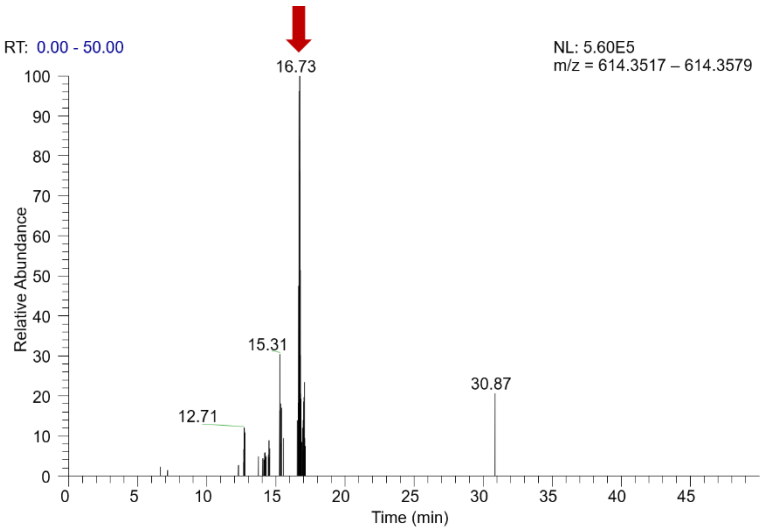
Dipeptide

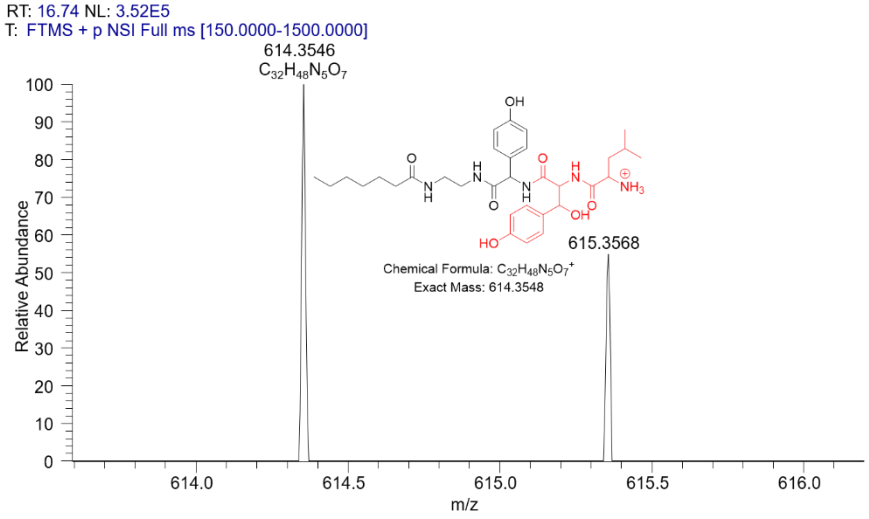


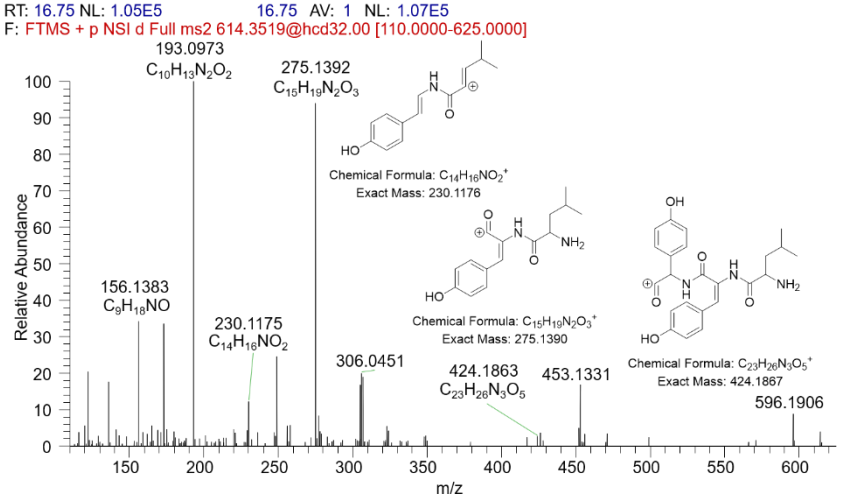


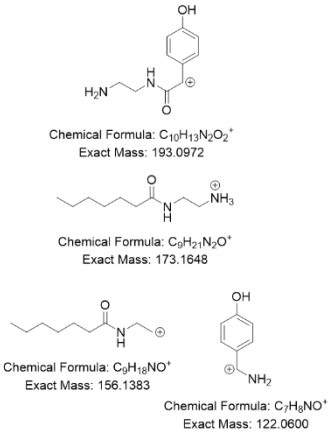


Tripeptide

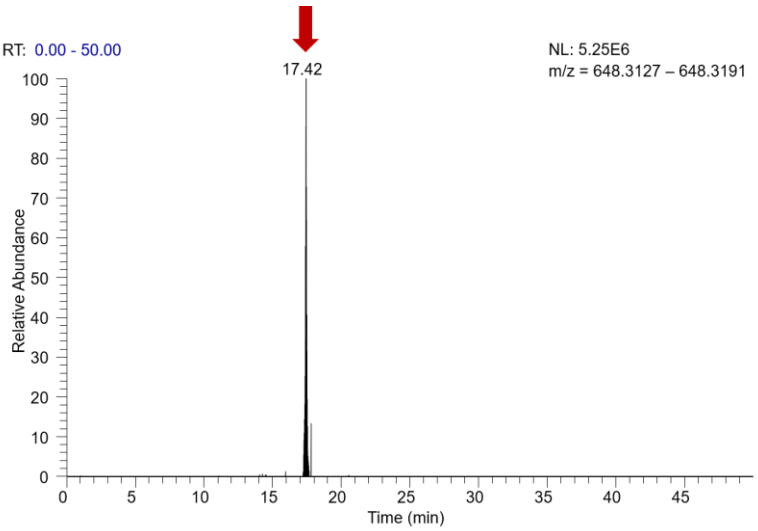

RT: 0.00 - 50.00
NL: 5.60E5
m/z = 614.3517 – 614.3579
16.73
15.31
30.87
12.71
Relative Abundance
Time (min)

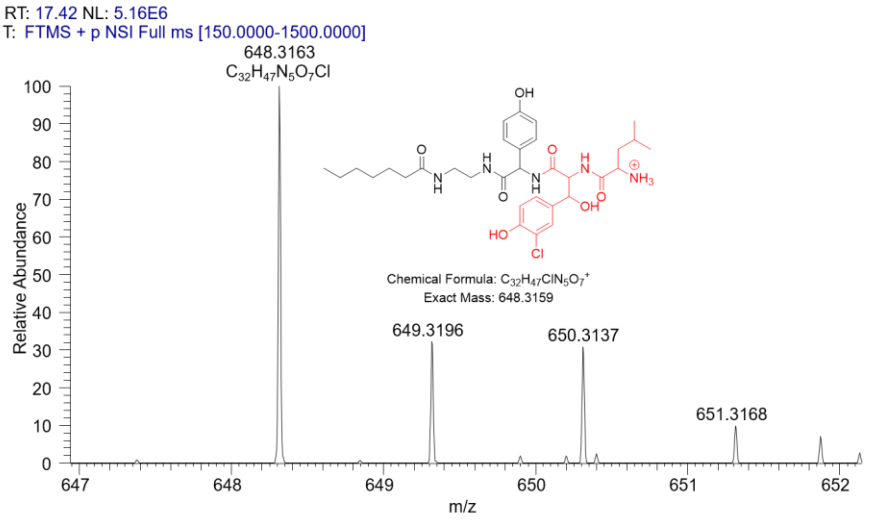

RT: 16.74 NL: 3.52E5
T: FTMS + p NSI Full ms [150.0000-1500.0000]
614.3546
C32H48N5O7
615.3568
Chemical Formula: C32H48N5O7+
Exact Mass: 614.3548
Relative Abundance
m/z

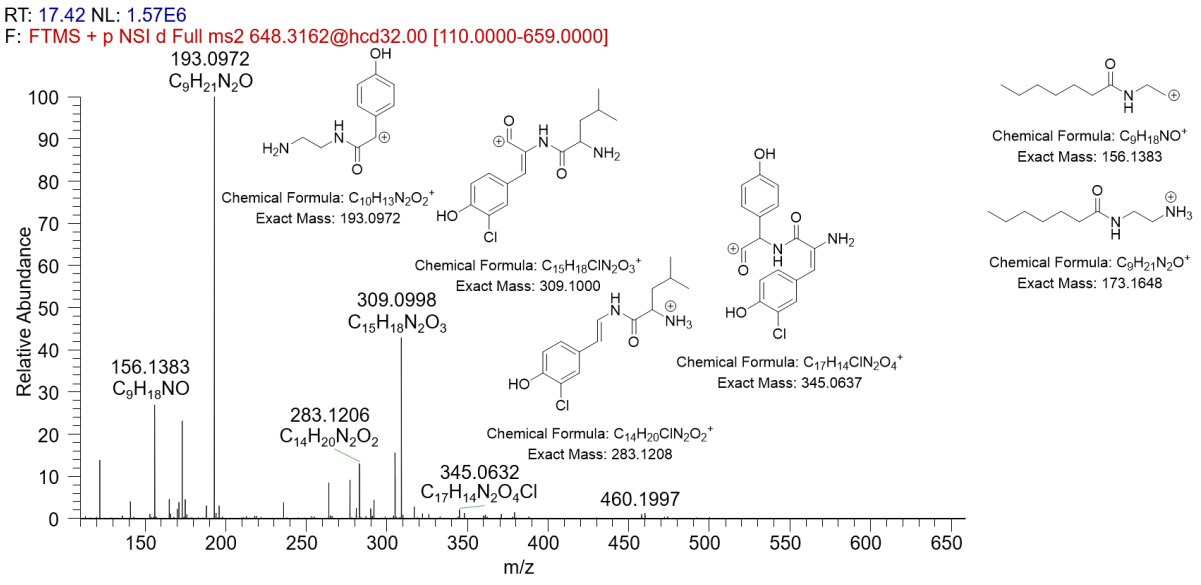

RT: 16.75 NL: 1.05E5
16.75 AV: 1 NL: 1.07E5
F: FTMS + p NSI d Full ms2 614.3519@hcd32.00 [110.0000-625.0000]
193.0973
C10H13N2O2
275.1392
C15H19N2O3
156.1383
C9H18NO
230.1175
C14H16NO2
306.0451
424.1863
C23H26N3O5
453.1331
596.1906
Chemical Formula: C14H16NO2+
Exact Mass: 230.1176
Chemical Formula: C15H19N2O3+
Exact Mass: 275.1390
Chemical Formula: C23H26N3O5+
Exact Mass: 424.1867
Relative Abundance
m/z


Chemical Formula: C10H13N2O2+
Exact Mass: 193.0972
Chemical Formula: C9H21N2O+
Exact Mass: 173.1648
Chemical Formula: C9H18NO+
Exact Mass: 156.1383
Chemical Formula: C7H8NO+
Exact Mass: 122.0600

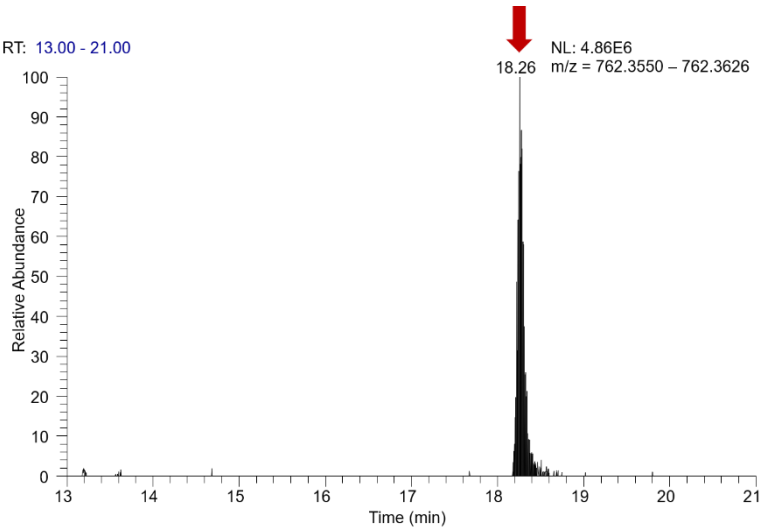
Chlorinated tripeptide

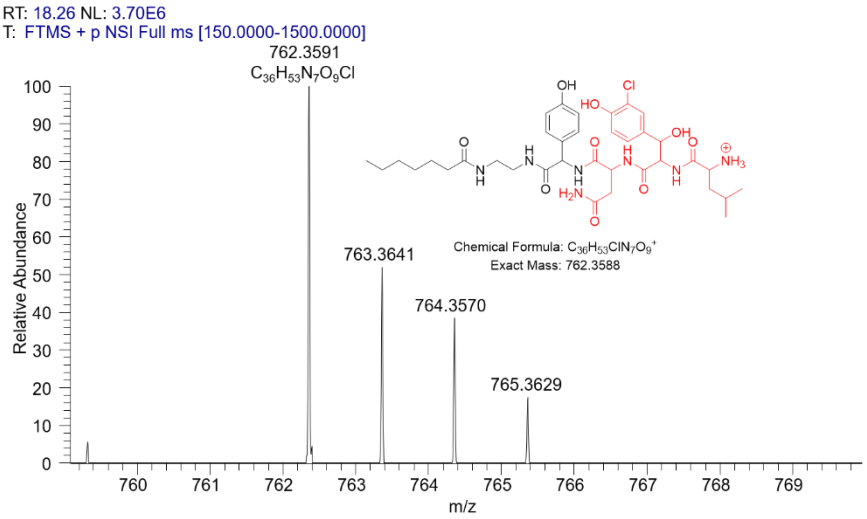


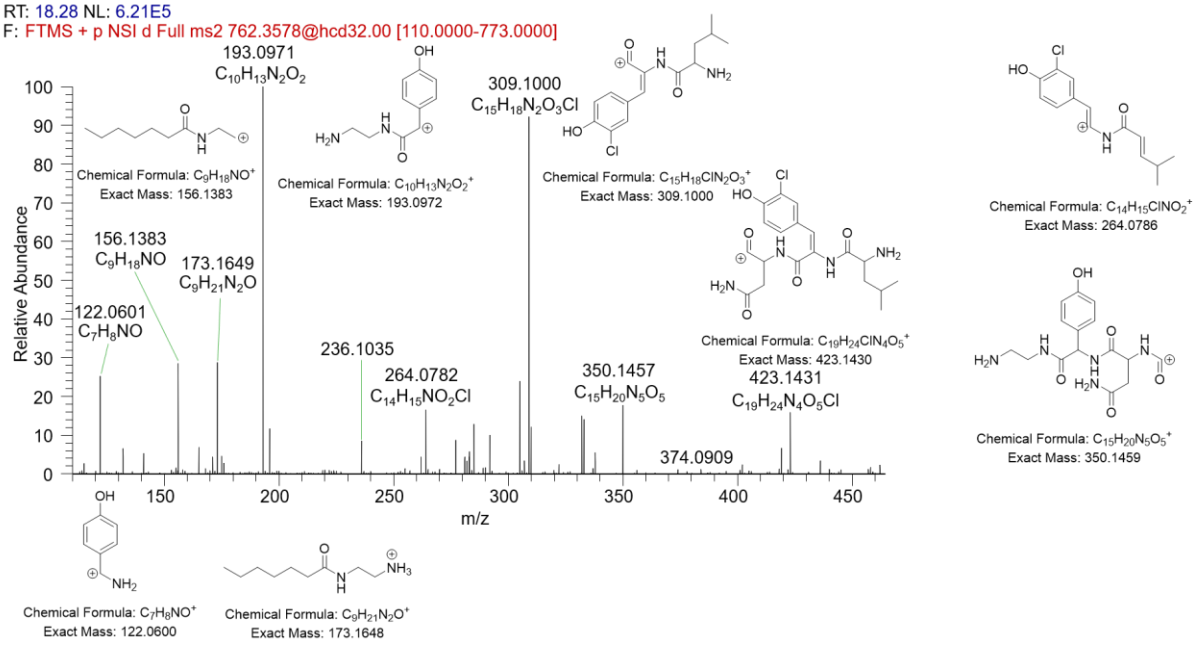




Chlorinated tetrapeptide

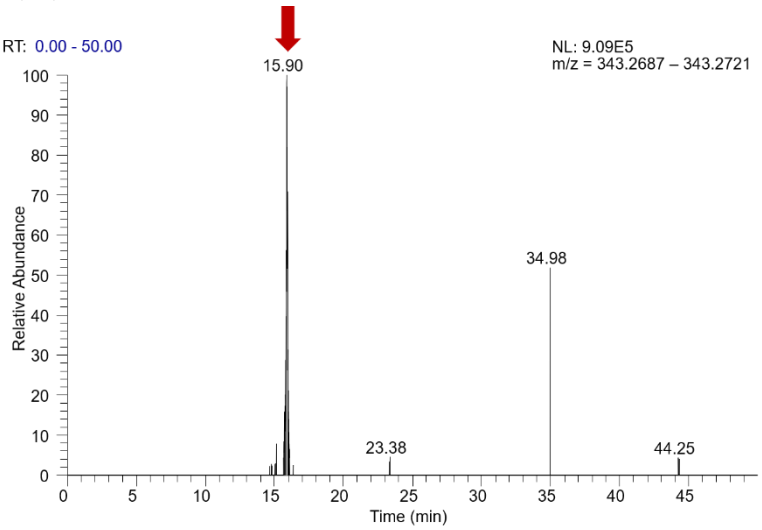


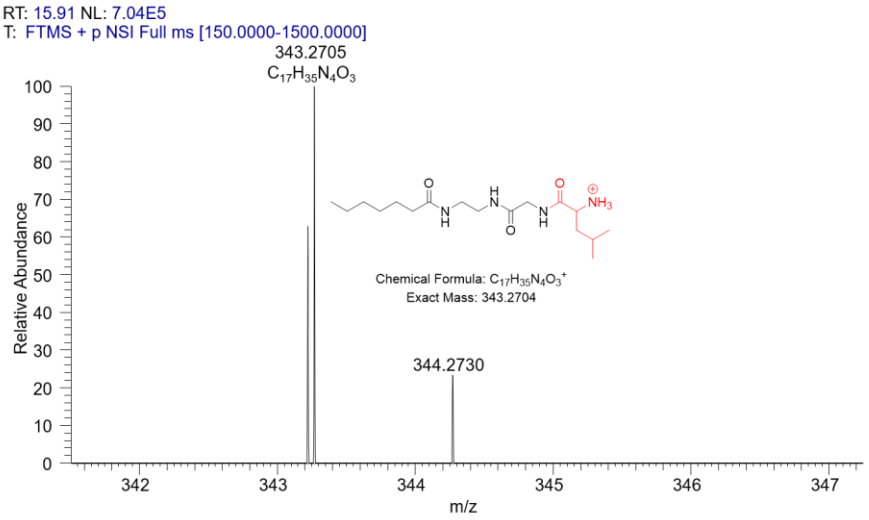


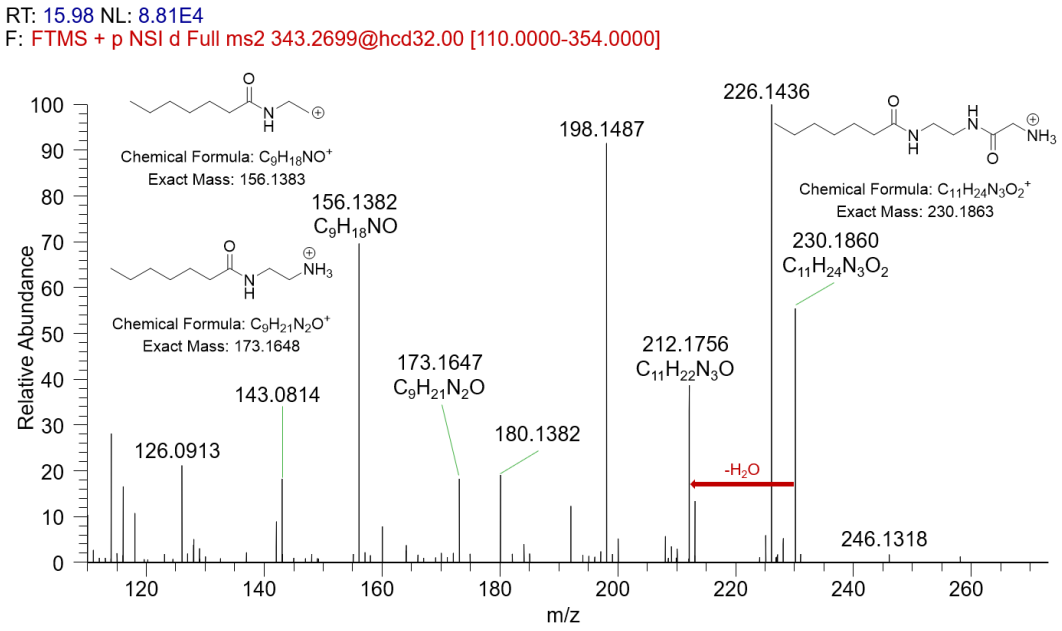


Capture of peptide intermediates from *A. orientalis* by glycine-based chain termination probe (16)

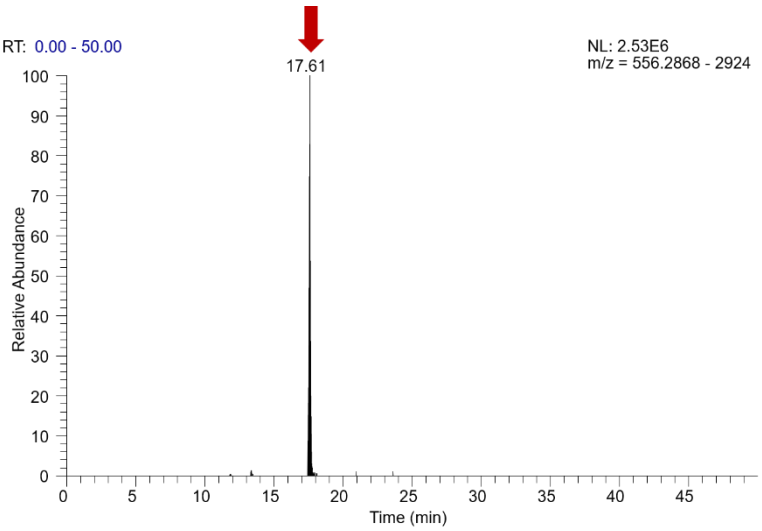
Dipeptide

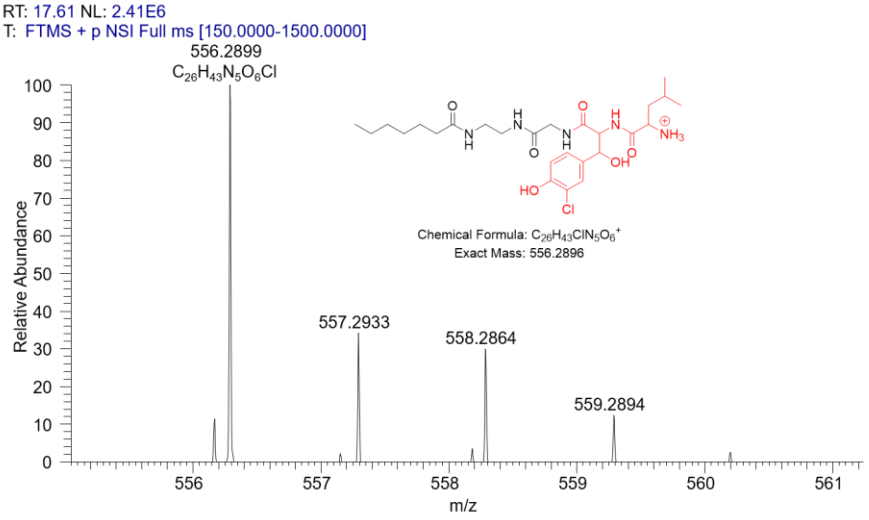


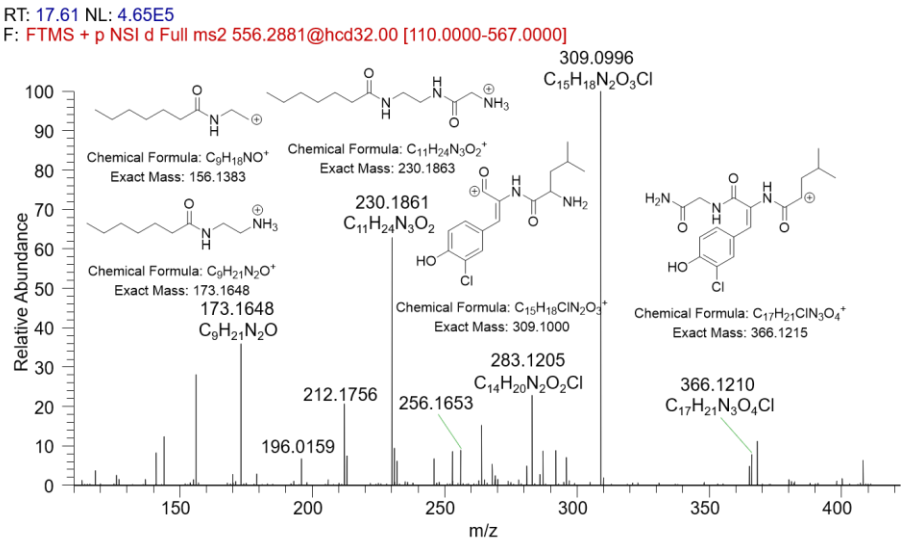


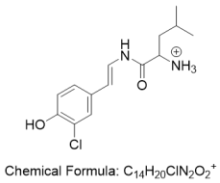


Tripeptide


RT: 0.00 - 50.00
17.61
NL: 2.53E6
m/z = 556.2868 - 2924
Relative Abundance
Time (min)

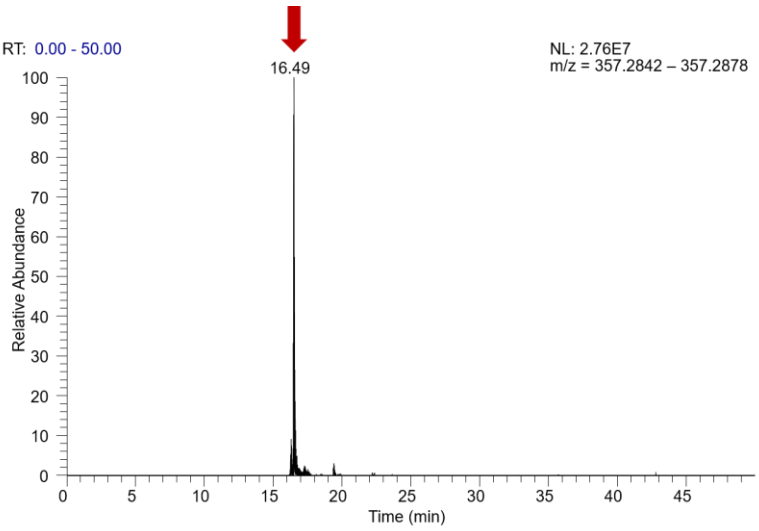

RT: 17.61 NL: 2.41E6
T: FTMS + p NSI Full ms [150.0000-1500.0000]
556.2899
C26H43N5O6Cl
Chemical Formula: C26H43ClN5O6+
Exact Mass: 556.2896
557.2933
558.2864
559.2894
Relative Abundance
m/z

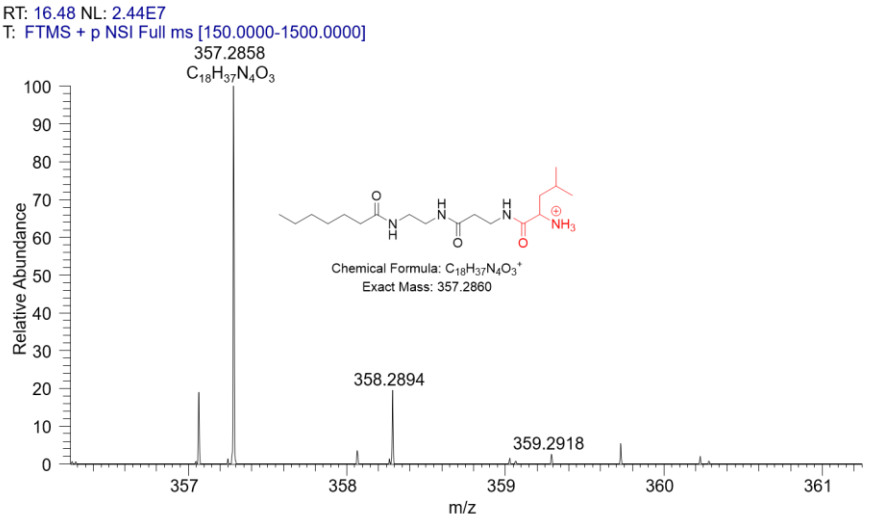

RT: 17.61 NL: 4.65E5
F: FTMS + p NSI d Full ms2 556.2881@hcd32.00 [110.0000-567.0000]
309.0996
C15H18N2O3Cl
Chemical Formula: C9H18NO+
Exact Mass: 156.1383
Chemical Formula: C11H24N3O2+
Exact Mass: 230.1863
230.1861
C11H24N3O2
Chemical Formula: C9H21N2O+
Exact Mass: 173.1648
173.1648
C9H21N2O
Chemical Formula: C15H18ClN2O3+
Exact Mass: 309.1000
Chemical Formula: C17H21ClN3O4+
Exact Mass: 366.1215
283.1205
C14H20N2O2Cl
366.1210
C17H21N3O4Cl
212.1756
256.1653
196.0159
Relative Abundance
m/z

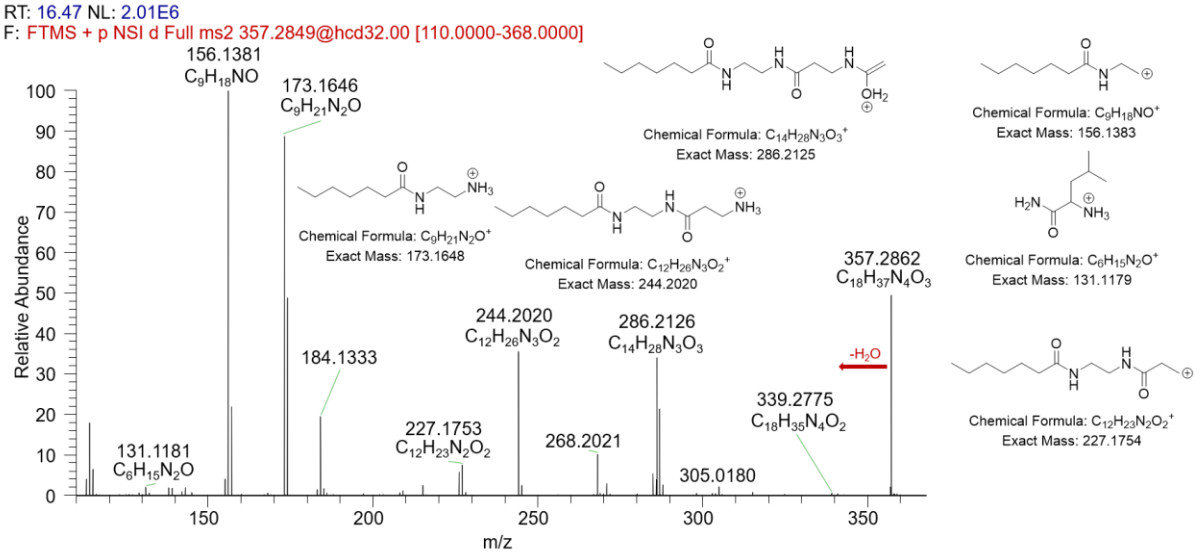

Chemical Formula: C14H20ClN2O2+
Exact Mass: 283.1208

Capture of peptide intermediates from *A. orientalis* by β-alanine-based chain termination probe (18)

Dipeptide

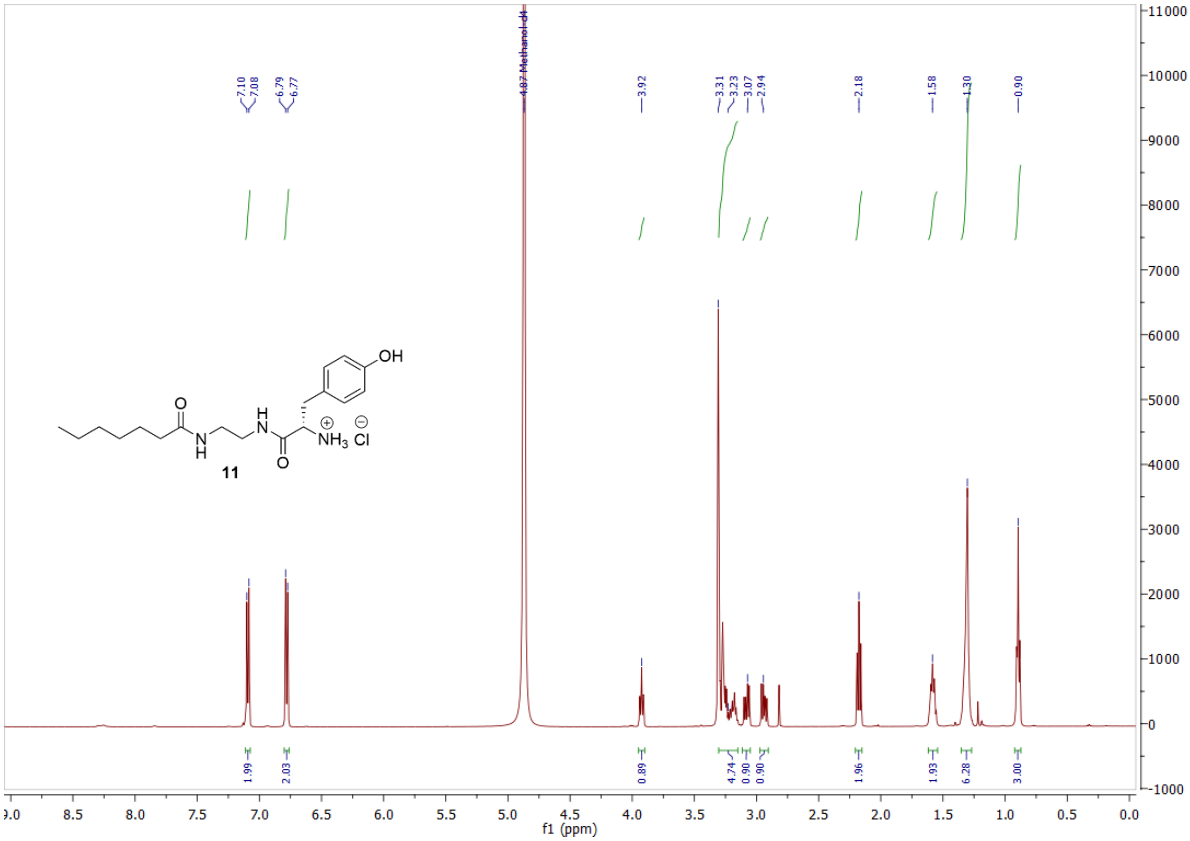




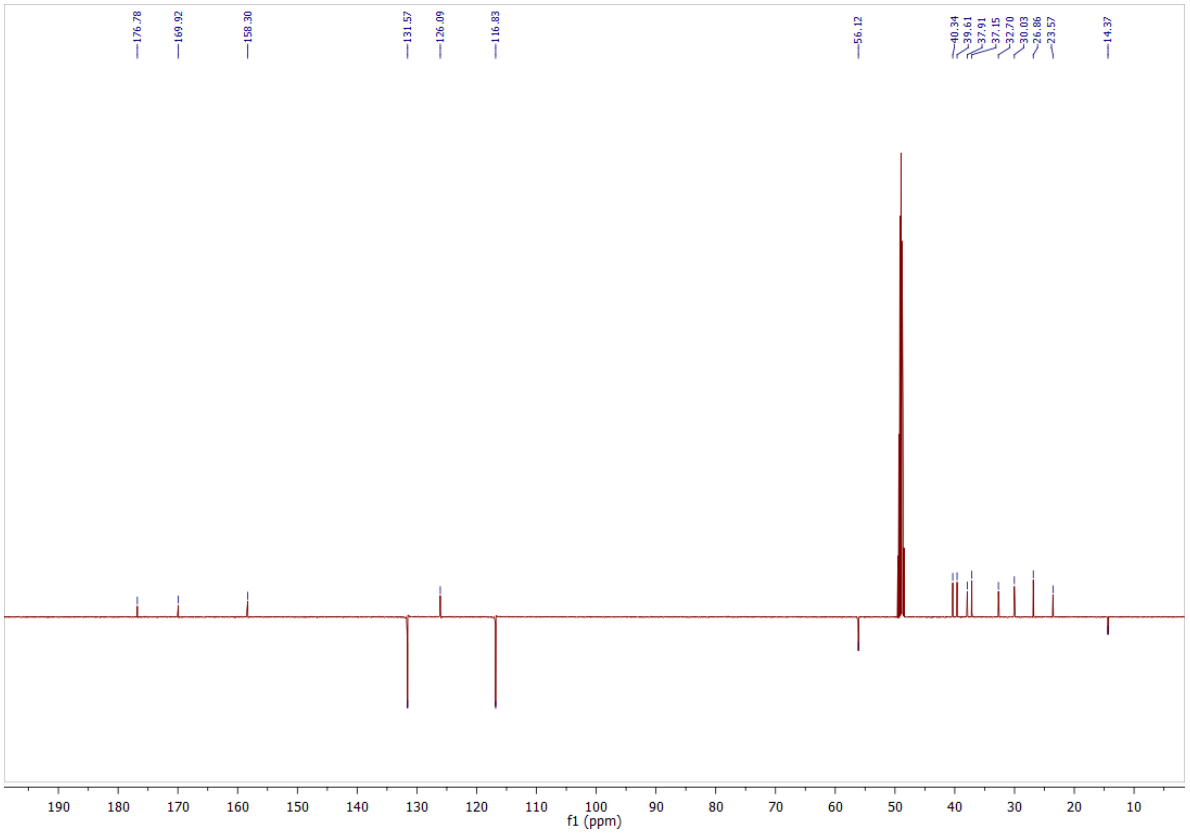


NMR Spectra of chemical probes 11, 12, 13, 14, 15, and 20

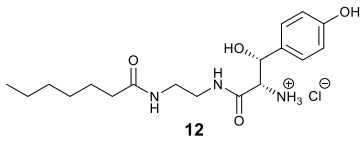
(*S*)-1-((2-heptanamidoethyl)amino)-3-(4-hydroxyphenyl)-1-oxopropan-2-aminium chloride (11)



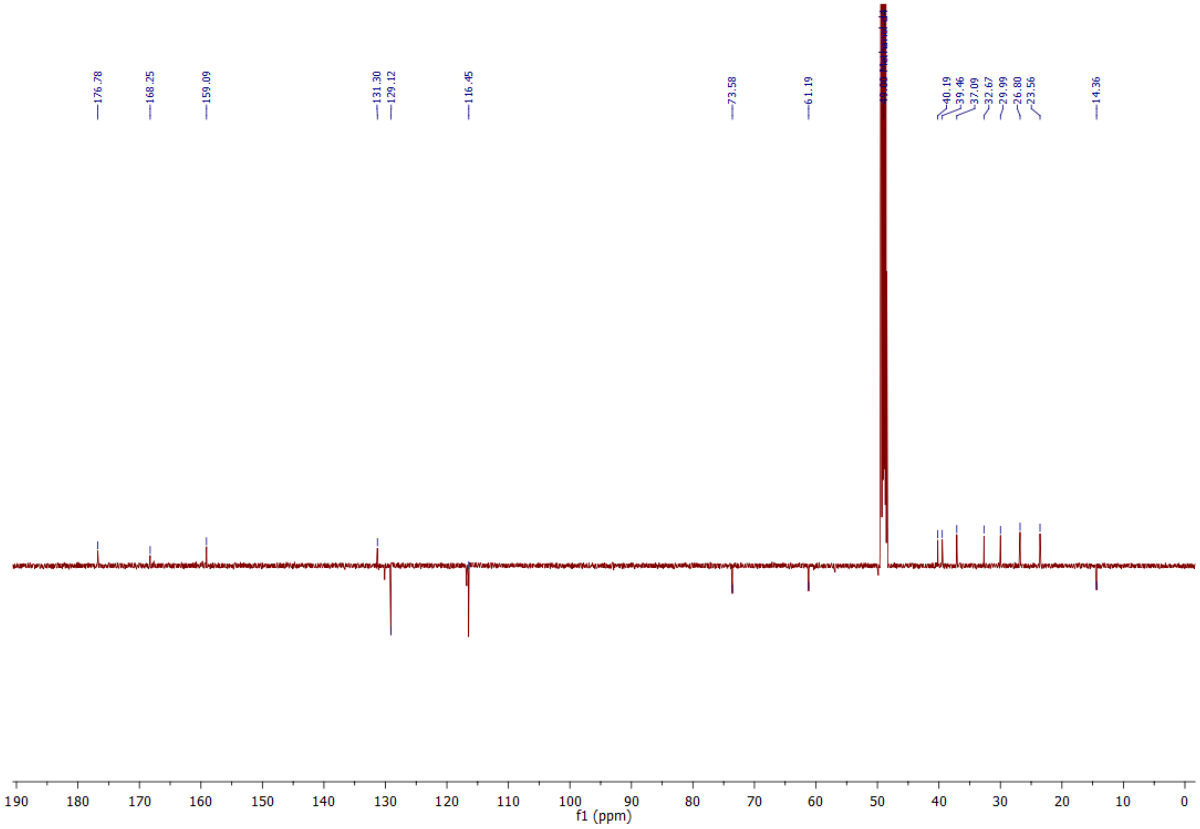
11



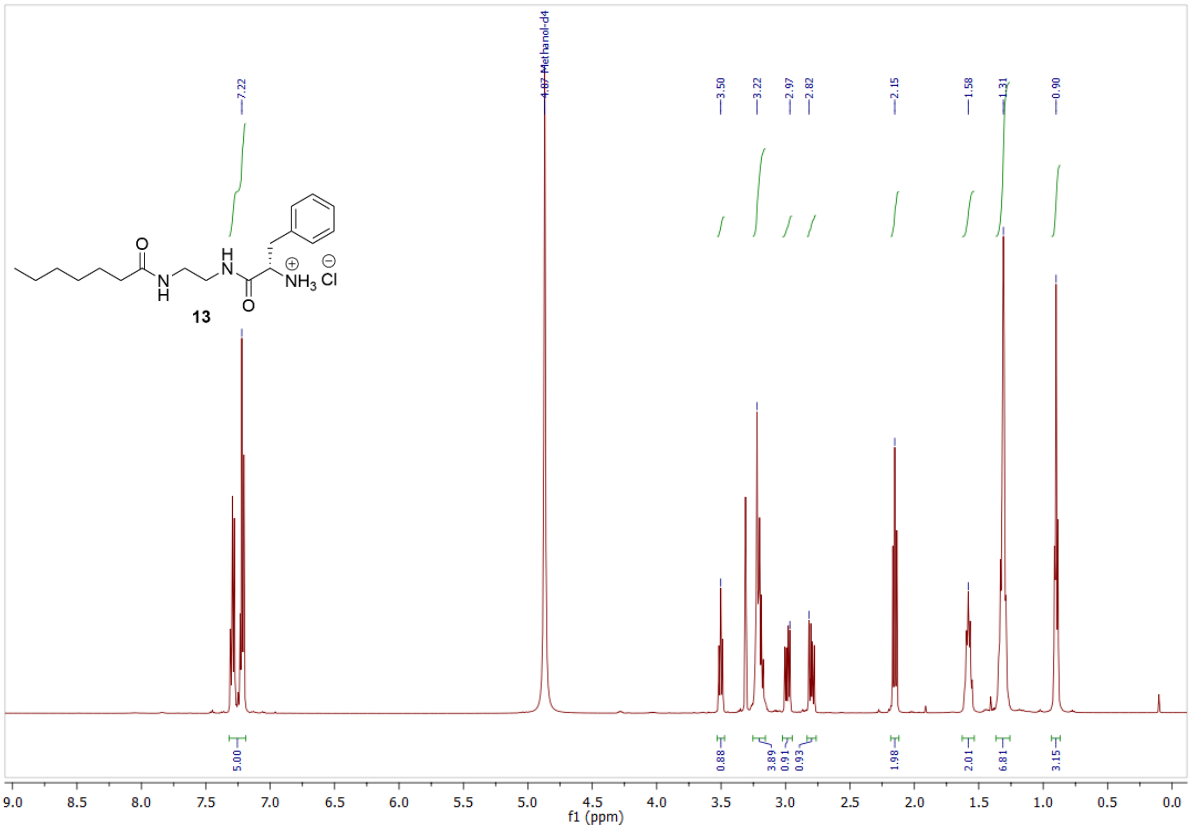
(2*S*,3*R*)-1-((2-heptanamidoethyl)amino)-3-hydroxy-3-(4-hydroxyphenyl)-1-oxopropan-2-aminium chloride (12)

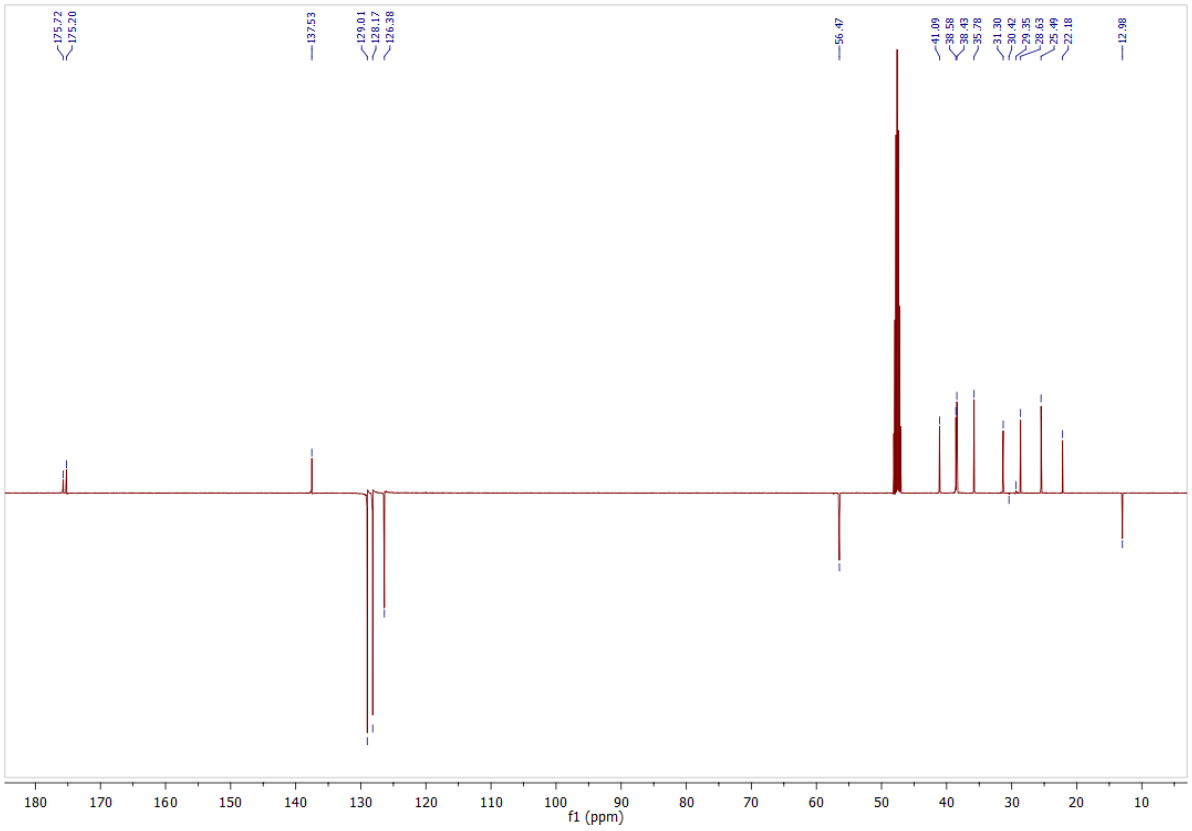


12

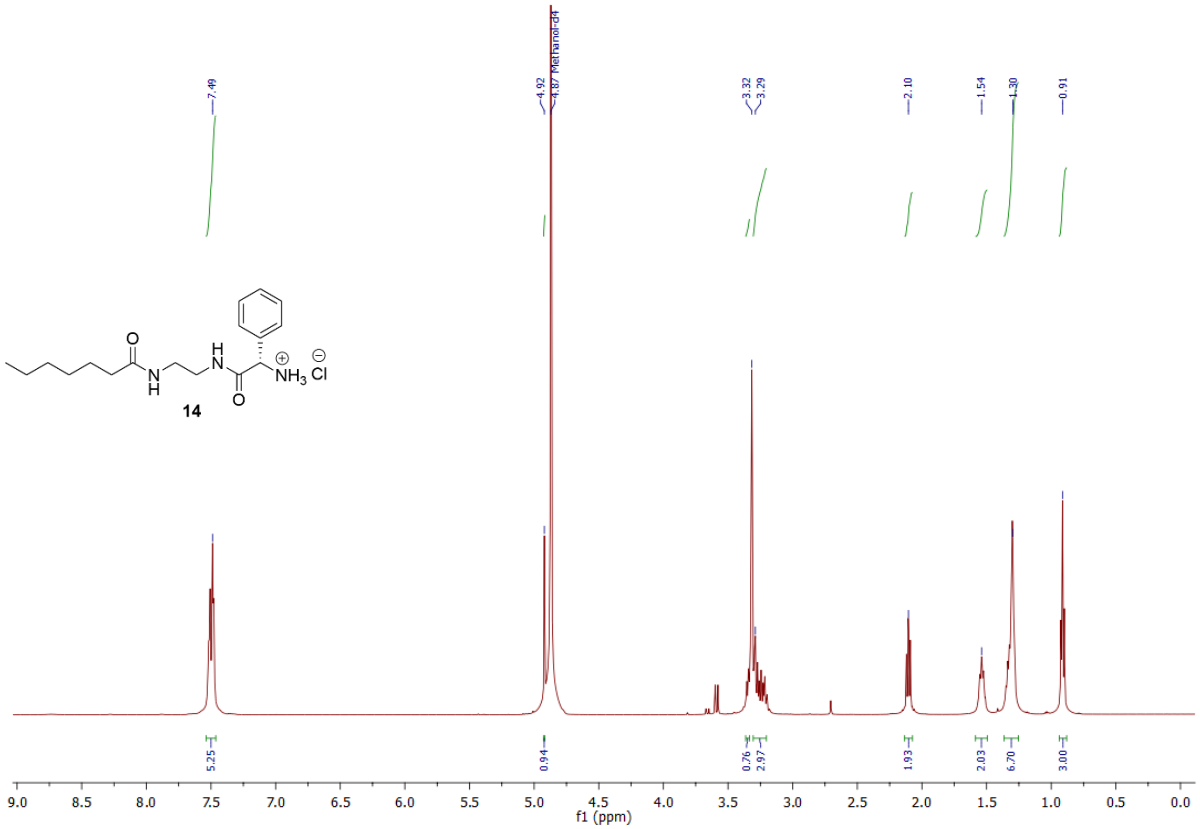


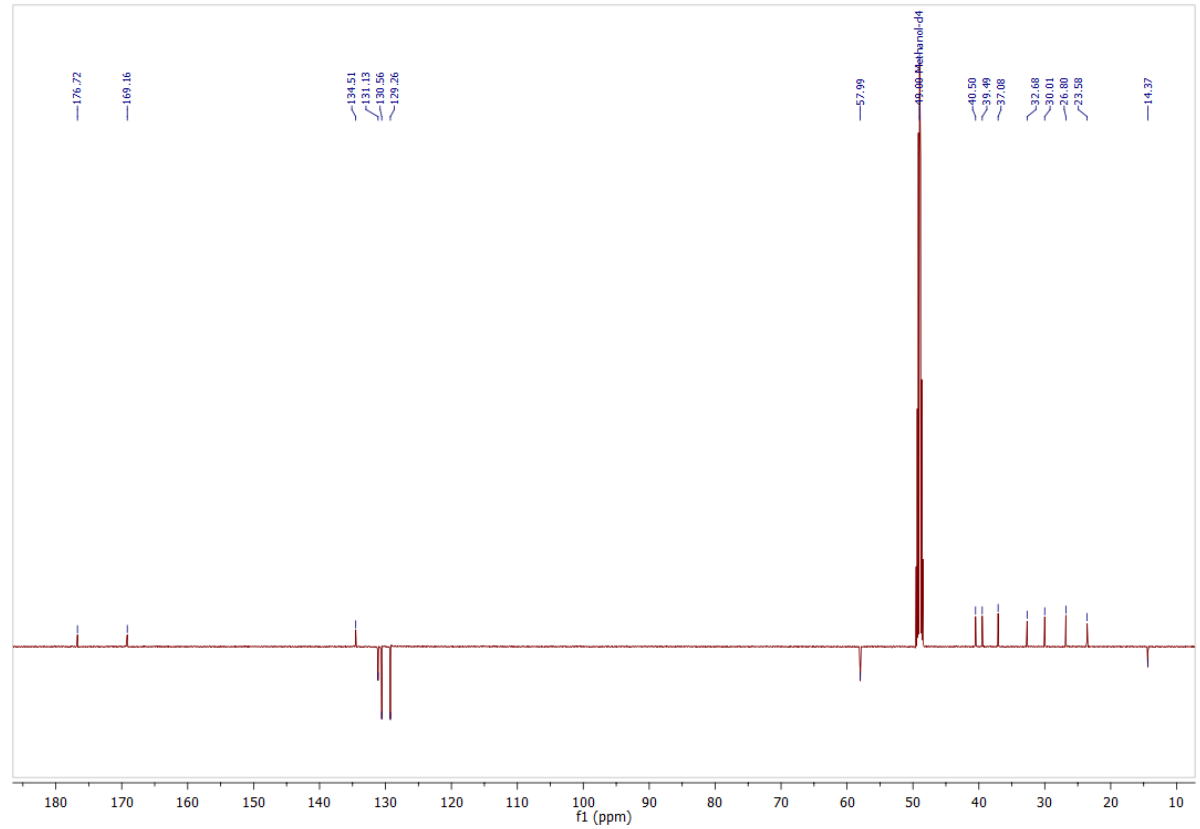
(*S*)-1-((2-heptanamidoethyl)amino)-1-oxo-3-phenylpropan-2-aminium chloride (13)



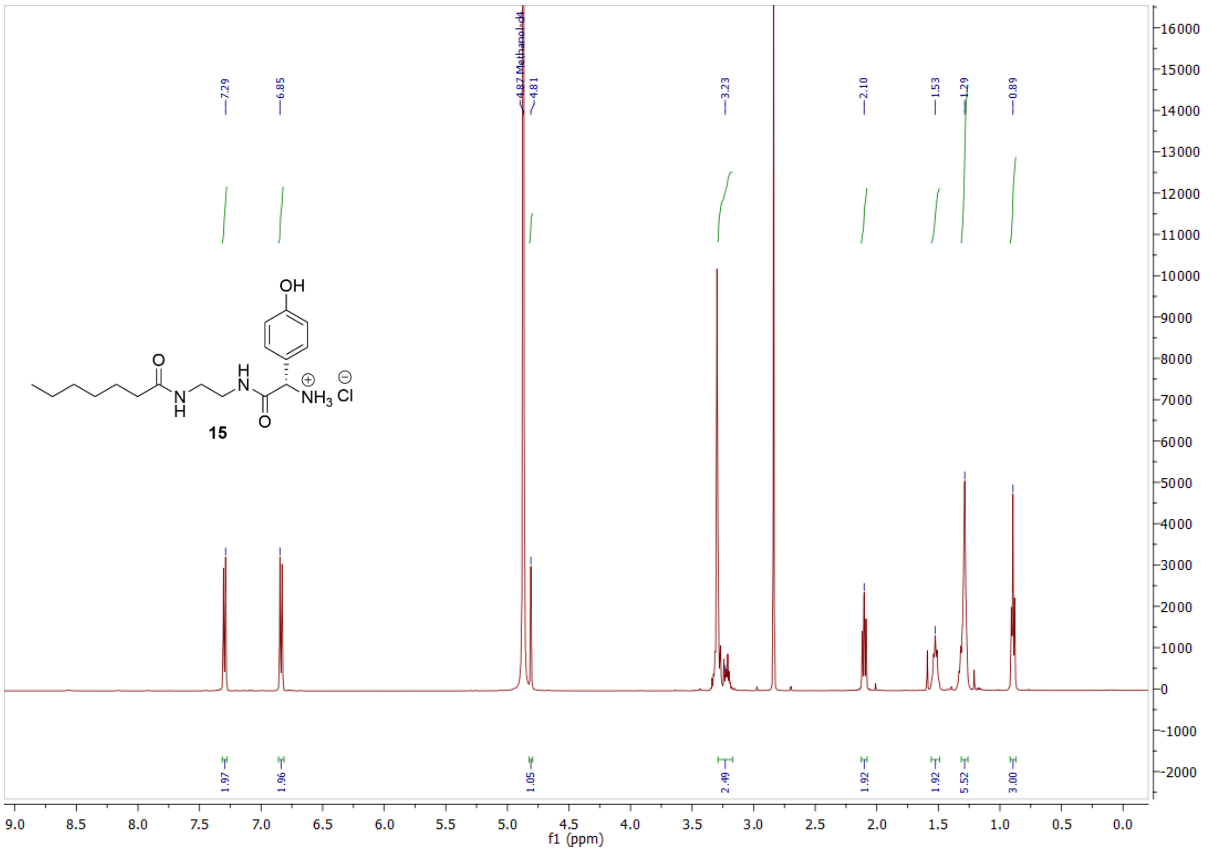


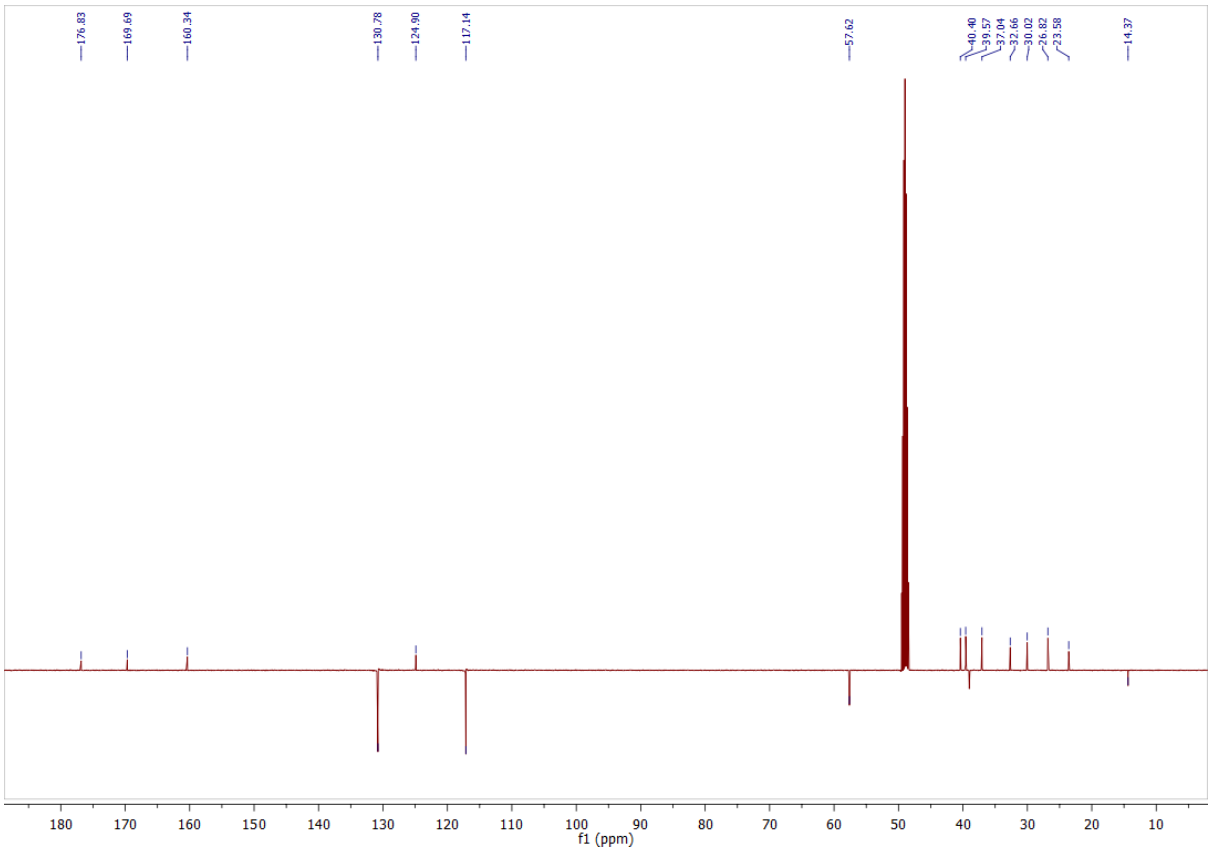
(*S*)-2-((2-heptanamidoethyl)amino)-2-oxo-1-phenylethan-1-aminium chloride (14)





(*S*)-2-((2-heptanamidoethyl)amino)-1-(4-hydroxyphenyl)-2-oxoethan-1-aminium chloride (15)





(*S*)-1-(3,5-dihydroxyphenyl)-2-((2-heptanamidoethyl)amino)-2-oxoethan-1-aminium chloride (20)