ELECTRONIC SUPPLEMENTARY INFORMATION

Searching for new cell-penetrating agents: hybrid cyclobutane-proline γ, γ –peptides.

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SUMMARY

General procedure for 5(6)-Carboxyfluorescein N^{γ}-terminal labeling and Boc group removal of compounds 1-6. Synthesis of peptides 7-12.

After Cbz removal by H_2/Pd(C) in peptides **1-6**, the unprotected peptide was solved in anhydrous DCM. CF (1.2 eq), PyBOP (1.2 eq) and DIEA (2.4 eq) were solved in minimum amount of NMP and added to the peptide. The reaction was followed by HPLC and 2-4h later, there was no sign of starting material. The reaction mixture was extracted with $NaHCO_3$ $5\%_{aq}$. The aqueous extracts were mixed together and then lyophilized. After CF-addition, peptide was solved in a TFA / DCM solution (4/6) and it was stirred for 1 h until total conversion. After that, solvent was removed. With the purpose to completely remove all TFA, co-evaporations with toluene (x3) were performed. Then, peptides were purified by semi-preparative HPLC.

Solid phase synthesis: general procedure.

γ-Hexapeptide backbone was prepared from amino acid (1*S*,3*R*)-3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2,2-dimethylcyclobutanecarboxylic acid (previously deprotected by reaction of the *tert*-butyl ester derivative **14** with a 40 % TFA solution in DCM), and the commercially available proline derivative (2*S*,4*S*)-4-((9H-fluoren-9-yl)methoxycarbonylamino)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid, using HBTU and HOBt as coupling agents. MBHA-polystyrene was chosen as the correct solid support to work using Fmoc/Boc chemistry. In order to deprotect the *N*-terminal group of the methylbenzhydrylamine, the resin was washed for 20 minutes with a 40 % TFA solution in DCM, followed by addition of 20 % DIPEA solution in DCM for 3 minutes. The reaction was monitored by the ninhydrin test. Then, the resin was washed with DMF in order to proceed attaching the first amino acid. All the coupling reactions were carried out for 2 hours approximately using 3 equivalents of the desired amino acid, 3 equivalents of HBTU and HOBT and 9 equivalents of DIPEA, using DMF as solvent and monitored by the ninhydrin test. The resin was washed with DMF (5 x 1 min) and DCM (5 x 1 min) after each coupling. Fmoc deprotection was achieved by washing the resin with a 50 % piperidine solution in DMF (2 x 10 min).

For N^{α}-acyl-γ-hexapeptides (**33-36**), after the N^{α}-Boc groups had been removed, acylation of the α-amino groups was carried out using 5-(Boc-amino)valeric acid (Boc-5-Ava-OH) (9 equiv, 3 equiv for each amine), HBTU and HOBt (9 equiv) in the presence of Et_3N (15 eq) in DMF for 2 h at 25 °C.

The resin was washed with DMF (5 x1 min) and DCM (5 x 1 min). The acylation was monitored by the chloranil test.

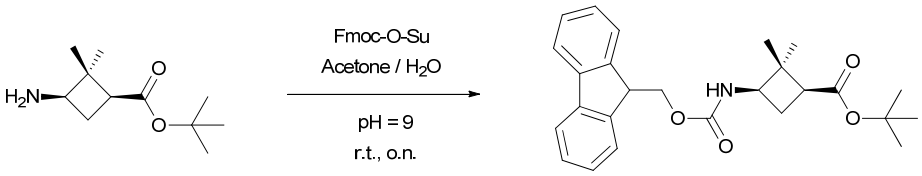
N^{α}-Alkyl-γ-peptides (**19-32**) were obtained via reductive amination using the corresponding aldehyde (15 eq) in 1% HOAc in DMF for 30 min followed by addition of $NaBH_3CN$ (15 eq) dissolved in MeOH for 2 hours. After that, the resin was washed with DMF (5 x 1 min) and DCM (5 x 1 min). The alkylation was monitored by the chloranil test.

For N^{α}-guanidylated-γ-peptides (**37-38**), the guanidinium group was introduced using *N*,*N*'-di-Boc-*1H*-pyrazole-1-carboxamidine (5 eq) in the presence of Et_3N (9 eq) in DCM and monitored by the chloranil test.

At the end of the synthesis (if necessary), after Fmoc removal, the fluorescent label 5(6)-carboxyfluorescein (CF, 5 eq) was introduced onto the *N*-terminal amino group using HBTU/HOBt (5 eq) as coupling reagents, in the presence of Et_3N (10 eq), followed by piperidine washes just before cleavage of the peptide from the resin.

Peptides were ultimately cleaved from the resin by acidolytic treatment with anhydrous HF. To proceed, the peptide resin was washed with MeOH (3 x 1 min), dried, and treated with HF in the presence of 10% anisole for 1 h at 0 °C. Peptides were precipitated with cold anhydrous MTBE, filtered, dissolved in an aqueous solution containing HOAc, and then lyophilized.

Synthesis of (1*R*,3*S*)-*tert*-butyl-1-((9H-fluoren-9-yl)methoxycarbonylamino)-2,2-dimethyl-cyclobutane-3-carboxylate 14:

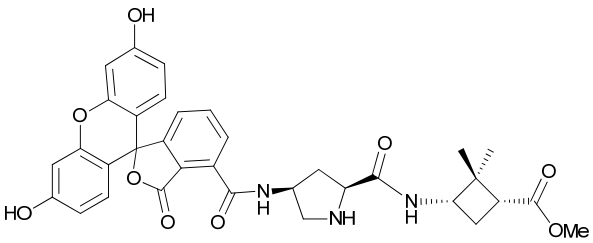


Hydrogenolysis of benzyl carbamate in **13** afforded a free amine (0.23 g, 1.15 mmol), which was dissolved in a mixture of acetone/water (40 mL, 1:1) and the pH was adjusted to 9 by addition of a saturated Na_2CO_3 solution. Fmoc-*O*-succinimide (0.39 g, 1.15 mmol, 1eq) was added over a period of 30 min. The mixture was stirred overnight and the value of the pH was maintained all this time at 9 by further addition of a few drops of saturated Na_2CO_3 solution. The mixture was diluted with ethyl acetate (50 mL) and acidified carefully with a 6 N HCl solution to pH 6-7. The phases were separated and the organic phase was washed with brine (3x30 mL) and dried with $MgSO_4$. After evaporation of the solvent under reduced pressure, the crude was purified by silica gel column

chromatography (2:1 hexane-ethyl acetate) to afford *N*-protected amine **14** as a white solid (0.32 g, 64 %).$[\alpha]_D$ = +20 (*c* = 1.0, CH_2Cl_2). IR (ATR): ν 3338, 3067, 2960, 1701, 1523, 1451, 1367, 1340 cm^{-1}. δ_H (250 MHz, $CDCl_3$) 0.94 (s, 3H, *trans-CH₃*), 1.29 (s, 3H, *cis-CH₃*), 1.46 (s, 9H, t-Bu), 1.95-2.16 (m, 1H, H_{4S}), 2.24-2.35 (m, 1H, H_3), 2.50 (m, 1H, H_{4R}), 3.88 (dd, $^2J_{H,H}$= 17.3 Hz, $^3J_{H,H}$= 8.6, 1H, H_1), 4.22 (m, 1H, H_{10}), 4.34-4.62 (c.s., 2H, C*H₂*), 5.09 (d, $^3J_{H\text{-}H}$= 8.5 Hz, 1H, N*H₇*), 7.28-7.77 (c.s., 8H, H_{Ar}); δ_C (62.5 MHz, $CDCl_3$) 16.8, 26.4, 28.3, 28.9, 43.8, 46.0, 47.4, 51.6, 66.6, 80.6, 120.0, 125.1, 127.1, 127.7 ($8C_{Ar}$), 141.4, 143.9, 144.0, 156.1, 172.08; *m/ z* (ESI): Found, 421.2252 $[M]^+$. Calcd. for $C_{26}H_{31}NO_4$: 421.2253.

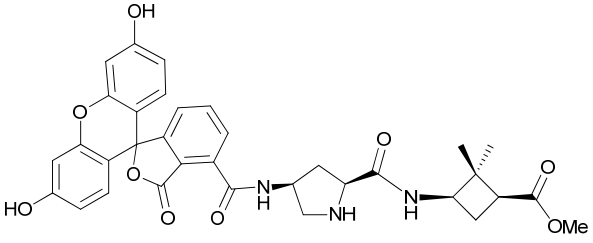
Peptide purification and characterization

Proline-cyclobutane γ,γ-(CF)dipeptide 7



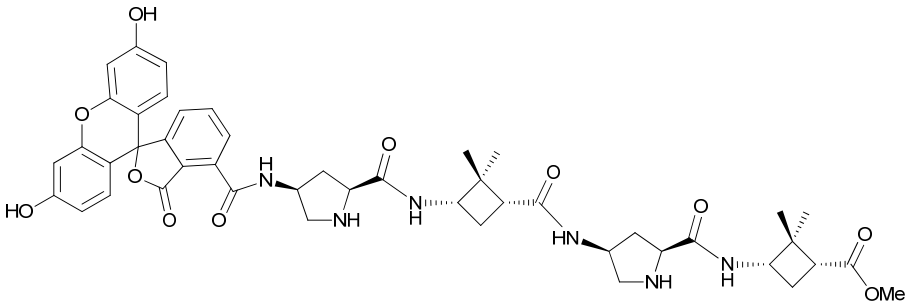
The crude was purified by semi-preparative HPLC-MS using a linear gradient (from 25 to 30% of MeCN in 12 min) of MeCN (containing 0.1% of TFA) and H_2O (containing 0.1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 95% pure. MS calcd for $C_{34}H_{34}N_3O_9$ $[M + H]^+$: 628.27. MALDI-TOF found: 628.29 $[M + H]^+$, 650.26 $[M + Na]^+$, 666.24 $[M + K]^+$. ESI found: 628.2, 314.6.

Proline-cyclobutane γ,γ-(CF)dipeptide 8



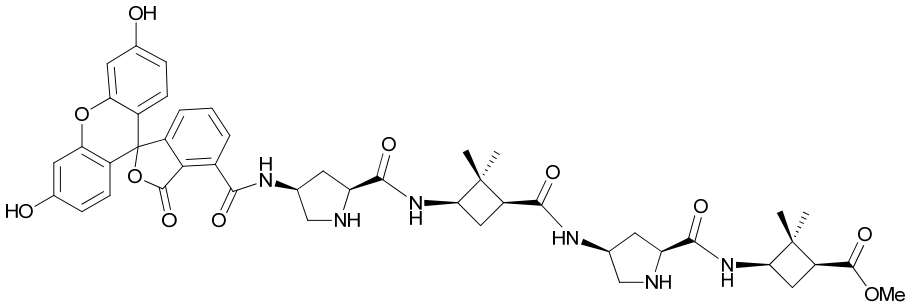
The crude was purified by semi-preparative HPLC-MS using a linear gradient (from 25 to 30% of MeCN in 12 min) of MeCN (containing 0.1% of TFA) and H_2O (containing 0.1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 93% pure. MS calcd for $C_{34}H_{34}N_3O_9$ $[M + H]^+$: 628.27. MALDI-TOF found: 628.09 $[M + H]^+$, 650.06 $[M + Na]^+$ 666.00 $[M + K]^+$. ESI found: 628.2, 314.7

Proline-cyclobutane γ,γ-(CF)tetrapeptide 9



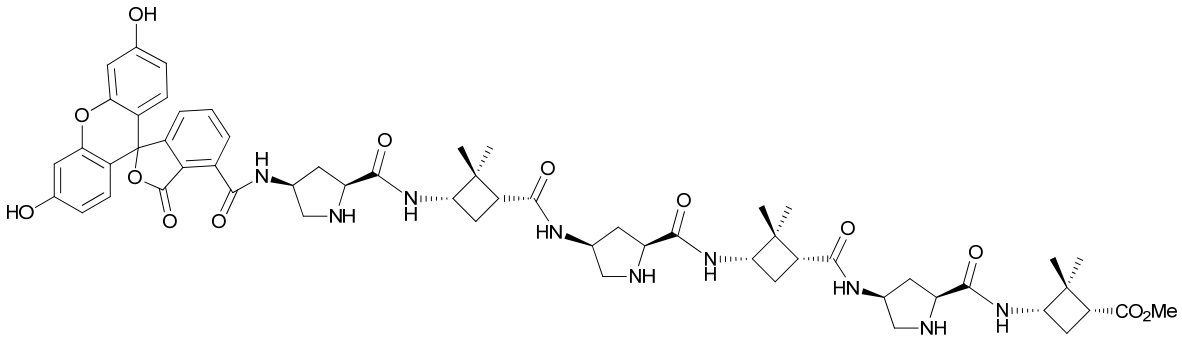
The crude was purified by semi-preparative HPLC-MS using a linear gradient (from 25 to 30% of MeCN in 12 min) of MeCN (containing 0.1% of TFA) and H_2O (containing 0.1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 99% pure. MS calcd for $C_{46}H_{53}N_6O_{11}$ $[M + H]^+$: 865.37. MALDI-TOF found: 865.48 $[M + H]^+$, 887.46 $[M + Na]^+$, 909.44 ESI found: 865.5, 433.3.

Proline-cyclobutane γ,γ-(CF)tetrapeptide 10



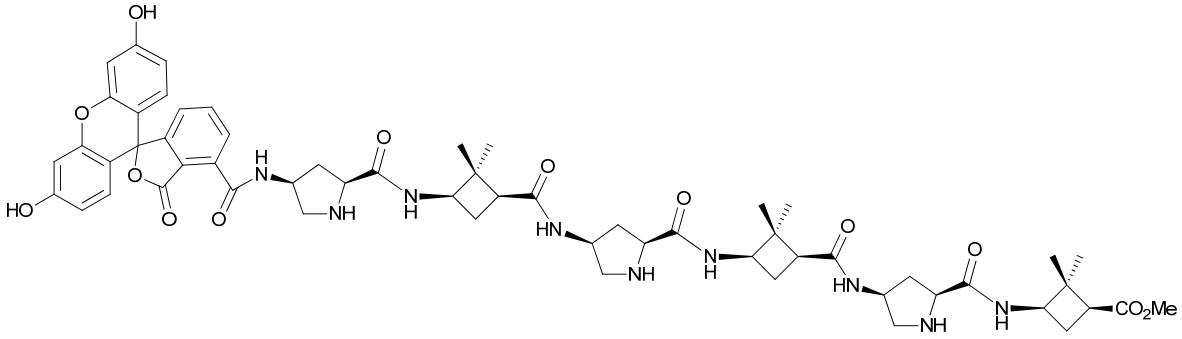
The crude was purified by semi-preparative HPLC-MS using a linear gradient (from 25 to 30% of MeCN in 12 min) of MeCN (containing 0.1% of TFA) and H_2O (containing 0.1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptides were 94% pure. MS calcd for $C_{46}H_{53}N_6O_{11}$ $[M + H]^+$: 865.37. MALDI-TOF found: 865.43 $[M + H]^+$, 887.41 $[M + Na]^+$, 903.37 $[M + K]^+$. ESI found: 865.8, 433.7.

Proline-cyclobutane γ,γ-(CF)hexapeptide 11



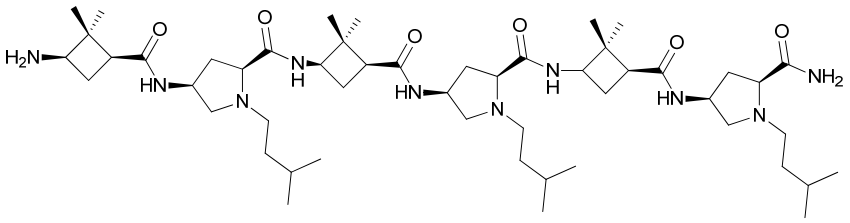
The crude was purified by semi-preparative HPLC-MS using a linear gradient (from 25 to 30% of MeCN in 12 min) of MeCN (containing 0.1% of TFA) and H_2O (containing 0.1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 99% pure. MS calcd for $C_{58}H_{72}N_9O_{13}$ $[M + H]^+$: 1102.52. MALDI-TOF found: 1102.65 $[M + H]^+$, 1124.63 $[M + Na]^+$, 1140.6 $[M + K]^+$. ESI found: 1102.8, 552.0, 368.3.

Proline-cyclobutane γ,γ-(CF)hexapeptide 12



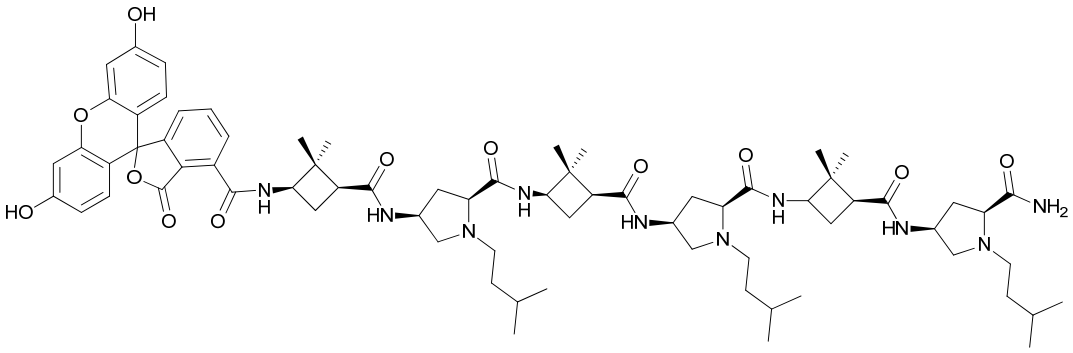
The crude was purified by semi-preparative HPLC-MS using a linear gradient (from 25 to 30% of MeCN in 12 min) of MeCN (containing 0.1% of TFA) and H_2O (containing 0.1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide were 97% pure. MS calcd for $C_{58}H_{72}N_9O_{13}$ $[M + H]^+$: 1102.52. MALDI-TOF found: 1102.69 $[M + H]^+$, 1124.65 $[M + Na]^+$. ESI found: 1103.0, 552.3.

Proline-cyclobutane γ,γ-hexapeptide 19



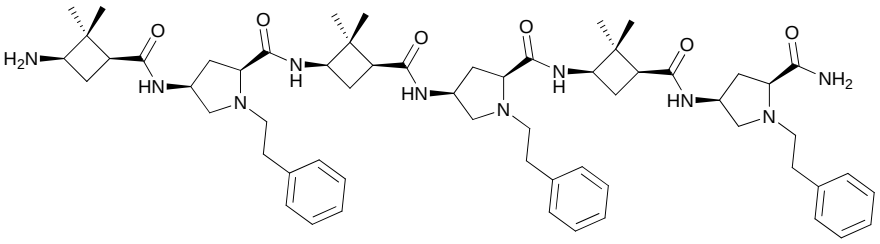
The crude peptide prepared according to the general procedure was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 2 min, increased to 20 % in 0.5 min, from 20 to 40 % in 6 min, increased to 100 % MeCN in 0.5 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). mp 148-150 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +20 (*c* = 0.4, CH_3OH). IR (ATR): ν 3263, 2915, 1738 cm^{-1}. δ_H (400 MHz, CD_3OD) 0.86-1.47 (c.s., 36H), 1.62 (c.s., 6H), 1.73 (c.s., 3H), 1.99-3.23 (c.s., 24H), 3.43-3.77 (c.s., 6H), 4.03-4.27 (c.s., 4H), 4.50 (c.s., 2H); δ_C (100 MHz, CD_3OD) 17.8, 22.3-30.84 (18C), 35.0-35.5 (3C), 36.9 (3C), 44.0-52.6 (9C), 55.0 (3C), 59.7-60.0 (3C), 68.4 (3C), 173.6-175.5 (6C).; *m/ z* (ESI): Found, 961.6943 $[M + Na]^+$. Calcd. for $C_{51}H_{90}N_{10}O_6Na$: 961.6937.

Proline-cyclobutane γ,γ-(CF)hexapeptide 20



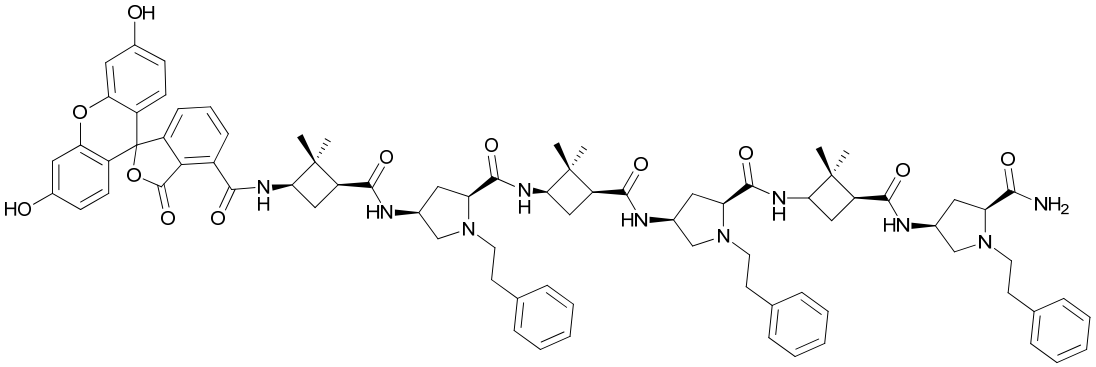
The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 3 min, increased to 30 % in 1 min, from 30 to 40 % in 7 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 99% pure. MS calcd for $C_{72}H_{101}N_{10}O_{12}$ $[M + H]^+$: 1297.76. MALDI-TOF found: 1297.59 $[M + H]^+$ and 1319.57 $[M + Na]^+$.

Proline-cyclobutane γ,γ-hexapeptide 21



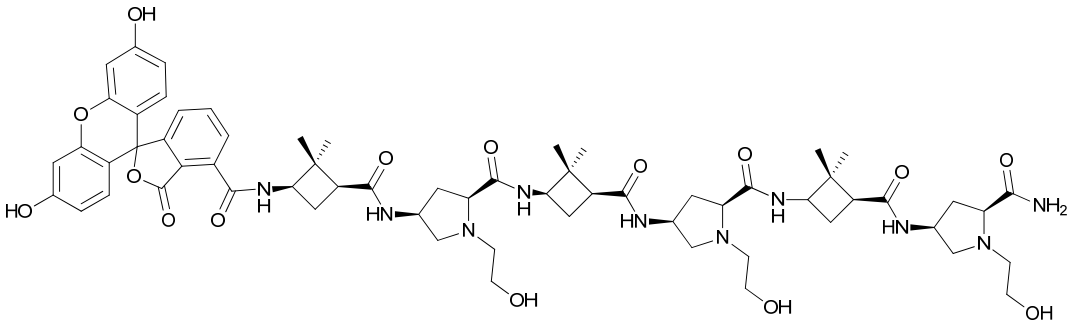
The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 2 min, increased to 20 % in 0.5 min, from 20 to 40 % in 6 min, increased to 100 % MeCN in 0.5 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). mp 129-131 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +37 (*c* = 0.1, CH_3OH). IR (ATR): ν 3273, 2917, 1667, 1537 cm^{-1}. δ_H (360 MHz, CD_3OD) 0.82-1.40 (c.s., 18H), 1.94-3.11 (c.s., 30H), 3.37-3.70 (c.s., 7H), 4.00-4.25 (c.s., 3H), 4.45 (c.s., 2H), 7.22-7.38 (c.s., 15H); δ_C (90 MHz, CD_3OD) 17.7 (3C), 25.1 (3C), 28.8-30.8 (6C), 33.0-33.4 (3C), 36.8 (3C), 43.9-47.4 (6C), 52.0-52.7 (3C), 57.4 (3C), 59.9 (3C), 68.6 (3C), 128.4-130.0 (15C), 137.3 (3C), 173.7-174.2 (6C); *m/z* (ESI): Found, 1063.6468 $[M + Na]^+$. Calcd. for $C_{60}H_{84}N_{10}O_6Na$: 1063.6467.

Proline-cyclobutane γ,γ-(CF)hexapeptide 22



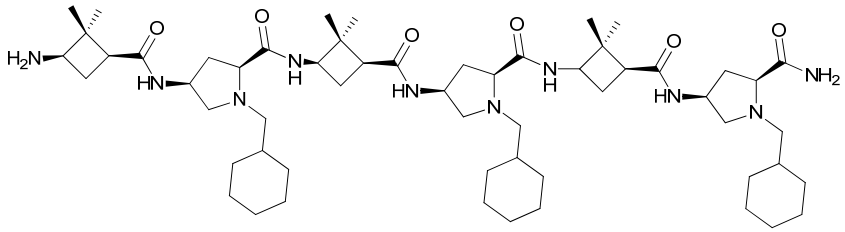
The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 3 min, increased to 30 % in 1 min, from 30 to 40 % in 7 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 98% pure. MS calcd for $C_{81}H_{95}N_{10}O_{12}$ $[M + H]^+$: 1400.68. MALDI-TOF found: 1400.54 $[M + H]^+$, 1423.53 $[M + Na]^+$, and 1437.49 $[M + K]^+$.

Proline-cyclobutane γ,γ-(CF)hexapeptide 24



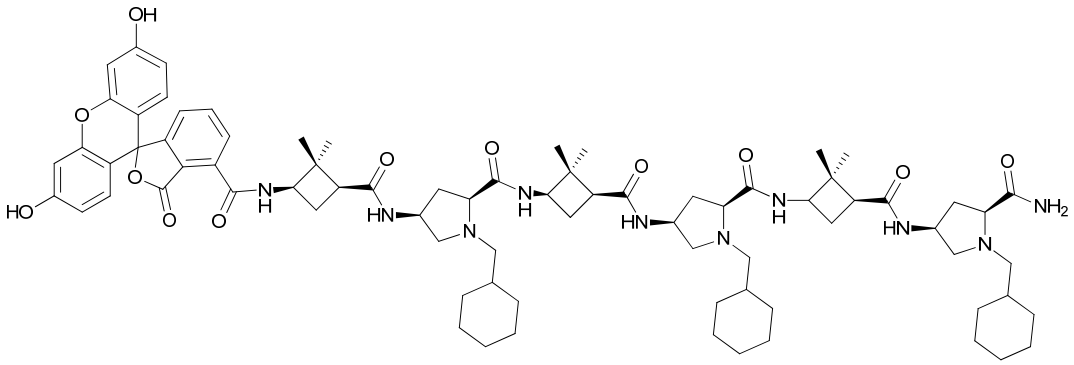
The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 3 min, increased to 20 % in 1 min, from 20 to 30 % in 7 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 100% pure. MS calcd for $C_{63}H_{82}N_{10}O_{16}$ $[M+H]^+$: 1220.39. MALDI-TOF found: 1220.49 $[M + H]^+$ and 1258.45 $[M + K]^+$.

Proline-cyclobutane γ,γ-hexapeptide 25



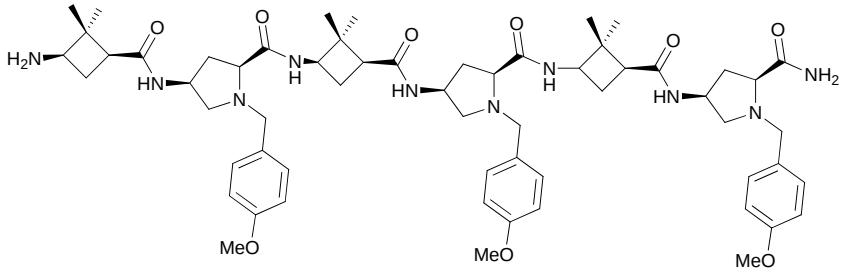
The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 3 min, increased to 30 % in 1 min, from 30 to 45 % in 7 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). mp 137-139 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +11 (*c* = 0.3, CH_3OH). IR (ATR): ν 3394, 2924, 1676 cm^{-1}. δ_H (400 MHz, CD_3OD) 0.90-2.10 (c.s., 39H), 2.16-3.12 (c.s., 24H), 3.43- 4.46 (c.s., 12H); δ_C (100 MHz, CD_3OD) 17.4-17.7 (3C), 23.7-40.3 (30C), 43.7-52.9 (6C), 59.8-71.1 (12C); *m/ z* (ESI): Found, 1017.7579 $[M + H]^+$. Calcd. for $C_{57}H_{97}N_{10}O_6$: 1017.7587.

Proline-cyclobutane γ,γ-(CF)hexapeptide 26



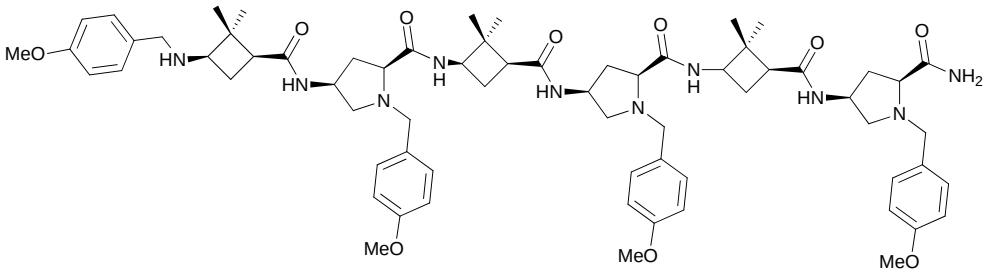
The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 4 min, increased to 30 % in 1 min, from 30 to 40 % in 7 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 100% pure. MS calcd for $C_{78}H_{107}N_{10}O_{12}$ $[M + H]^+$: 1375.81. MALDI-TOF found: 1375.69 $[M + H]^+$ and 1397.67 $[M + Na]^+$.

Proline-cyclobutane γ,γ-hexapeptide 27



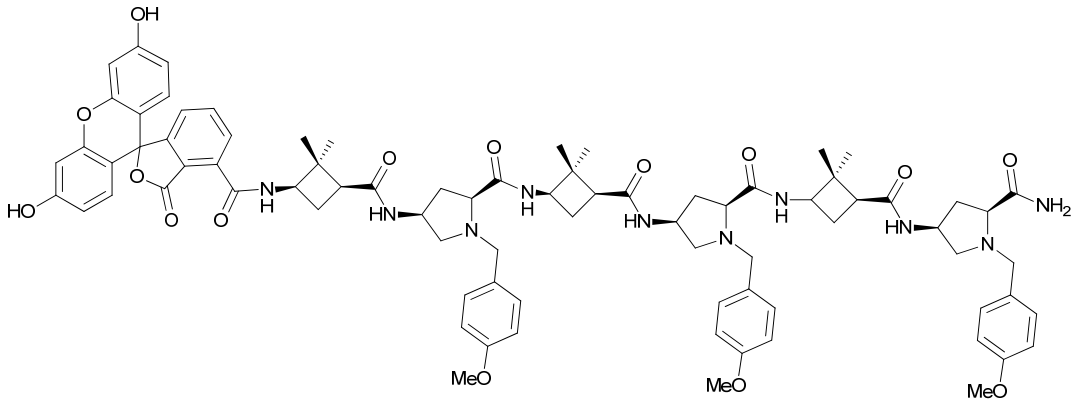
The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 2 min, increased to 25 % in 3 min, from 25 to 30 % in 7 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). mp 144-146 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +12 (*c* = 0.3, CH_3OH). IR (ATR): ν 3260, 3068, 2917, 2850, 1729, 1667, 1547, 1536, 1516 cm^{-1}. δ_H (400 MHz, CD_3OD) 0.66-1.26 (c.s., 18H), 1.96-2.96 (c.s., 25H), 3.39-3.69 (c.s., 6H, 3CH_2Bn), 3.80-3.85 (c.s., 9H), 4.07-4.59 (c.s., 5H), 7.00 (c.s., 6H), 7.44 (c.s., 6H); δ_C (100 MHz, CD_3OD) 17.3-37.4 (12C), 44.5-44.9 (3C), 47.0-51.5 (6C), 55.9 (3C), 58.8-67.0 (12C), 115.6 (6C), 123.9 (3C), 132.7-132.9 (6C), 162.3 (3C); *m/z* (ESI): Found, 1089.6493 $[M + H]^+$. Calcd. for $C_{60}H_{85}N_{10}O_9$: 1089.6496.

Proline-cyclobutane γ,γ-hexapeptide 28



The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 2 min, increased to 25 % in 3 min, from 25 to 30 % in 7 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). mp 133-135 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +16 (*c* = 0.3, CH_3OH). IR (ATR): ν 3278, 3068, 2915, 2849, 1730, 1667, 1613, 1546, 1516 cm^{-1}. δ_H (360 MHz, CD_3OD) 0.66 (c.s., 6H), 1.17 (c.s., 6H), 1.32 (c.s., 6H), 1.78-2.91 (c.s., 21H), 3.75-3.92 (c.s., 16H), 4.00-4.50 (c.s., 13H), 6.96 (c.s., 8H), 7.71 (c.s., 8H); δ_C (100 MHz, CD_3OD) 17.1-17.4 (3C), 24.1-25.4 (3C), 29.0-33.0 (6C), 37.1-37.6 (3C), 44.4-51.6 (7C), 55.9 (4C), 59.0-67.1 (12C), 115.4-115.7 (8C), 123.9 (4C), 132.6-132.8 (8C), 162.4 (4C); *m/z* (ESI): Found, 1209.7065 $[M + H]^+$. Calcd. for $C_{68}H_{93}N_{10}O_{10}$: 1209.7071.

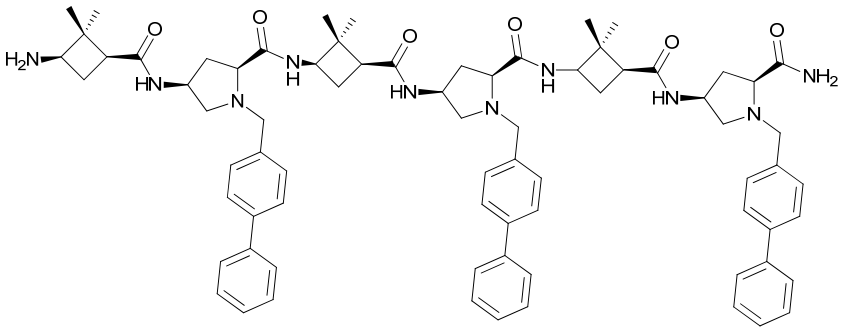
Proline-cyclobutane γ,γ-(CF)hexapeptide 29



The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 5 min, increased to 20 % in 1 min, from 20 to 30 % in 13 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and

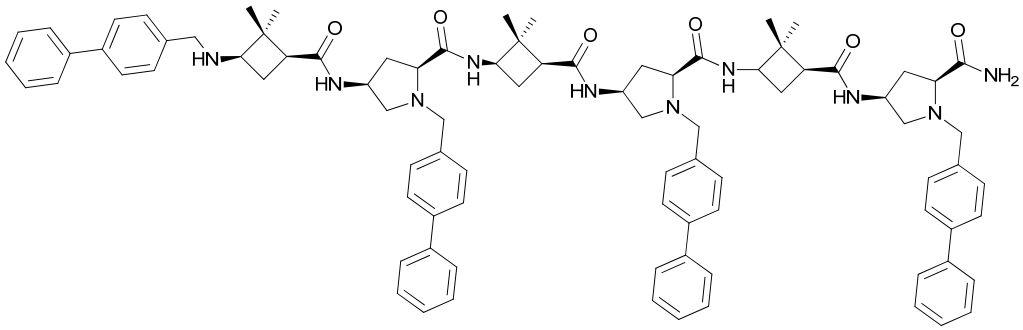
MALDI-TOF and showed that the peptide was 100% pure. MS calcd for $C_{81}H_{95}N_{10}O_{15}$ $[M + H]^+$: 1447.70. MALDI-TOF found: 1447.54 $[M + H]^+$, 1471.51 $[M + Na]^+$, and 1487.49 $[M + K]^+$.

Proline-cyclobutane γ,γ-hexapeptide 30



The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 3 min, increased to 40 % in 1 min, from 40 to 55 % in 8 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). mp 66-69 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +10 (*c* = 0.3, CH_3OH). IR (ATR): ν 3276, 3059, 2918, 2850, 1655, 1535 cm^{-1}. δ_H (360 MHz, CD_3OD) 0.51-1.42 (c.s., 18H), 1.90-3.18 (c.s., 21H), 3.43-3.70 (c.s., 4H), 3.77- 4.56 (c.s., 11H), 7.27-7.77 (c.s., 27H); δ_C (90 MHz, CD_3OD) 17.0 -17.5 (3C), 23.7-26.0 (3C), 28.7-33.1 (6C), 37.0 (3C), 44.6-51.6 (6C), 59.0-61.0 (9C), 66.7-67.4 (3C), 128.0-132.3 (27C), 141.2-144.0 (9C), 173.45 (6C); *m/ z* (ESI): Found, 1227.7119 $[M + H]^+$. Calcd. for $C_{75}H_{91}N_{10}O_6$: 1227.7118.

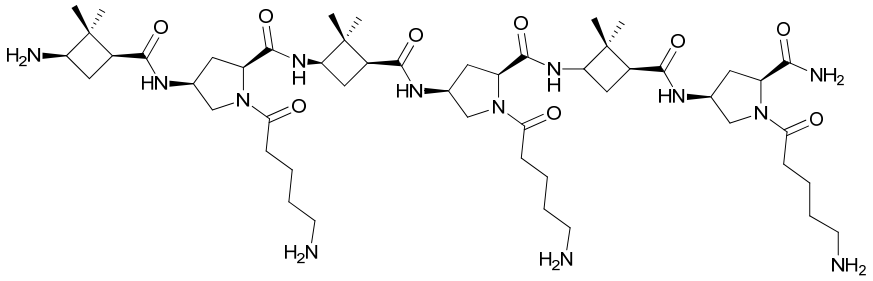
Proline-cyclobutane γ,γ-hexapeptide 31



The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 3 min, increased to 40 % in 1 min, from 40 to 55 % in 8 min, increased to 100 % MeCN in 1 min, and finally the original conditions were re-established) of MeCN (containing 1% of TFA) and H_2O (containing 1% of TFA). mp 141-143 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +10 (*c* = 0.3,

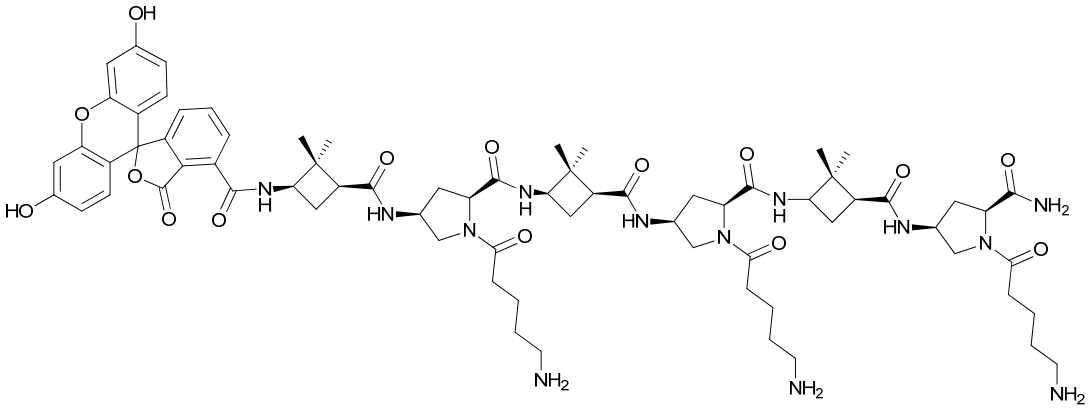
CH_3OH). IR (ATR): ν 3276, 3059, 2918, 2850, 1655, 1535 cm^{-1}. δ_H (400 MHz, CD_3OD) 0.63-1.40 (c.s., 18H), 1.84-3.28 (c.s., 22H), 3.46-4.53 (c.s., 16H), 7.27-7.81 (c.s., 36H); δ_C (100 MHz, CD_3OD) 17.2-17.5 (3C), 23.6-26.0 (3C), 29.1-33.1 (6C), 37.7 (3C), 44.4-45.7 (3C), 47.1-51.6 (4C), 59.0-60.3 (8C), 64.1, 66.5-71.2 (3C), 127.9-131.6 (36C), 141.3-144.0 (12C), 173.1-175.5 (6C); *m/ z* (ESI): Found, 1393.7910 $[M + H]^+$. Calcd. for $C_{88}H_{101}N_{10}O_6$: 1393.7900.

Proline-cyclobutane γ,γ-hexapeptide 33



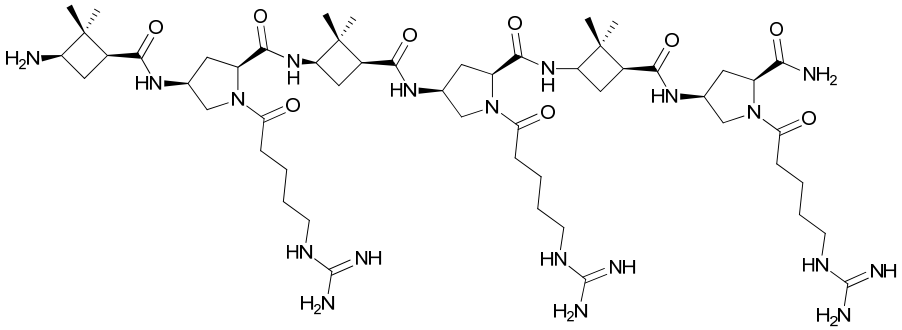
The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 2 min, increased to 10 % in 0.5 min, from 10 to 20 % in 6.5 min, increased to 100 % MeCN in 0.5 min, and finally the original conditions were re-established) of MeCN and H_2O. The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 98% pure. MS calcd for $C_{51}H_{88}N_{13}O_9$ $[M+H]^+$: 1220.39. MALDI-TOF found: 1220.49 $[M + H]^+$ and 1258.45 $[M + K]^+$. mp 58-61 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +32 (*c* = 0.2, CH_3OH). IR (ATR): ν 3279, 3058, 2958, 1669, 1628, 1533 cm^{-1}. δ_H (400 MHz, CD_3OD) 0.98-1.37 (c.s., 18H), 1.72 (c.s., 12H), 1.80-2.77 (c.s., 24H), 2.97 (c.s., 4H), 3.42 (c.s., 3H), 3.98 (c.s., 5H), 4.37-4.56 (c.s., 6H); δ_C (100 MHz, CD_3OD) 17.0-17.3 (3C), 22.0-22.3 (3C), 24.9-25.7 (3C), 28.0-29.7 (6C), 33.8-36.0 (6C), 40.4 (3C), 43.9-47.7 (6C), 50.0-54.1 (6C), 59.7-60.5 (6C), 162.9-163.2 (2C), 172.9-174.4 (6C), 177.26; *m/ z* (ESI): Found, 513.8451 $[(M + 2H)/2]^+$. Calcd. for $(C_{51}H_{89}N_{13}O_9)/2$: 513.8451.

Proline-cyclobutane γ,γ-(CF)hexapeptide 34



The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 2 min, increased to 20 % in 0.5 min, from 20 to 40 % in 7 min, increased to 100 % MeCN in 0.5 min, and finally the original conditions were re-established) of MeCN and H_2O. The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 97% pure. MS calcd for $C_{72}H_{98}N_{13}O_{15}$ $[M + H]^+$: 1384.73. MALDI-TOF found: 1385.05 $[M + H]^+$, 1406.97 $[M + Na]^+$, and 1422.98 $[M + K]^+$.

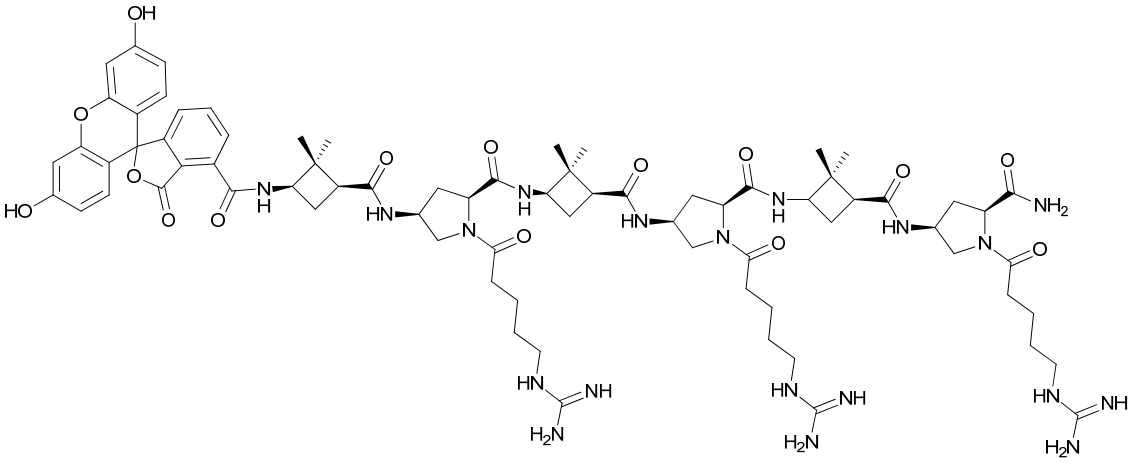
Proline-cyclobutane γ,γ-hexapeptide 35



The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 2 min, increased to 30 % in 0.5 min, from 30 to 32 % in 6.5 min, increased to 100 % MeCN in 0.5 min, and finally the original conditions were re-established) of MeCN and H_2O. The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 99% pure. MS calcd for $C_{54}H_{94}N_{19}O_9$ $[M + H]^+$: 1152.75. MALDI-TOF found: 1152.67 $[M + H]^+$, 1174.65 $[M + Na]^+$, and 1194.71 $[M +3H^+ + K]^{4+}$. mp 154-156 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = -25 (c = 0.3, CH_3OH). IR (ATR): ν 3264, 3185, 3067, 2950, 2873, 1620, 1561, 1544, 1535, 1460, 1439, 1431, 1405 cm^{-1}. δ_H (360 MHz, CD_3OD) 0.96-1.36 (c.s., 18H), 1.64 (c.s., 12H), 1.85-2.70 (c.s., 24H), 2.99-3.25 (c.s., 4H), 3.38 (c.s., 3H), 3.93 (c.s., 5H), 4.35-4.57 (c.s., 6H); δ_C (90

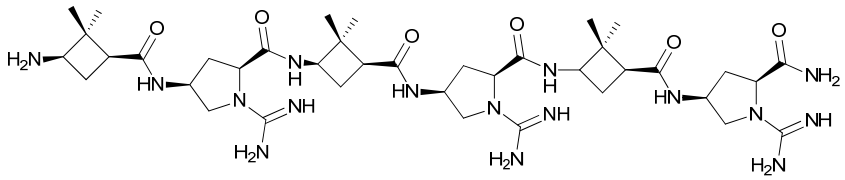
MHz, CD_3OD) 16.8-17.4 (3C), 22.7 (3C), 24.0-25.4 (3C), 28.8-30.7 (6C), 34.0-36.5 (6C), 42.2 (3C), 44.7-47.7 (6C), 50.0-54.5 (6C), 60.0-60.2 (6C), 158.7 (3C) 173.0-174. 6 (2C), 177.0-177.3 (6C), 180.0 (1C); *m/z* (ESI): Found, 384.9218 $[(M + 3H)/3]^+$. Calcd. for $(C_{54}H_{96}N_{19}O_9)/3$: 384.9207

Proline-cyclobutane γ,γ-(CF)hexapeptide 36



The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 2 min, increased to 20 % in 0.5 min, from 20 to 30 % in 6 min, increased to 100 % MeCN in 0.5 min, and finally the original conditions were re-established) of MeCN and H_2O. The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 90% pure. MS calcd for $C_{75}H_{104}N_{19}O_{15}$ $[M + H]^+$: 1510.74. MALDI-TOF found: 1510.99 $[M + H]^+$, 1532.96 $[M + Na]^+$, and 1548.93 $[M + K]^+$.

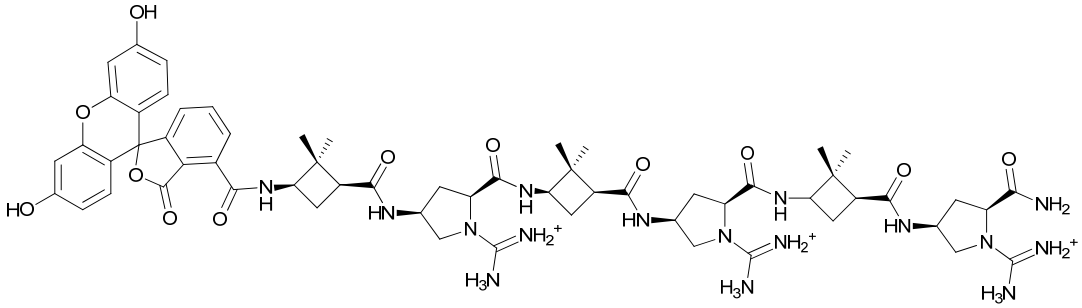
Proline-cyclobutane γ,γ-hexapeptide 37



The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 3 min, increased to 18 % in 1 min, maintained at 18 % for 11 min, increased to 100 % MeCN in 1 min, 3 min at 100 % in MeCN and finally the original conditions were re-established) of MeCN and H_2O containing 0.1 % of TFA. The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 97% pure. MS calcd for $C_{39}H_{67}N_{16}O_6$ $[M + H]^+$: 855.54. MALDI-TOF found: 855.48 $[M + H]^+$, 877.47 $[M + Na]^+$, and 893.45 $[M + K]^+$. mp

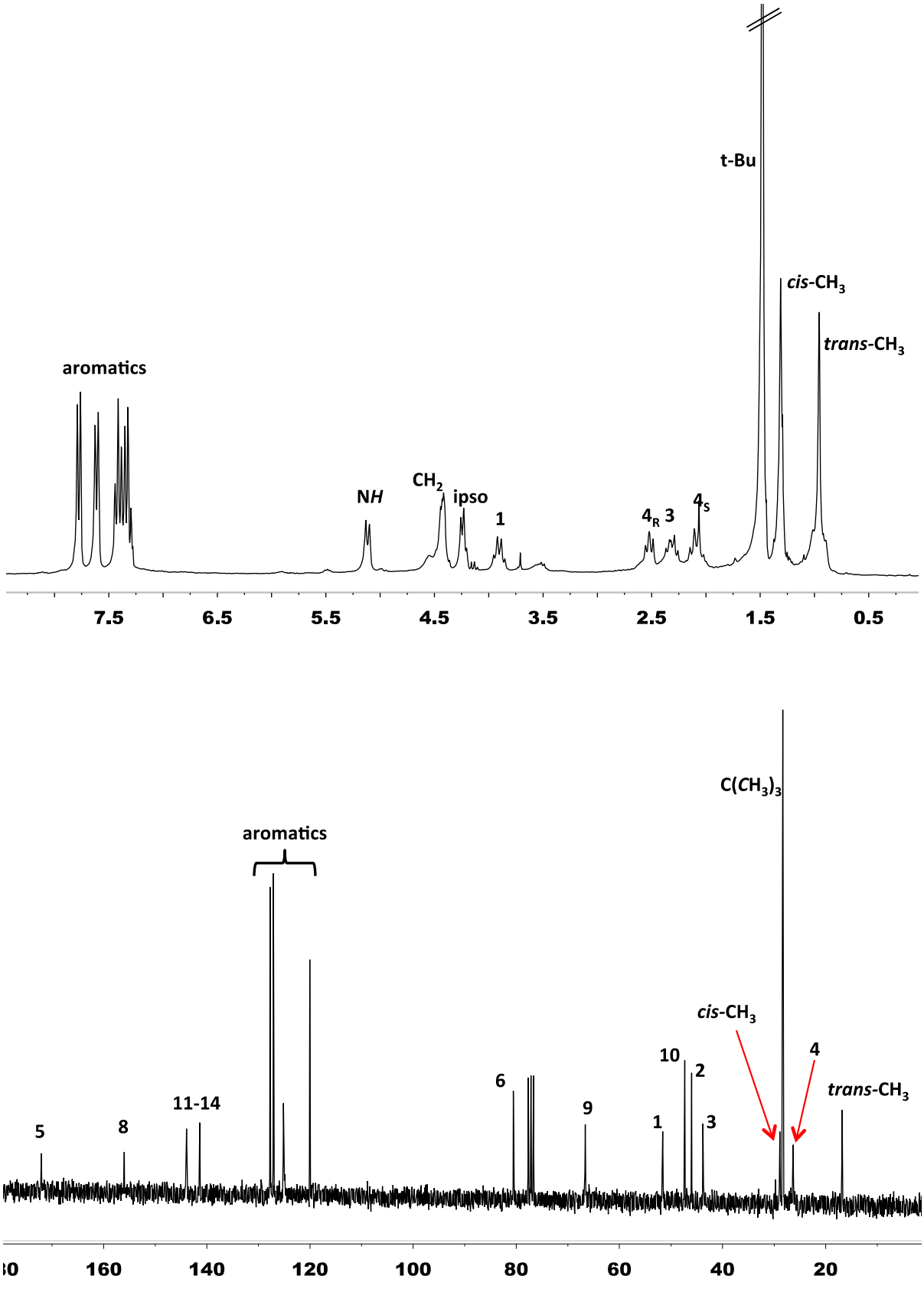
167-169 ºC (from CH_3CN / H_2O); $[\alpha]_D$ = +17 (*c* = 0.1, CH_3OH). IR (ATR): ν 3187, 2963, 1688, 1658, 1641, 1631, 1611 cm^{-1}. δ_H (360 MHz, CD_3OD) 0.90-1.33 (c.s., 18H), 1.61 (c.s., 3H), 1.93-2.33 (c.s., 12H), 2.47-3.06 (c.s., 10H), 3.59-4.04 (c.s., 6H), 4.48 (c.s., 2H); *m/ z* (ESI): Found, 428.2756 $[(M + 2H)/2]^+$. Calcd. for $(C_{39}H_{68}N_{16}O_6)/2$: 428.2748

Proline-cyclobutane γ,γ-(CF)hexapeptide 38

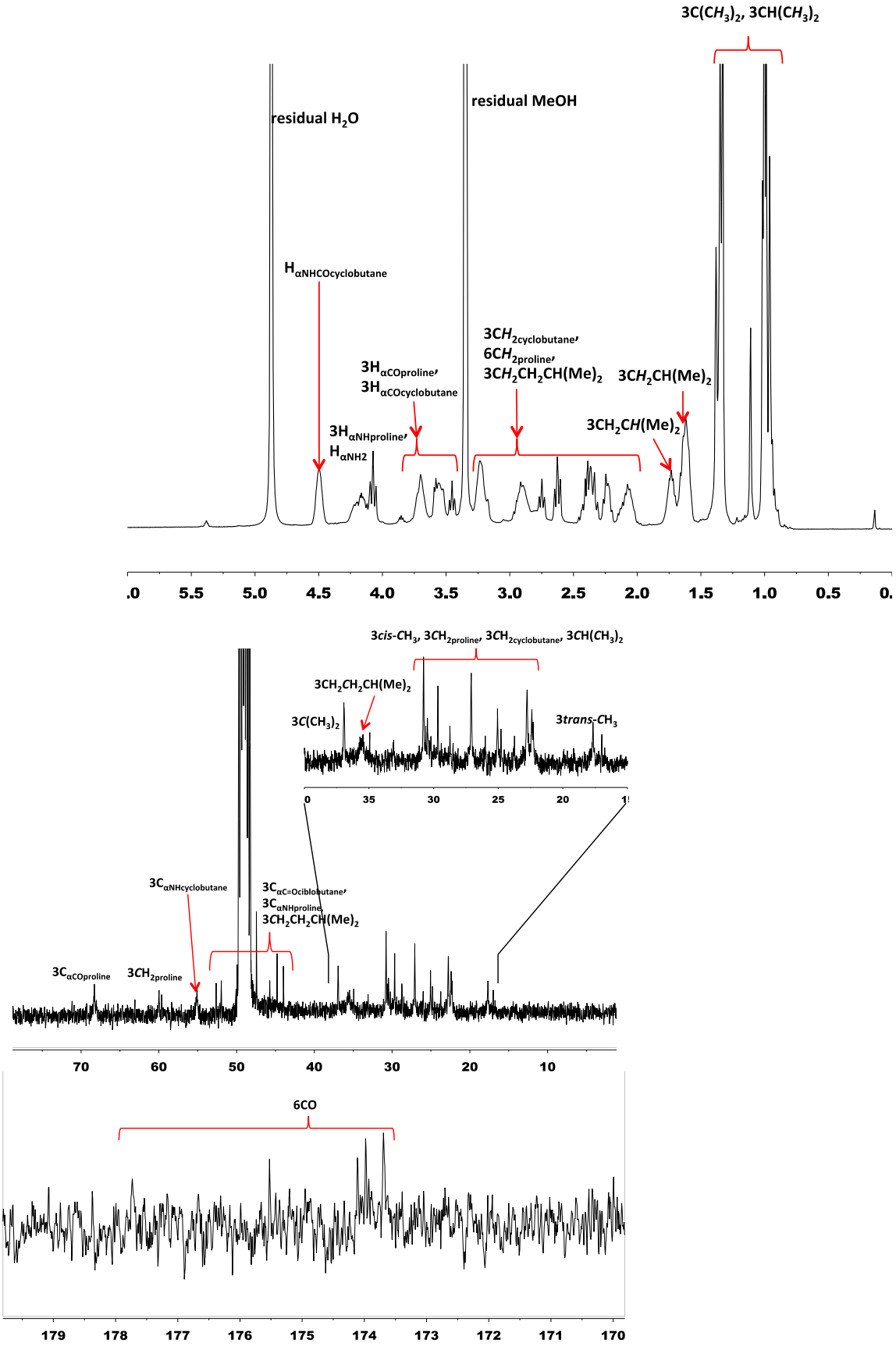


The crude peptide was purified by semi-preparative HPLC-MS using a non-linear gradient (5 % MeCN for 3 min, increased to 35 % in 1 min, from 35 to 38 % in 11 min, increased to 100 % MeCN in 1 min, 3 min at 100 % in MeCN and finally the original conditions were re-established) of MeCN and H_2O containing 0.1 % of TFA. The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 99% pure. MS calcd for $C_{60}H_{80}N_{16}O_{12}$ $[M + H]^+$: 1216.61. MALDI-TOF found: 1216.40 $[M + H]^+$.

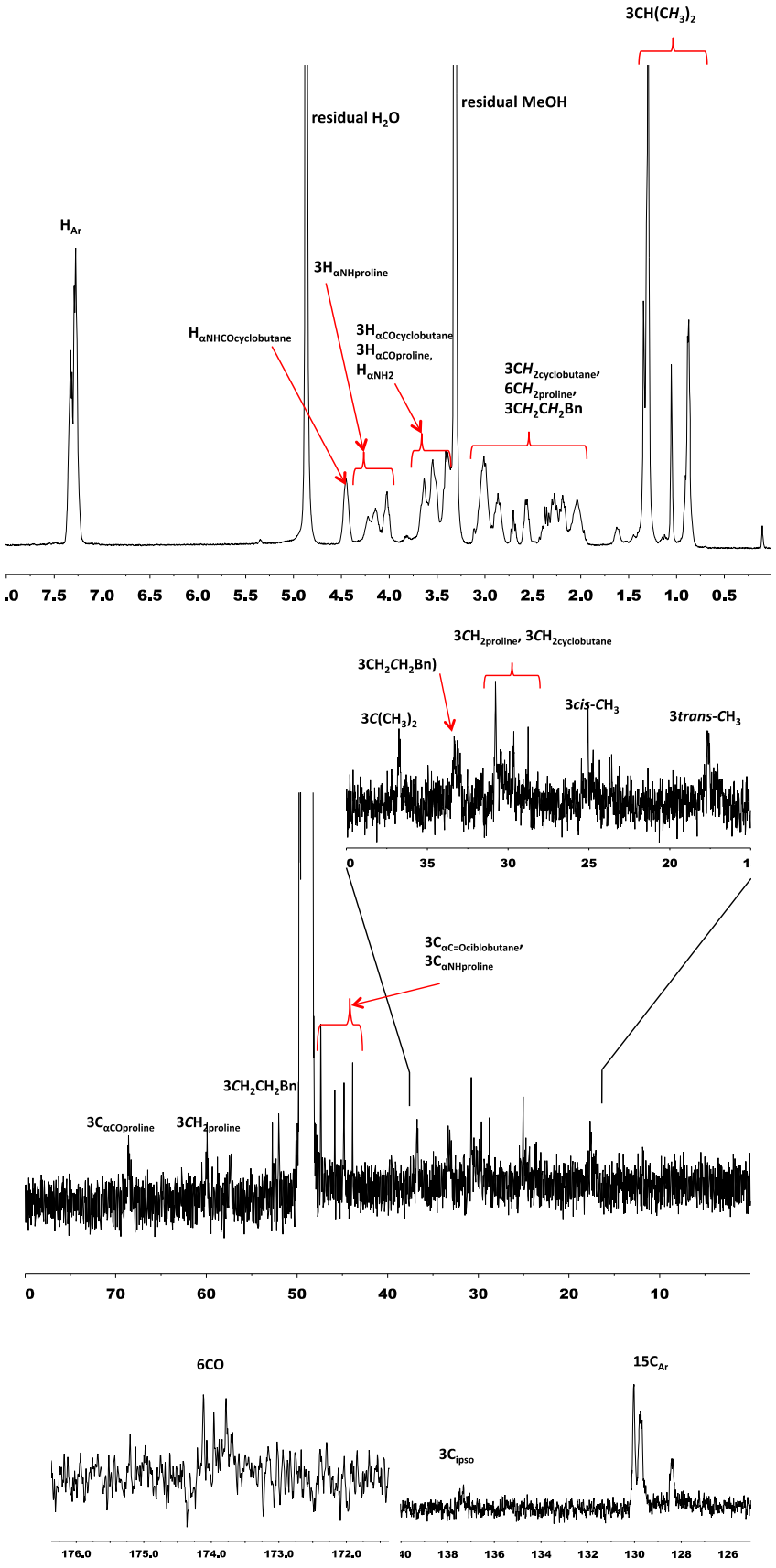
^{1}H-NMR ($CDCl_3$, 250 MHz) and ^{13}C-NMR ($CDCl_3$, 62.5 MHz) spectra for compound 14



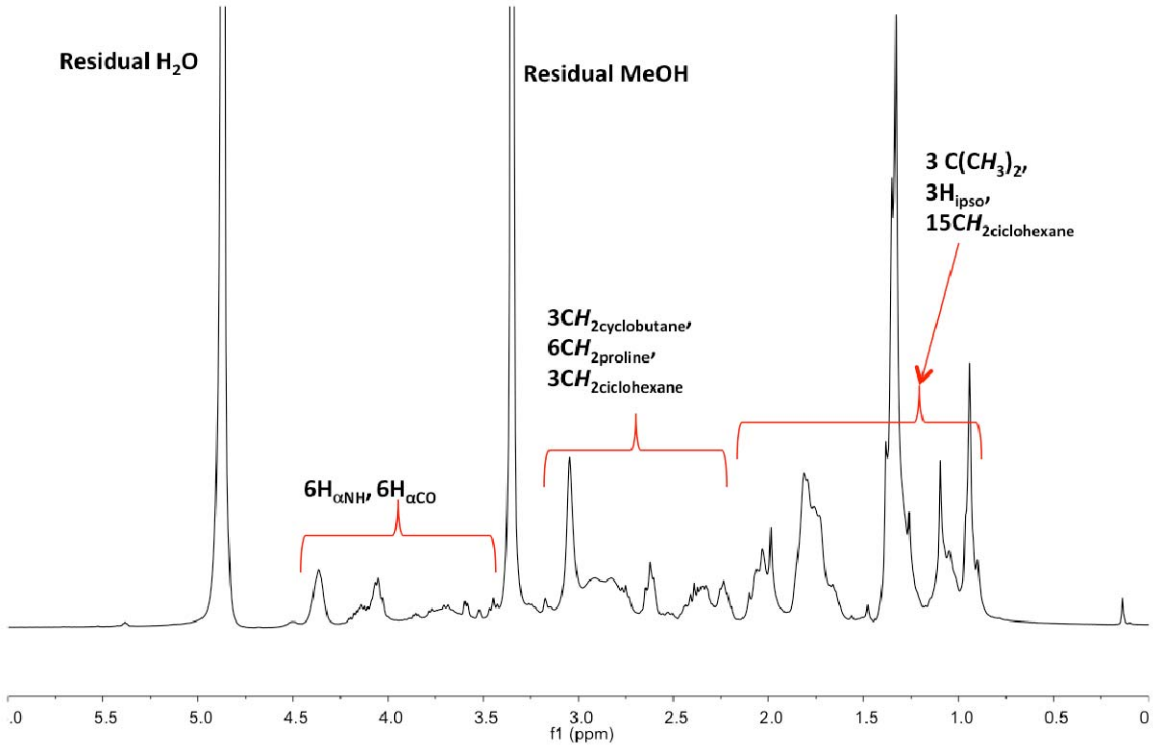
^{1}H-NMR (Methanol-d_4, 400 MHz) and ^{13}C-NMR (Methanol-d_4, 90 MHz) spectra for compound 19

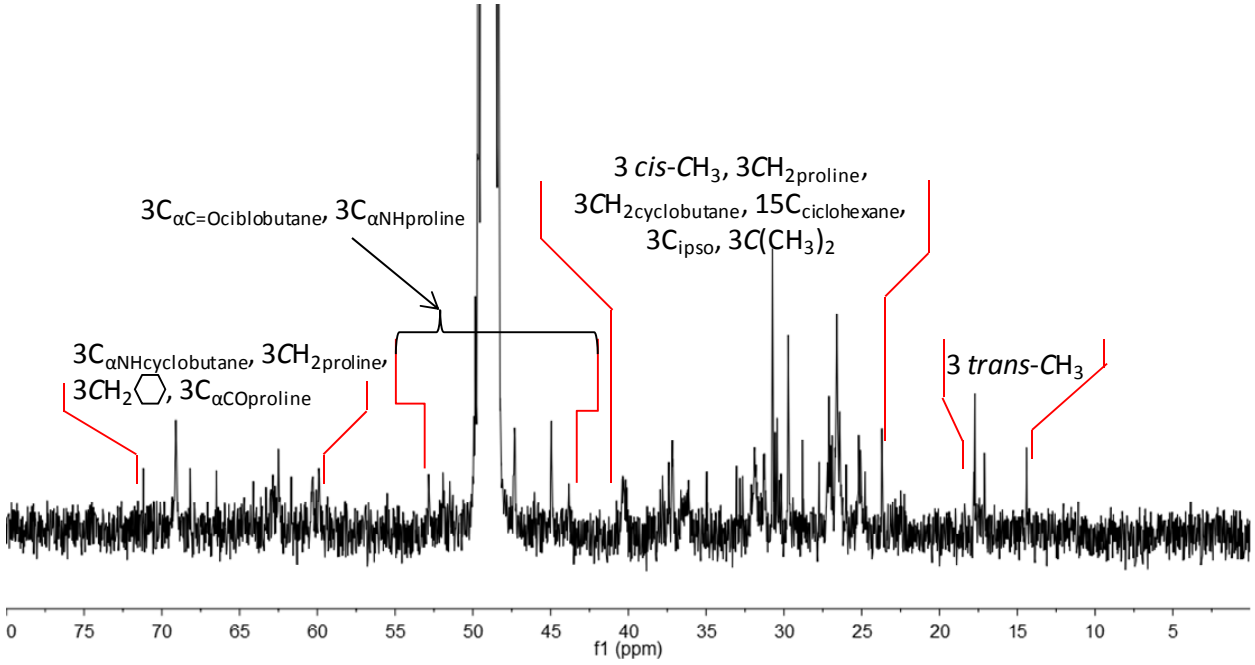


^{1}H-NMR (Methanol-d_4, 400 MHz) and ^{13}C-NMR (Methanol-d_4, 90 MHz) spectra for compound 21

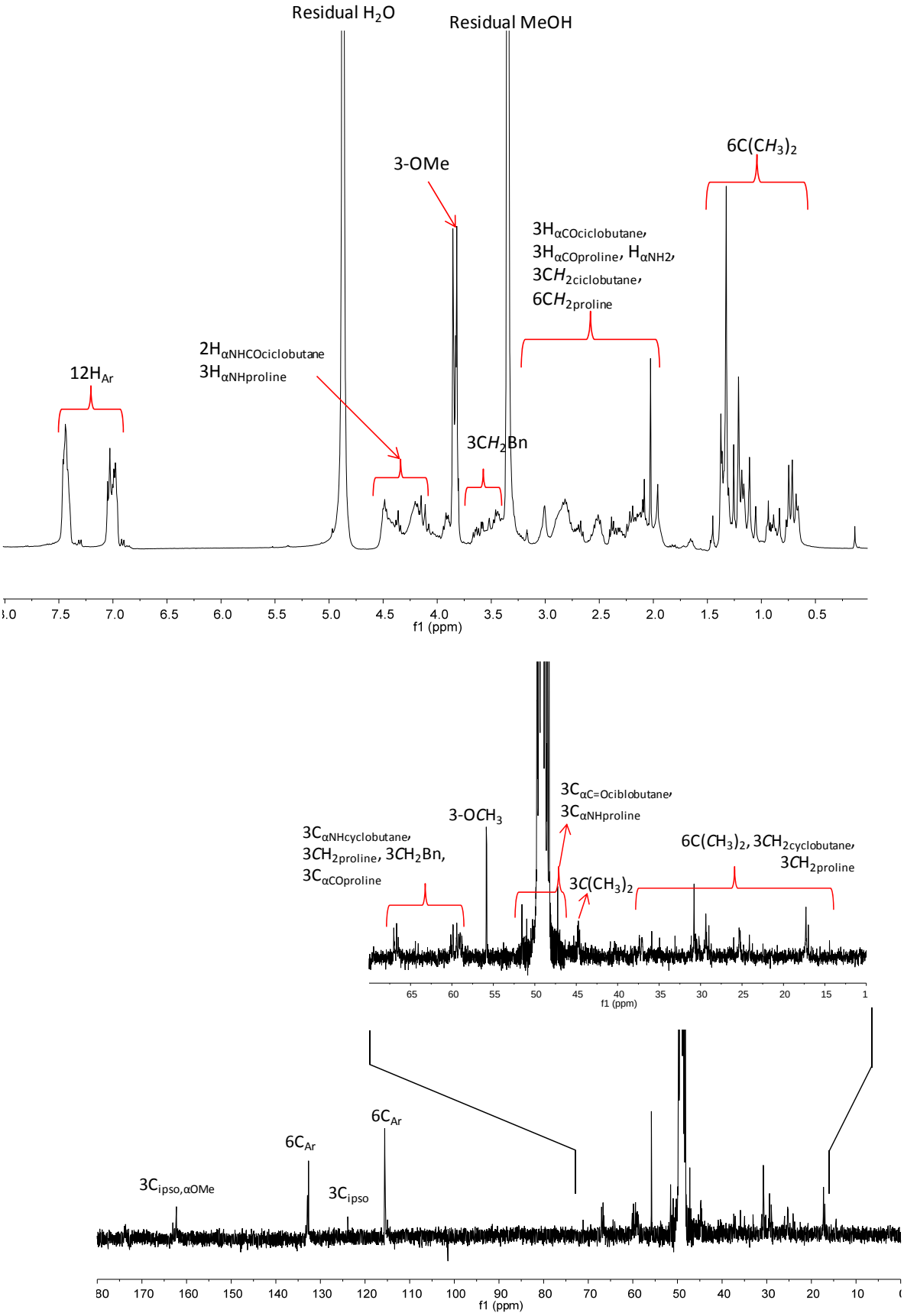


^{1}H-NMR (Methanol-d_4, 400 MHz) and ^{13}C-NMR (Methanol-d_4, 100 MHz) spectra for compound 25

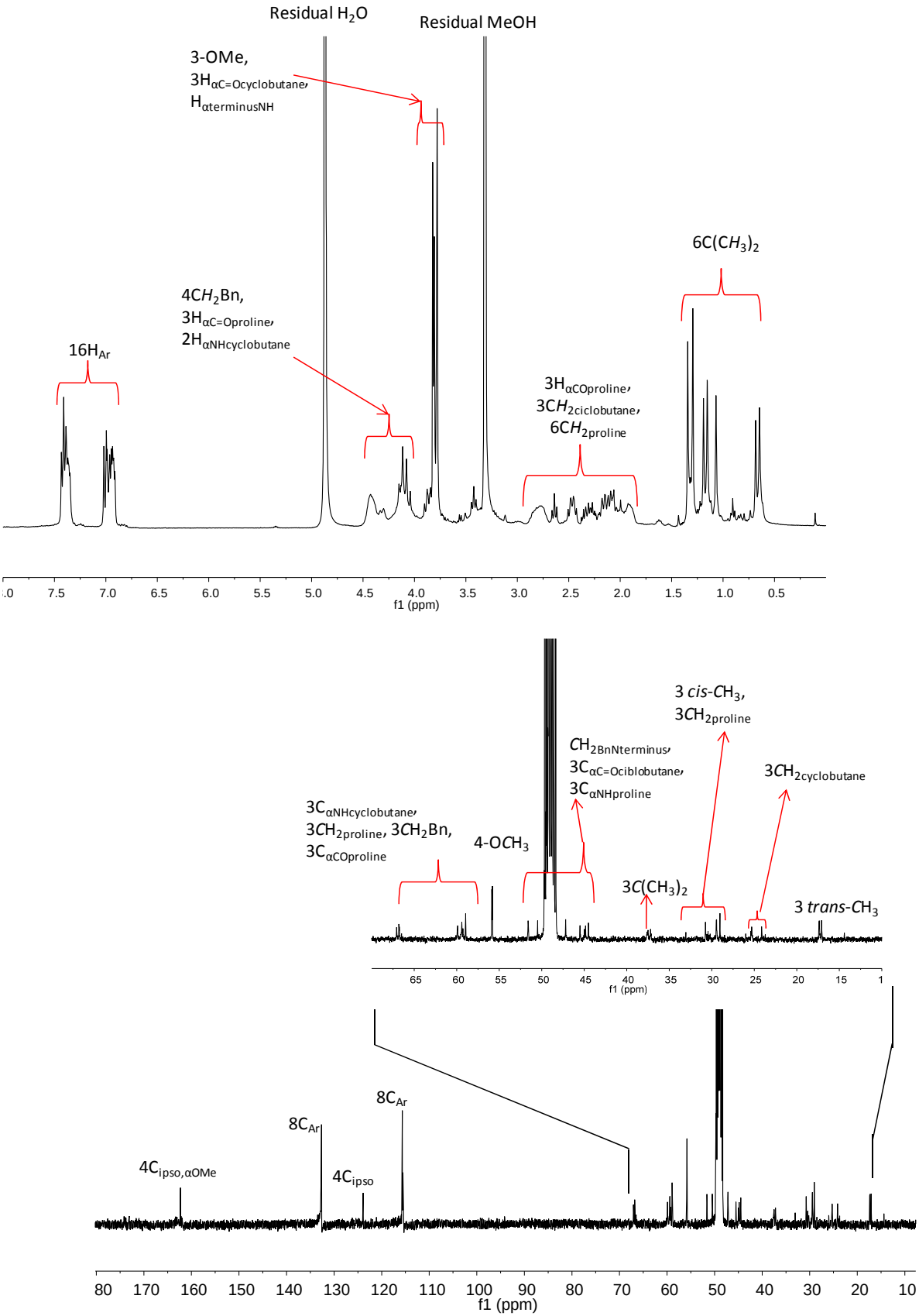




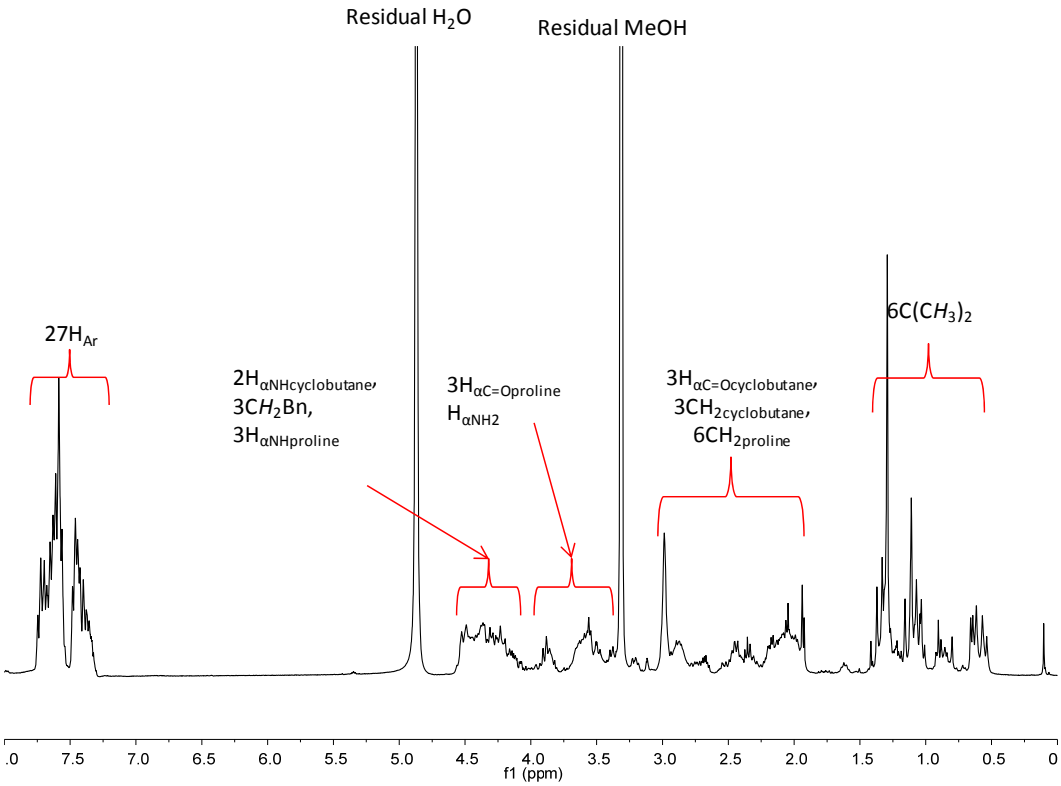
^{1}H-NMR (Methanol-d_4, 400 MHz) and ^{13}C-NMR (Methanol-d_4, 100 MHz) spectra for compound 27

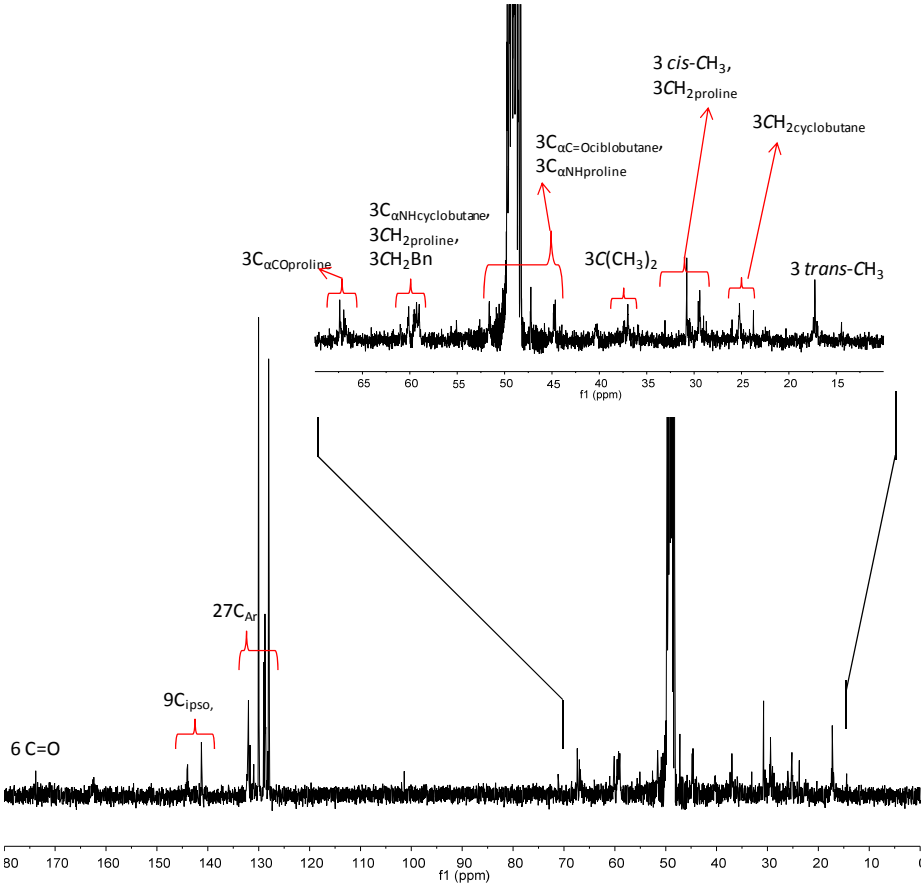


^{1}H-NMR (Methanol-d_4, 360 MHz) and ^{13}C-NMR (Methanol-d_4, 100 MHz) spectra for compound 28

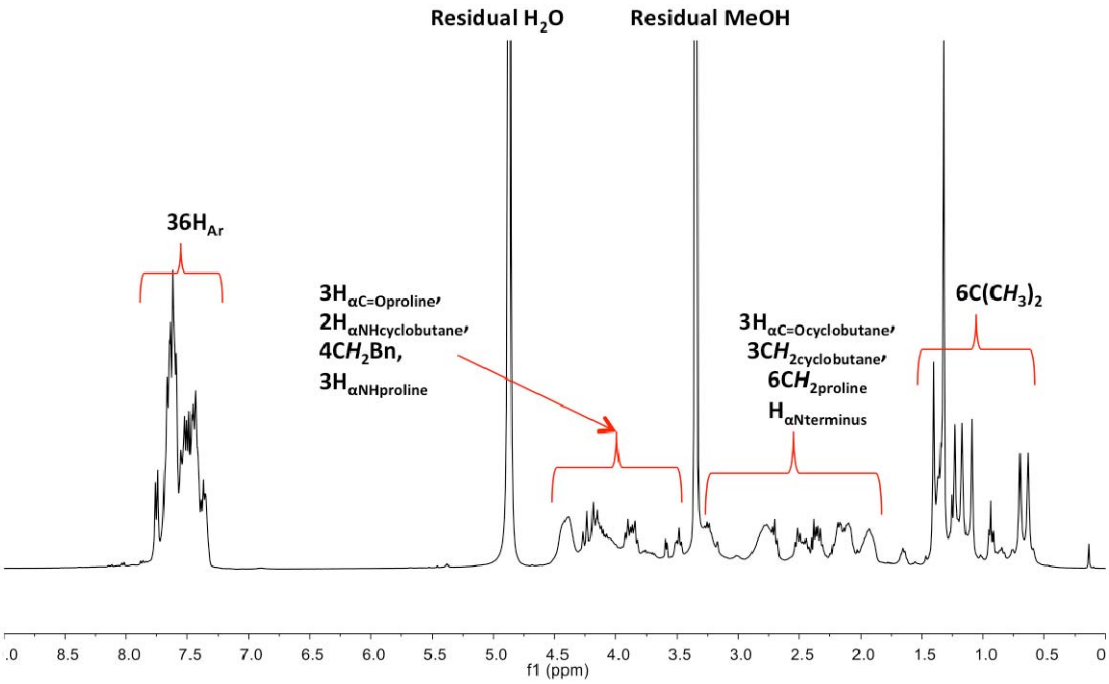


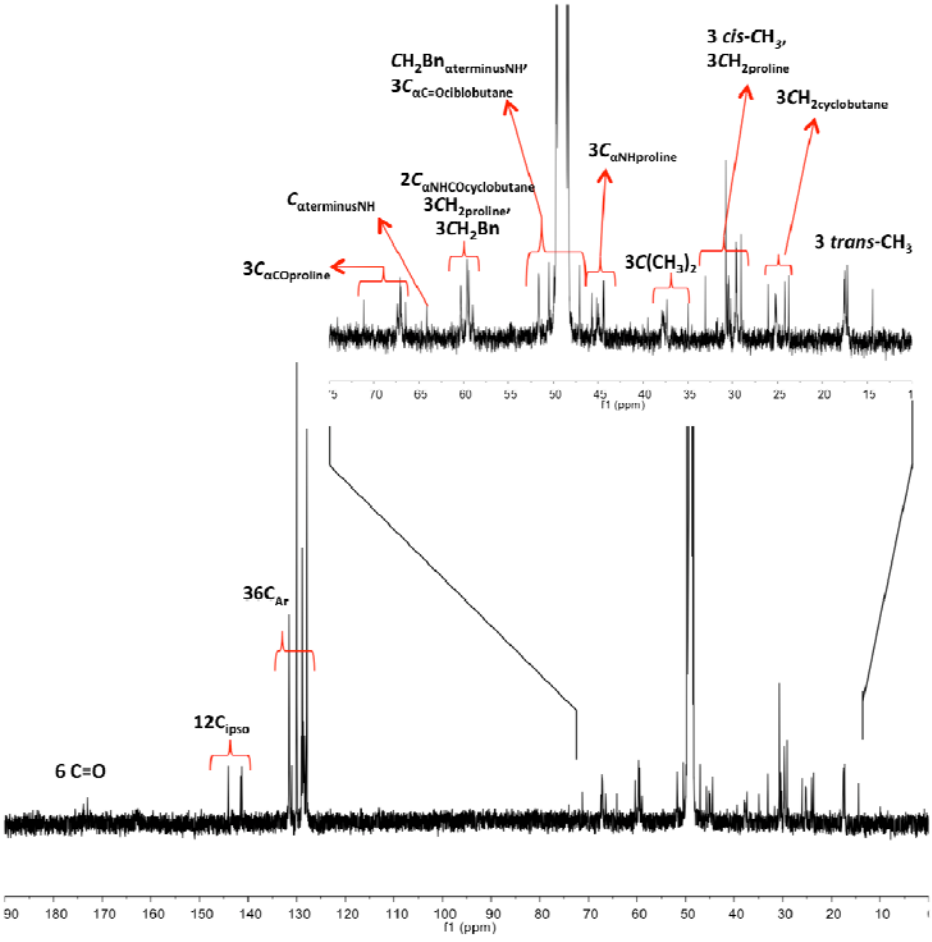
^{1}H-NMR (Methanol-d_4, 360 MHz) and ^{13}C-NMR (Methanol-d_4, 100 MHz) spectra for compound 30



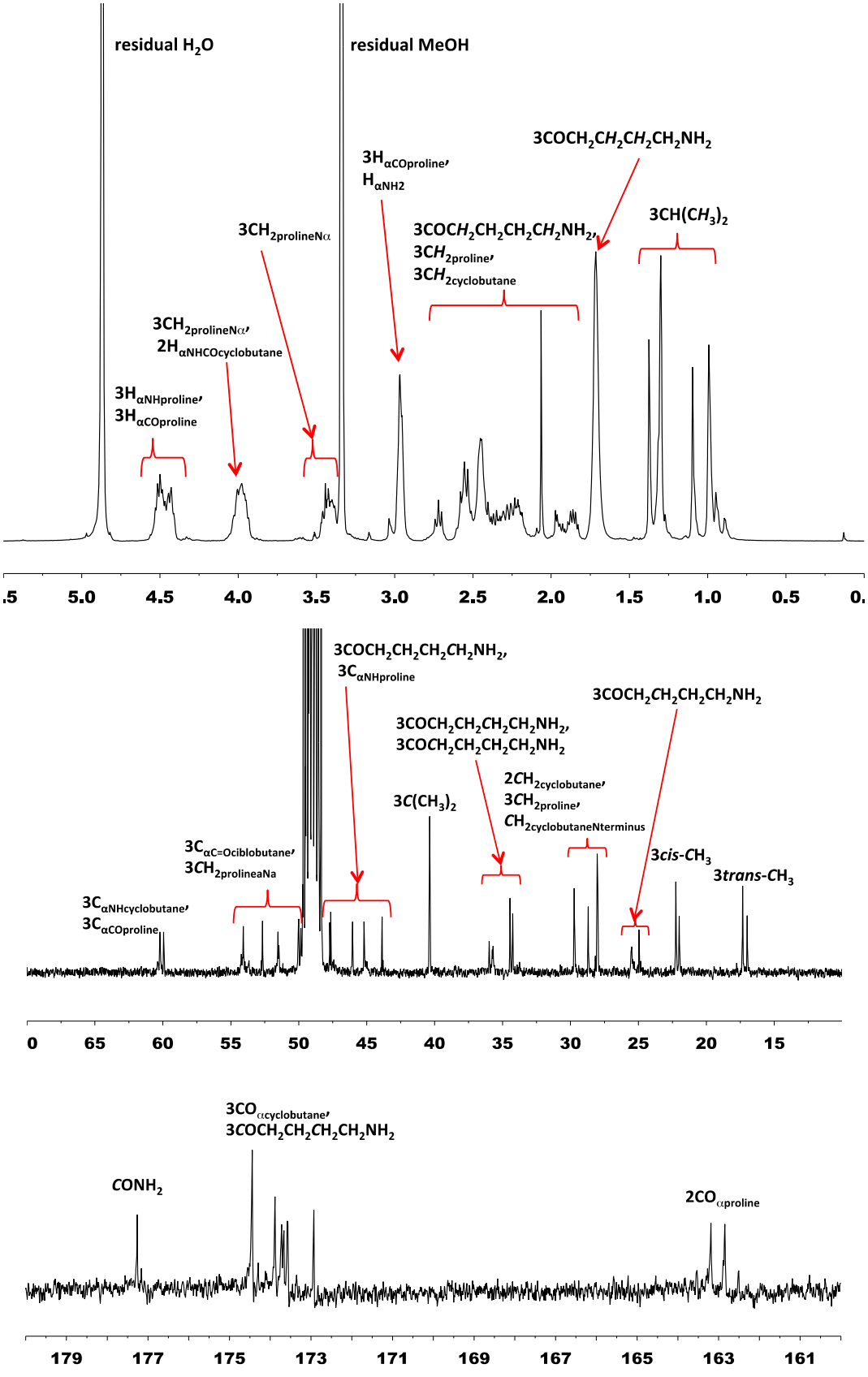


^{1}H-NMR (Methanol-d_4, 400 MHz) and ^{13}C-NMR (Methanol-d_4, 100 MHz) spectra for compound 31

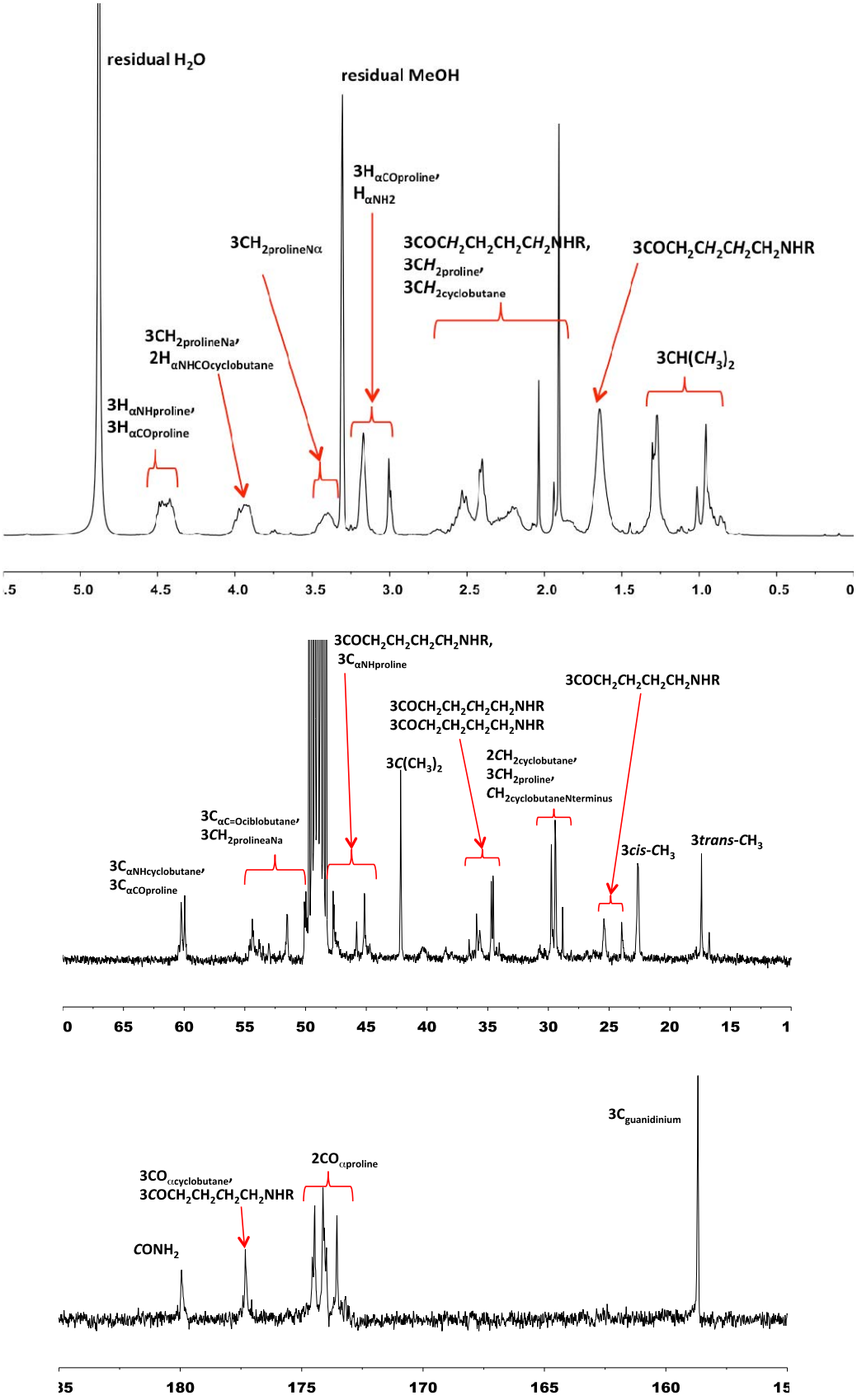




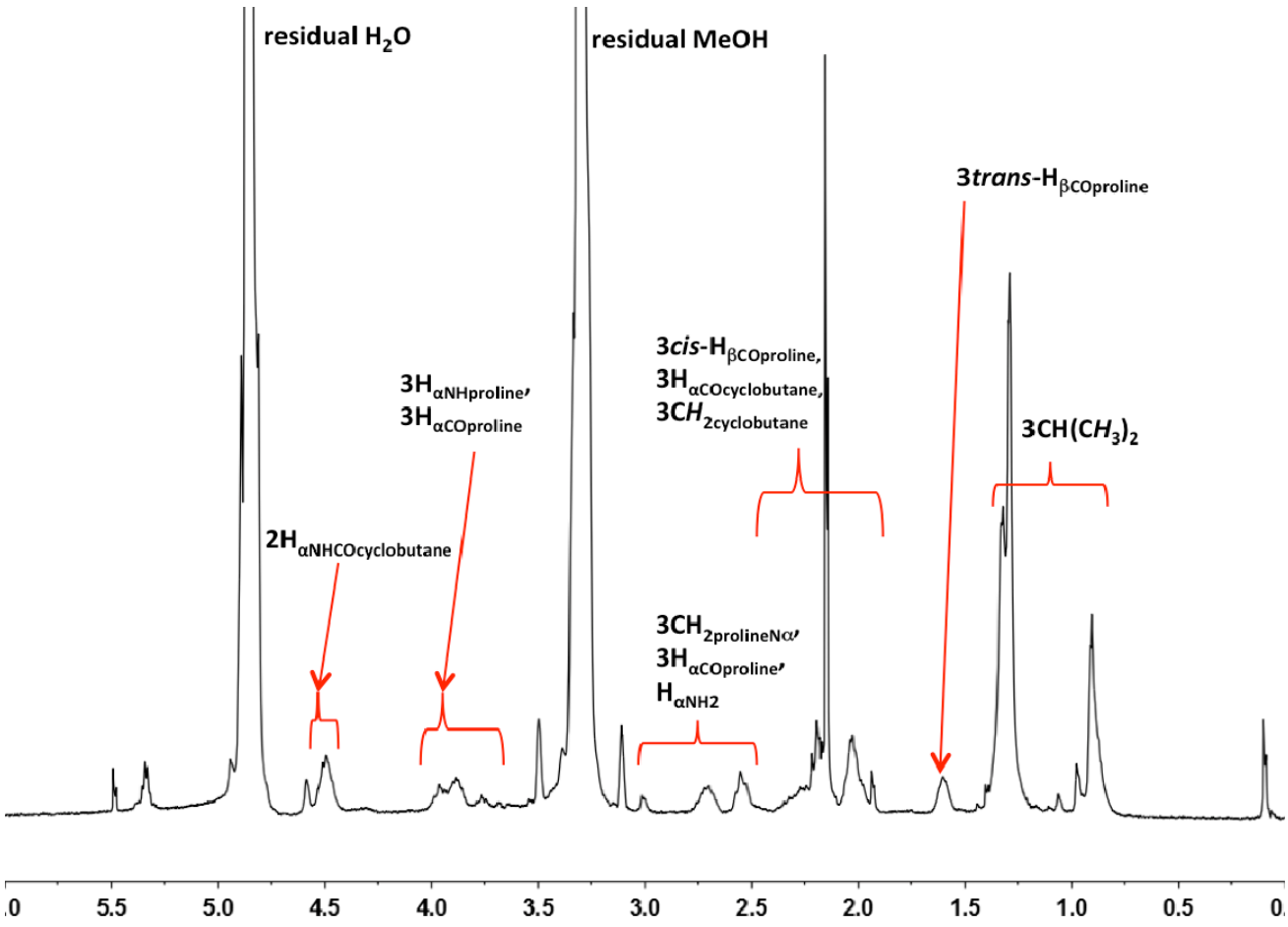
^{1}H-NMR (Methanol-d_4, 400 MHz) and ^{13}C-NMR (Methanol-d_4, 100 MHz) spectra for compound 33



^{1}H-NMR (Methanol-d_4, 360 MHz) and ^{13}C-NMR (Methanol-d_4, 90 MHz) spectra for compound 35



^{1}H-NMR (Methanol-d_4, 360 MHz) spectrum for compound 37



Cytotoxicity and cell-uptake properties of peptides **7-12**

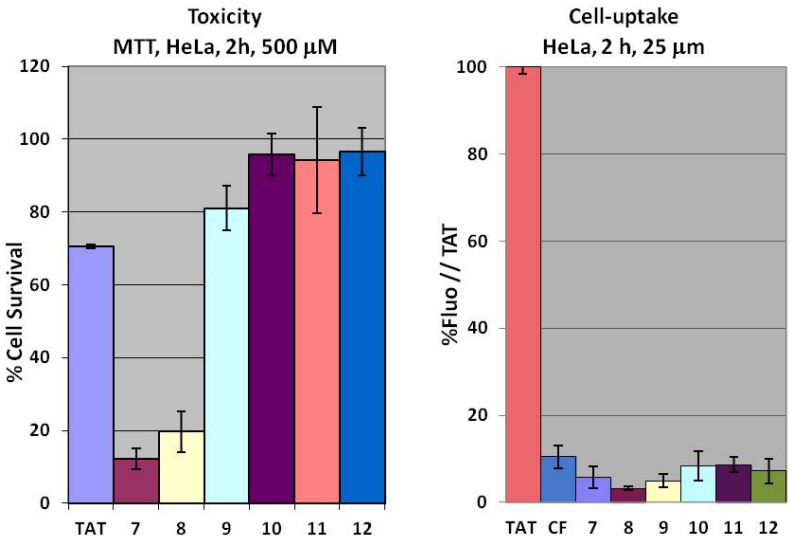


Figure S1 Representation of the cytotoxicity and cell-uptake properties of hybrid peptides **7-12** in relation to the reference peptide TAT.

Cytotoxicity of peptides**, 20, 22, 24, 26, 29, 34, 36,** and **38**

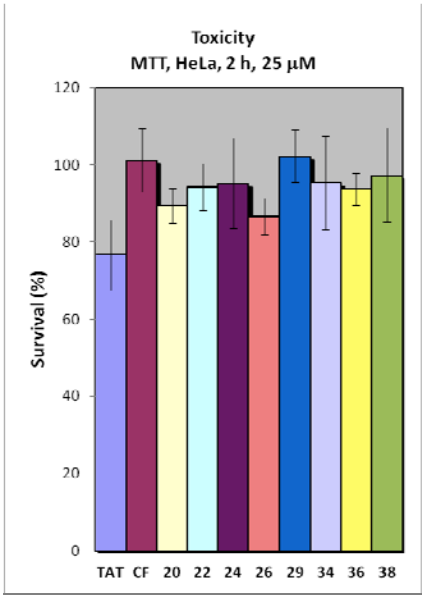


Figure S2. Cytotoxicity of the different γ-hexapeptides as monitored in HeLa cell lines. Cell death was quantified using the MTT assay after 2 h of incubation using 25 µM peptide concentration. Error bars represent standard deviation (SD) from the mean value of three independent experiments of each peptide**.**