Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.
This journal is © The Royal Society of Chemistry 2014

Bidirectional Macrocyclization of Peptides by Double Multicomponent Reactions

Manuel G. Ricardo,[a] Fidel. E. Morales,[a,b] Hilda Garay,[b] Osvaldo Reyes,[b] Dimitar Vasilev,[c] Ludger A. Wessjohann,[c]* and Daniel G. Rivera,[a]*

[a]Center for Natural Products Research, Faculty of Chemistry, University of Havana, Zapata y G, 10400, La Habana, Cuba. Tel.: +537 8792331. Email: dgr@fq.uh.cu

[b]Laboratory of Peptide Synthesis, Physical-Chemistry Division, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, La Habana, Cuba.

[c]Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120, Halle/Saale, Germany. Email: wessjohann@ipb-halle.de

Experimental Section

General solution-phase peptide coupling: The Boc-protected aminoacid (1.0 mmol, 1.0 equiv.), HOBt (149 mg, 1.1mmol, 1.1 equiv.), EDC (241 mg, 1.1 mmol, 1.1 equiv.) and the *C*-methylesteraminoacid hydrochloride are suspended in dry CH_2Cl_2 (10 mL). Et_3N (0.15 mL, 1.1mmol, 1.1 equiv.) is syringed in one portion and the resulting solution is stirred at room temperature overnight (~12 h). The reaction mixture is then diluted with 100 mL EtOAc, transferred to a separatory funnel and sequentially washed with 0.5 M aqueous solution of citric acid (2×50 mL) and saturated aqueous suspension $NaHCO_3$ (2×50 mL). The organic phase is dried over $MgSO_4$, filtered and concentrated under reduced pressure.

Bidirectional solution-phase peptide coupling: The Boc-protected amino acid acid (*N*-Boc-Asp-OH or *N*-Boc-Glu-OH) (1.0 mmol, 1.0 equiv.) was bidirecctionally coupled to *C*-methyl ester amino acid (dipeptide, tripeptide) hydrochloride (2 equiv.) in the presence of HOBt (198 mg, 2.2 mmol, 2.2 equiv.), EDC (482 mg, 2.2 mmol, 2.2 equiv.) and Et_3N (0.30 mL, 2.2 mmol, 2.2 equiv.) according to the general peptide coupling procedure.

General carboxylic acid deprotection procedure: The peptide is dissolved in THF/H_2O (2:1, 15 mL) and LiOH (6 eq.) was added at 0 °C. After completion of the deprotection, as confirmed by TLC and ESI-MS analysis, the reaction mixture is treatedwith aq. 10% $NaHSO_4$ to pH 3. The resulting phases were separated and the aqueous phase was additionally extracted with EtOAc (2×20 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure.

General Boc removal procedure: The crude peptide is exposed to high vacuum for 1 hour before dissolving it in a 4 M HCl solution in dioxane (2 mL) for Boc removal. As the material dissolved, gas evolution could be detected and the pressure that built up inside the reaction flask is regularly relieved by opening the reaction flask. After 30 min, usually no starting material is detected by thin layer chromatography and the reaction is concentrated under a stream of dry N_2 for about 30 min. The volatiles are then fully removed by concentrating the resulting thick oily residue under reduced pressure in the rotary evaporator and then placing the flask under high vacuum. If required, the hydrochloride salt can be crystallized from frozen diethyl ether.

General peptide *N*-terminal acetylation: The peptide (1 mmol, 1 equiv) is dissolved in CH_2Cl_2 (3 mL, 3:1) and treated with Ac_2O (85μL, 1.5 equiv) and Et_3N (430 μL, 3 equiv). The reaction mixture was stirred for 2 h and the volatiles were evaporated under reduced pressure. The crude product was dissolved in EtOAc (50 mL) and washed with aq. 10% HCl (2×10 mL) and aq.10%

$NaHCO_3$ (2×30 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford the *N*-acetyl peptide.

Ac-Glu-Ala-Phe-Glu-Leu-OMe (1): Boc-Glu(OBzl)-OH (337 mg,1.0 mmol) was coupled to HCl·Leu-OMe (181mg, 1.0mmol) according to the general peptide coupling procedure, following by deprotection of the *N*-terminus by Boc removal. The same protocol was employed for the sequential coupling of *N*-Boc-Phe-OH (265 mg, 1.0mmol), *N*-Boc-Ala-OH (189mg, 1.0mmol) and *N*-Boc-Glu(OBzl)-OH (337mg, 1.0mmol). Flash column chromatography purification (CH_2Cl_2/EtOAc 3:1) furnished pentapeptide Boc-Glu(Bzl)-Ala-Phe-Glu(Bzl)-Leu-OMe (478 mg, 53%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$): δ = 7.39-7.33 (m, 10H), 7.25-7.16(m, 5H), 5.13 (s, 2H), 5.09 (s, 2H), 4.68 (dd, *J* = 7.0, 4.2 Hz, 1H), 4.50 (m, 2H), 4.45 (m, 1H), 4.30 (m, 1H), 4.10 (m, 1H), 3.93 (m, 1H), 3.71 (s, 3H), 3.25 (dd, *J* = 14.0, 4.2 Hz, 1H), 3.05 (dd, *J* = 14.0, 9.1 Hz,1H), 2.55 – 2.32 (m, 4H), 2.27 (m, 2H), 2.07 (m, 1H), 1.87 (m, 1H), 1.75 (m, 1H), 1.63 – 1.57 (m, 3H), 1.40 (s, 9H), 1.28 (d, *J* = 6.6 Hz, 3H), 0.92-0.86 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$) δ= 173.7, 173.4, 172.9, 172.3, 171.6, 171.5, 170.5, 155.0 (CO), 136.6, 135.6 (C), 129.2, 128.8, 128.5, 128.2, 127.9 (CH) 79.8 (C), 66.8 (CH_2), 56.0, 55.6, 55.0, 54.3, 54.1 (CH), 52.4(CH_3), 41.9, 39.4, 34.4, 33.2, 29.5, 29.0 (CH_2), 28.4 (CH_3), 25.8 (CH), 24.2, 18.5 (CH_3). ESI-MS *m/z*: 924.45 $[M+Na]^+$, calcd. for$C_{48}H_{63}O_{12}N_5Na$: 924.43. This product (350 mg, 0.47 mmol) was subjected to Boc removal and *N*-terminal acetylation according to the general procedures. The resulting crude *N*-acetyl peptide was dissolved inAcOEt (20 mL) and 10% Pd(C) (60 mg) was added. The reaction mixture was treated successively with hydrogen and vacuum and finally stirred under hydrogen atmosphere for 24 h. Completion of the hydrogenation process was confirmed by TLC and ESI-MS analysis. The catalyst was then removed by filtration over Celite and the resulting solution

was evaporated under reduced pressureto furnish peptide **1** (253 mg, 72%).This product was recrystalized from Et_2O/AcOEt (5 mL, 5:1) and used forward without further purification. ESI-MS *m/z*: 662.31 $[M-H]^-$, calcd. for $C_{31}H_{44}O_{11}N_5$: 662.30.

General solid-phase peptide synthesis: Peptides were synthesized manually on MBHA resin (250 mg, 0.45 mmol/mg) by a stepwise Fmoc/*t*Bu strategy. Amino acids were coupled using DIC/HOBt or TBTU/HOBt/DIEA activation, and completion of the coupling reactionwas monitored by the ninhydrin test. Fmoc-deprotection was carried out using 20% piperidine solution in DMF. *N*-terminal acetylation was accomplished with 20% Ac_2O in DMF and DIEA for 30 min. Peptides were cleaved from the resin with the cocktail TFA/TIPS/water (95:2.5:2.5), precipitated from frozen diethyl ether, then taken up in 1:1 acetonitrile/water and lyophilized. Peptides were analyzed by HPLC in a reverse-phase (RP) C18 column (Vydac, 4.6 × 150 mm, 5μm)to prove purity >90 and characterized by ESI-MS.

Ac-Phe-Val-Glu-Ile-Pro-Asn-Glu-Ala-NH_2 (4): The peptide (101 mg) was obtained in 92% purity by the general solid-phase peptide synthesis. R_t= 16.0 min. ESI-MS *m/z*: 959.49 $[M+H]^+$, calcd. for $C_{44}H_{67}O_{14}N_{10}$: 959.48.

Ac-Leu-Glu-Ala-Asn-Gly-Glu-Phe-Ala-NH_2 (6):The peptide (95 mg) was obtained in 89% purity by the general solid-phase peptide synthesis. R_t = 13.3 min. ESI-MS *m/z*: 891.42 $[M+H]^+$, calcd. for $C_{39}H_{59}O_{14}N_{10}$: 891.42.

Ac-Leu-Glu-Phe-Asn-Gly-Leu-Glu-Ala-NH_2 (9): The peptide (91 mg) was obtained in 87% purity by the general solid-phase peptide synthesis. R_t = 16.9 min. ESI-MS *m/z*: 933.46 $[M+H]^+$, calcd. for $C_{42}H_{65}O_{14}N_{10}$: 936.47.

Ac-Asp-Ile-Leu-OH (11): *N*-Boc-Ile-OH (231 mg, 1.0 mmol) was coupled to HCl·Leu-OMe (181 mg, 1.0 mmol) according to the general peptide coupling procedure, following by deprotection of the *N*-terminus by Boc removal. The same protocol was employed for the coupling of *N*-Boc-Asp(Bzl)-OH (323 mg, 1.0 mmol). Flash column chromatography purification (CH_2Cl_2/EtOAc 5:1) furnished tripeptide *N*-Boc-Asp(Bzl)-Ile-Leu-OMe (422 mg, 75%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ = 7.39–7.26 (m, 5H), 5.07 (s, 2H), 4.72 (dd, *J* = 6.4, 5.2 Hz, 1H), 4.53 (m, 1H), 4.25 (d, *J* = 6.1 Hz, 1H), 3.67 (s, 3H), 2.76 (dd, *J* = 13.8, 5.2 Hz, 1H), 2.69 (dd, *J* = 13.8, 6.3 Hz, 1H), 1.85 (m, 1H), 1.60 (m, 1H),1.55 (m, 2H), 1.40 (s, 9H), 1.28 (m, 1H), 1.19 (m, 1H), 0.90–0.84 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD) δ = 172.3, 171.3, 170.7, 170.5, 155.8 (CO), 136.5 (C), 128.5, 128.1, 127.9 (CH), 79.5 (C), 66.2 (CH_2), 60.0, 54.5, 54.3 (CH), 52.5 (CH_3), 41.5, 39.2 (CH_2), 36.9 (CH), 28.5 (CH_3), 26.4 (CH_2), 25.3 (CH), 23.4, 15.9, 12.6 (CH_3). ESI-MS *m/z*: 586.30 $[M+Na]^+$, calcd. for $C_{29}H_{45}O_8N_3Na$: 586.31. This product (393 mg, 0.70 mmol) was subjected to Boc removal, *N*-terminal acetylation and removal of the methyl and benzyl esters according to the general procedures to afford peptide **11** (227 mg, 81%). This product was recrystalized from Et_2O/AcOEt (10 mL, 5:1) and used forward without further purification. ESI-MS *m/z*: 400.22 $[M-H]^-$, calcd. for $C_{18}H_{30}O_7N_3$:400.20.

Ac-Asp-Val-Ile-Leu-OH (13): *N*-Boc-Ile-OH (231 mg, 1.0 mmol) was coupled to HCl·Leu-OMe (181 mg, 1.0 mmol) according to the general peptide coupling procedure, following by deprotection of the *N*-terminus by Boc removal. The same protocol was employed for the sequential coupling of *N*-Boc-Val-OH (217 mg, 1.0 mmol) and *N*-Boc-Asp(Bzl)-OH (323 mg, 1.0 mmol). Flash column chromatography purification (CH_2Cl_2/EtOAc 5:1) furnished tetrapeptide *N*-Boc-Asp(Bzl)-Val-Ile-Leu-OMe (449 mg, 68%) as a white amorphous solid. ^{1}H

NMR (400 MHz, $CDCl_3/CD_3OD$) δ = 7.39 – 7.27 (m, 5H), 5.07 (s, 2H), 4.59 (dd, *J* = 6.2, 5.2 Hz, 1H), 4.36 (m, 1H), 4.27–4.15 (m, 2H), 3.69 (s, 3H), 2.82 (dd, *J* = 14.1, 5.2 Hz, 1H), 2.70 (dd, *J* = 14.1, 6.2 Hz, 1H), 2.01 (m, 1H), 1.82 (m, 1H), 1.62 (m, 1H), 1.56 – 1.51 (m, 2H), 1.40 (s, 9H), 1.35 (m, 1H), 1.26 (m, 1H), 0.97–0.88 (m, 9H), 0.82–0.74 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$) δ = 173.6, 172.1, 171.9, 171.7, 171.6, 155.5 (CO), 136.3 (C), 128.7, 128.3, 127.9 (CH), 80.0 (C), 66.0 (CH_2), 62.1, 59.6, 55.5, 52.9 (CH), 52.5 (CH_3), 42.2, 39.9 (CH_2), 37.9, 31.9 (CH), 28.5 (CH_3) 27.2 (CH_2), 25.7 (CH), 24.3, 19.6, 16.4, 12.2 (CH_3).ESI-MS *m/z*: 685.34 $[M+Na]^+$, calcd. for $C_{34}H_{54}O_9N_4Na$: 685.38. This product (396 mg, 0.60 mmol) was subjected to removal of the methyl and benzyl esters according to the general procedure to afford peptide **13** (258 mg, 86%). This product was recrystalized from Et_2O/AcOEt (10 mL, 5:1) and used forward without further purification. ESI-MS *m/z*: 499.30 $[M-H]^-$, calcd. for $C_{23}H_{39}O_8N_4$: 499.28.

Ac-Glu(Val-OH)-Val-OH (15): *N*-Boc-Glu-OH (247 mg, 1.0mmol) was coupled to HCl·Val-OMe (334 mg, 2.0mmol) according to the bidirectional peptide coupling procedure. Flash column chromatography purification (CH_2Cl_2/EtOAc 5:1) furnished tripeptide Boc-Glu(Val-OMe)-Val-OMe (355 mg, 75%) as a white amorphous solid. ^1H NMR (400 MHz, $CDCl_3/CD_3OD$) δ= 4.67 (d, *J* = 6.1 Hz, 1H), 4.60 (d, *J* = 6.0 Hz, 1H), 4.02 (m, 1H), 3.72 (s, 3H), 3.70 (s, 3H), 2.36 (m, 1H), 2.27–2.13 (m, 3H), 1.93 (m, 1H), 1.41 (s, 9H), 0.98 - 0.89 (m, 12H).^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$) δ = 172.6, 171.9, 171.1, 170.8, 155.7 (CO), 79.8 (C), 59.9, 59.6, 55.7 (CH), 52.5, 52.3 (CH_3), 33.5 (CH_2), 32.4, 31.7 (CH), 30.3 (CH_2), 28.4, 19.8, 19.6 (CH_3).ESI-MS *m/z*: 474.29 $[M+H]^+$, calcd. for $C_{22}H_{40}O_8N_3$: 474.28. This product (316 mg, 0.67 mmol) was subjected to Boc removal, *N*-terminal acetylation and removal of the methyl esters according to the general procedures to afford peptide **15** (200 mg, 77%). This product was

recrystalized from Et_2O/AcOEt (7 mL, 5:1) and used forward without further purification. ESI-MS *m/z*: 386.23 $[M-H]^-$, calcd. for $C_{17}H_{28}O_7N_3$: 386.19.

Ac-Asp(Ala-Phe-OH)-Ala-Phe-OH (17): *N*-Boc-Ala-OH (416mg, 2.2mmol) was coupled to HCl·Phe-OMe (473mg, 2.2 mmol) according to the general coupling procedure. The resulting dipeptide was then subjected to Boc removal and crystallization of its hydrochloride salt from frozen diethyl ether. This latter dipeptide HCl·Ala-Phe-OMe (572 mg, 2.0 mmol) was coupled to *N*-Boc-Asp-OH (323 mg, 1 mmol) according to the bidirectioanal procedure. Flash column chromatography purification (CH_2Cl_2/EtOAc 5:1) furnished the pure pentapeptide Boc-Asp(Ala-Phe-OMe)-Ala-Phe-OMe (508 mg, 73%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ = 7.25-7.13 (m, 10H), 4.69 (m, 2H), 4.55 (dd, *J* = 9.1, 4.3 Hz, 1H), 4.42 (m, 1H), 4.36 (m, 1H), 3.69 (s, 6H), 3.14 (m, 2H), 2.90 (m, 2H), 2.69 (dd, *J* = 13.5, 4.3 Hz, 1H), 2.61 (dd, *J* = 13.5, 9.1 Hz, 1H), 1.41 (s, 9H), 1.31 (d, *J* = 6.8 Hz, 3H), 1.29 (d, *J* = 6.7 Hz, 3H).^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD) δ = 174.4, 173.0, 171.4, 170.7, 168.1, 167.9, 155.6 (CO), 136.2 (C), 128.6, 128.1, 127.9 (CH), 79.8 (C), 56.9, 55.2, 53.1 (CH), 52.4 (CH_3), 50.6, 49.8 (CH), 40.3, 39.8, 39.2 (CH_2), 28.8, 18.1, 17.8 (CH_3). ESI-MS *m/z*: 720.38 $[M+Na]^+$, calcd. for $C_{35}H_{47}O_{10}N_5Na$: 720.32. This product (418 mg, 0.60mmol) was subjected to Boc removal, *N*-terminal acetylation and removal of the methyl esters according to the general procedures to afford peptide **17** (261 mg, 71%). This product was recrystalized from Et_2O/AcOEt (10 mL, 5:1) and used forward without further purification. ESI-MS *m/z*: 610.31 $[M-H]^-$, calcd. for $C_{30}H_{36}O_9N_5$: 610.25.

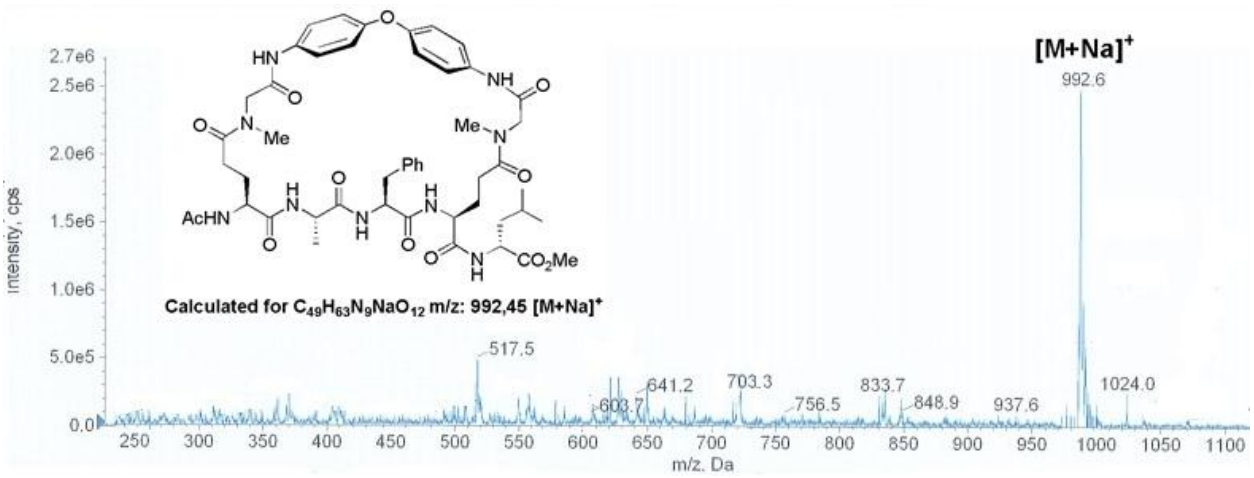

Figure 1. ESI-MS of macrocycle **3**.

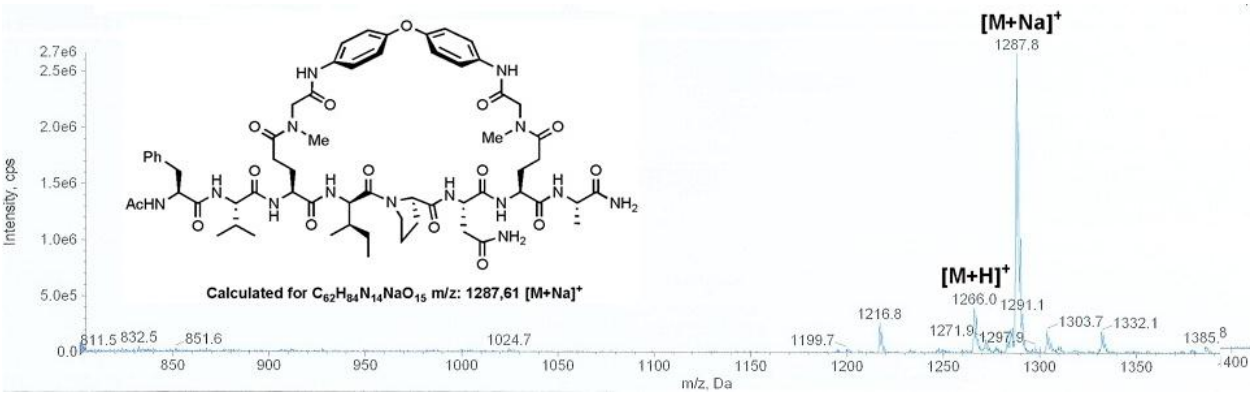

Figure 2. ESI-MS of macrocycle **5**.

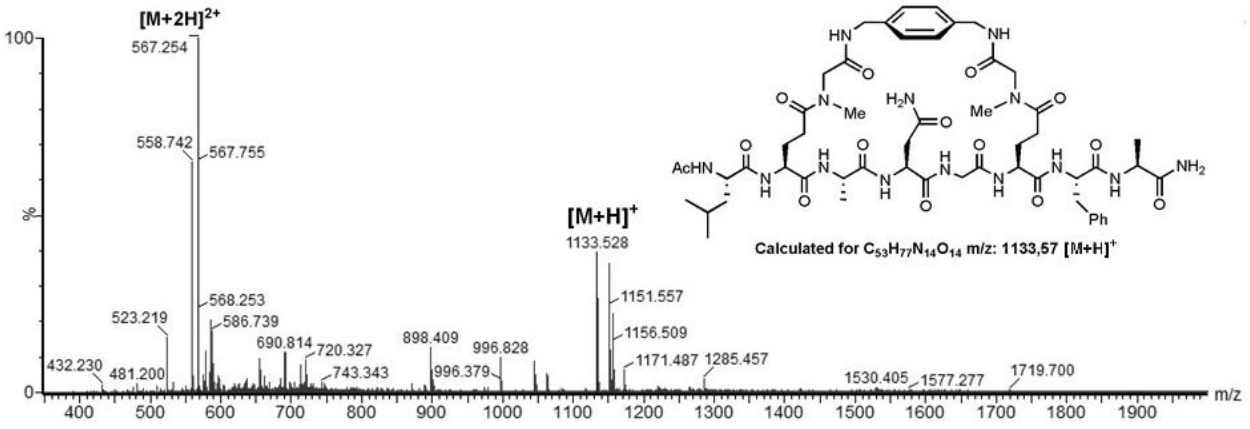

Figure 3. ESI-MS of macrocycle **8**.

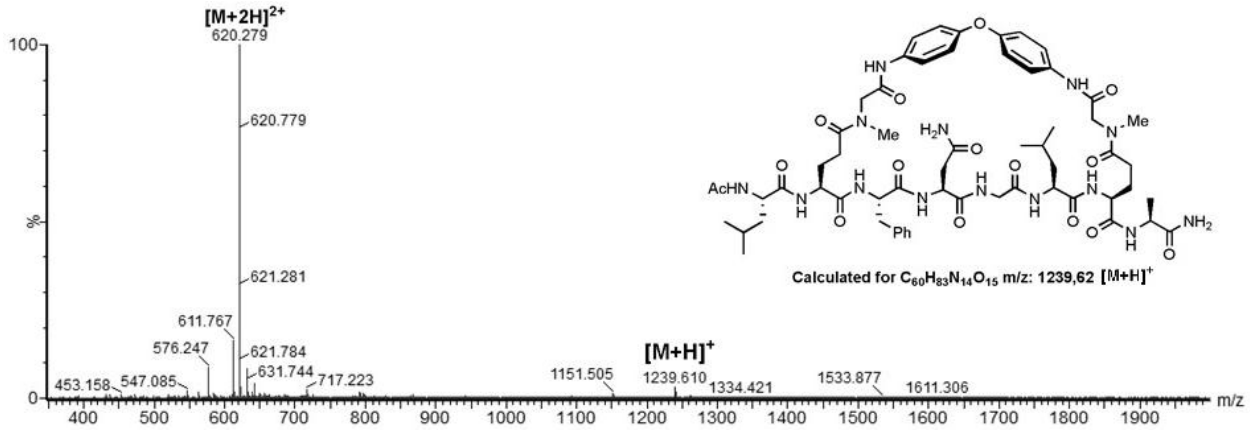

Figure 4. ESI-MS of macrocycle **10**.

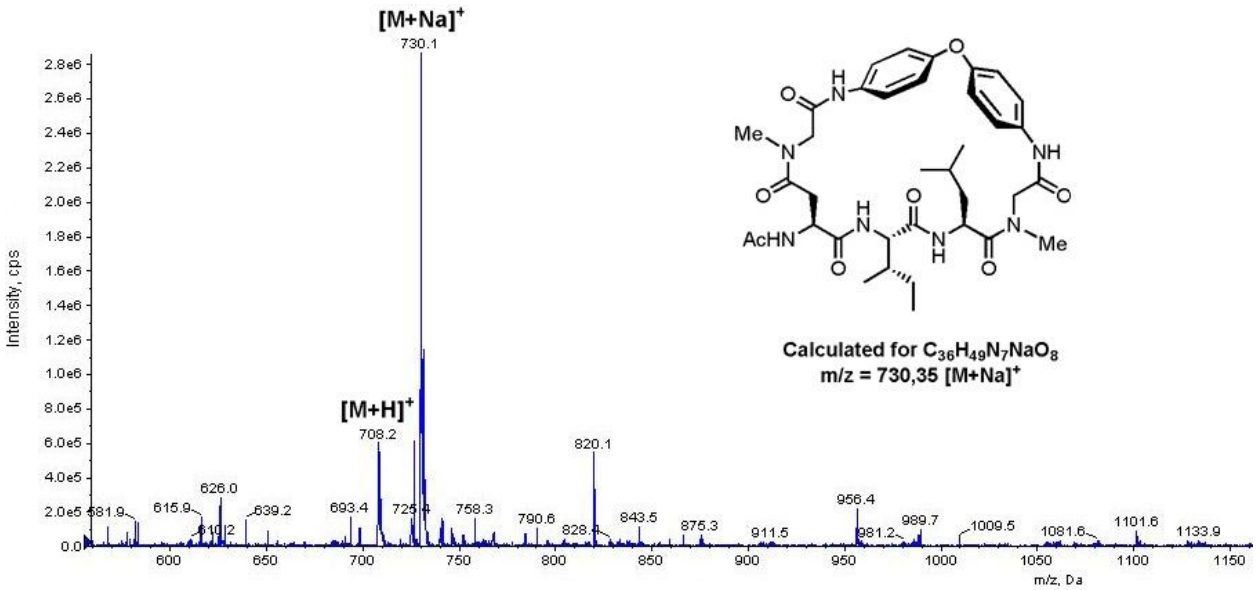

Figure 5. ESI-MS of macrocycle **12**.

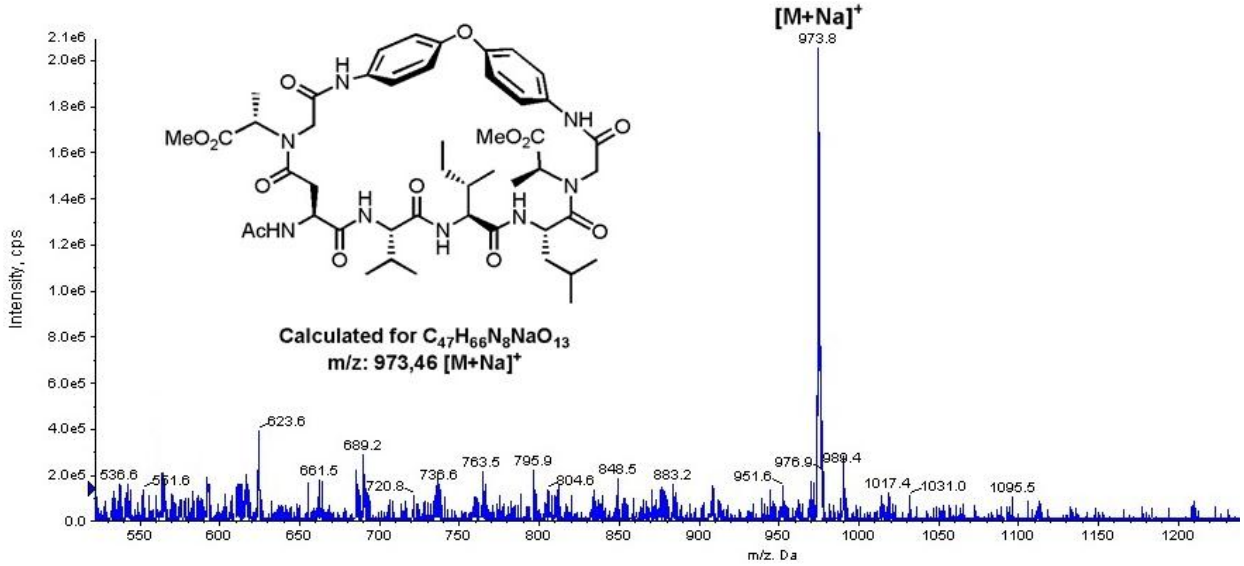

Figure 6. ESI-MS of macrocycle **14**.

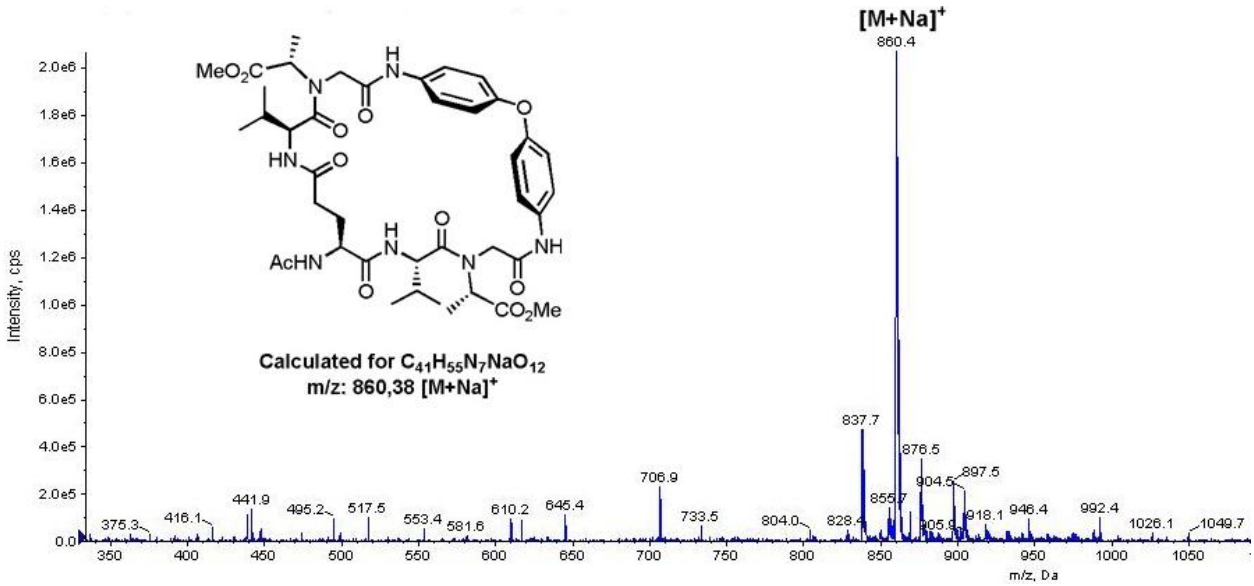

Figure 7. ESI-MS of macrocycle **16**.

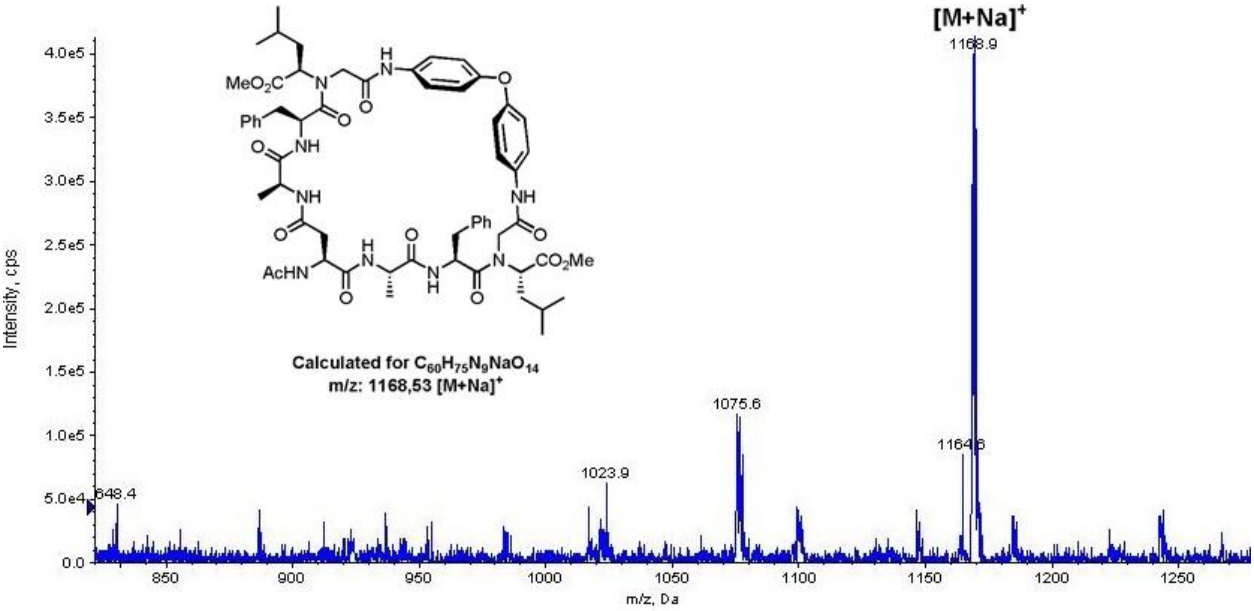

Figure 8. ESI-MS of macrocycle **18**.

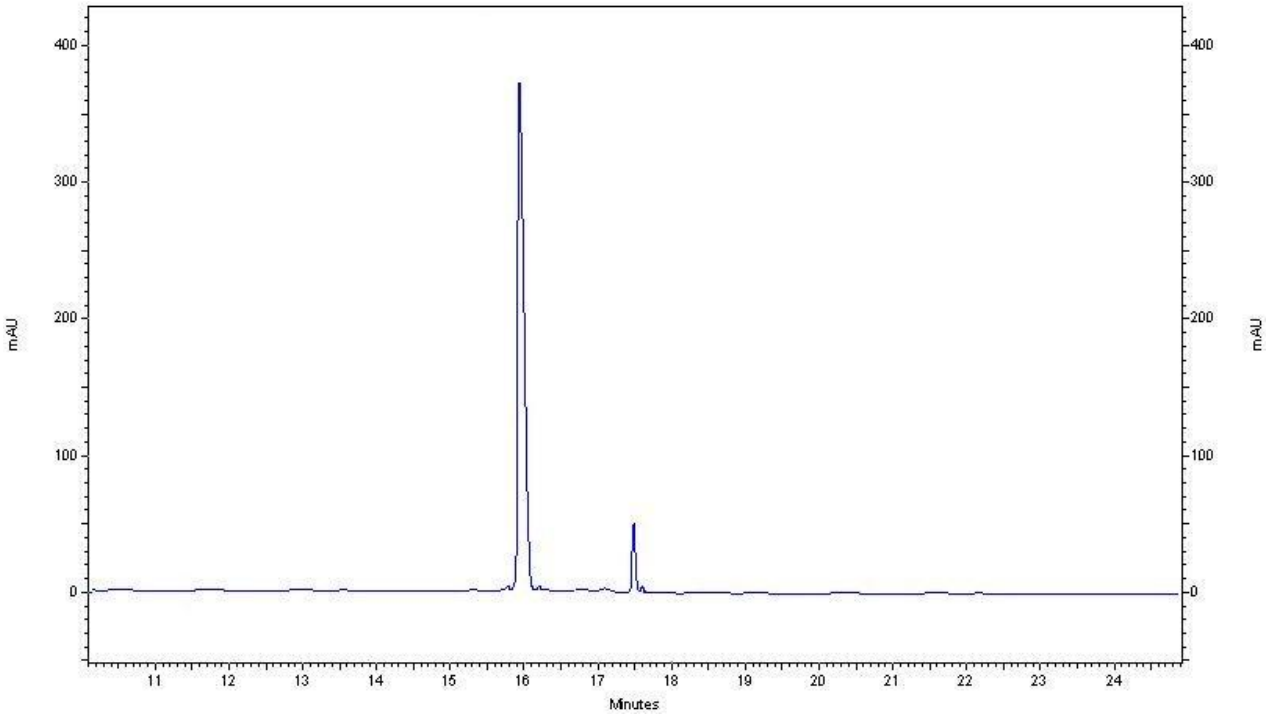

Figure 9. HPLC chromatogram of crude peptide **4** as obtained by solid-phase peptide synthesis.

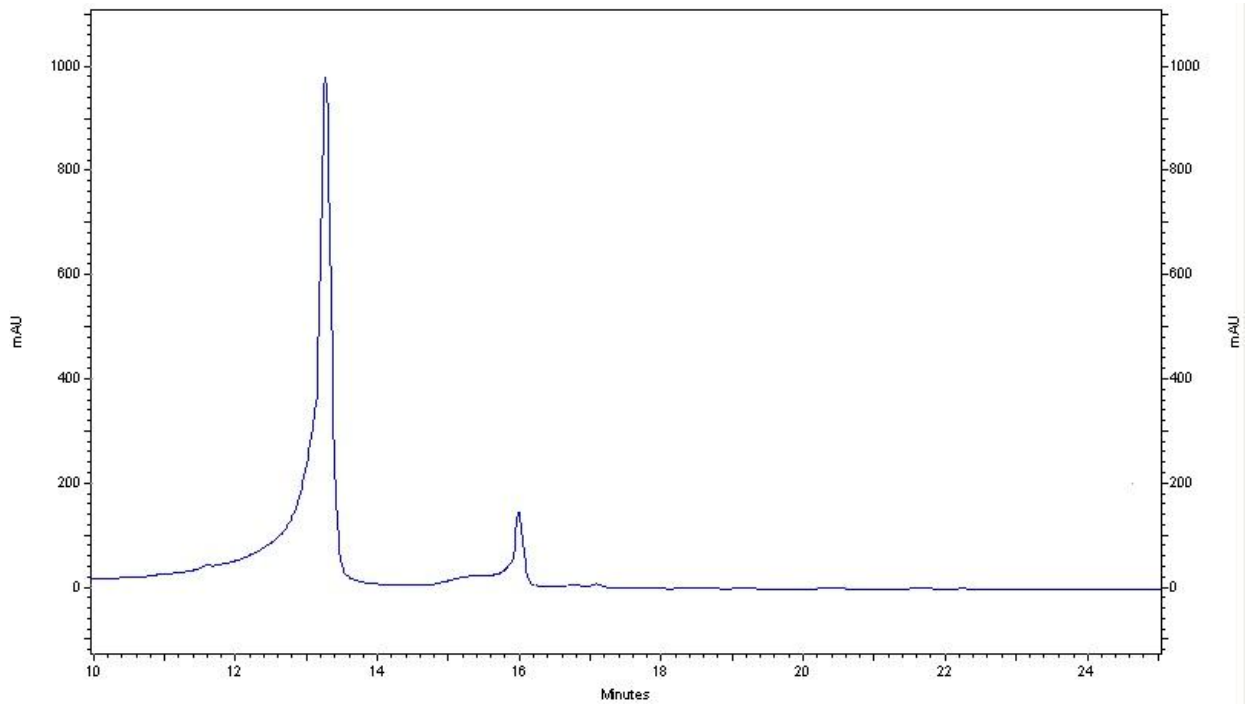

Figure 10. HPLC chromatogram of the crude peptide **6** as obtained by solid-phase peptide synthesis.

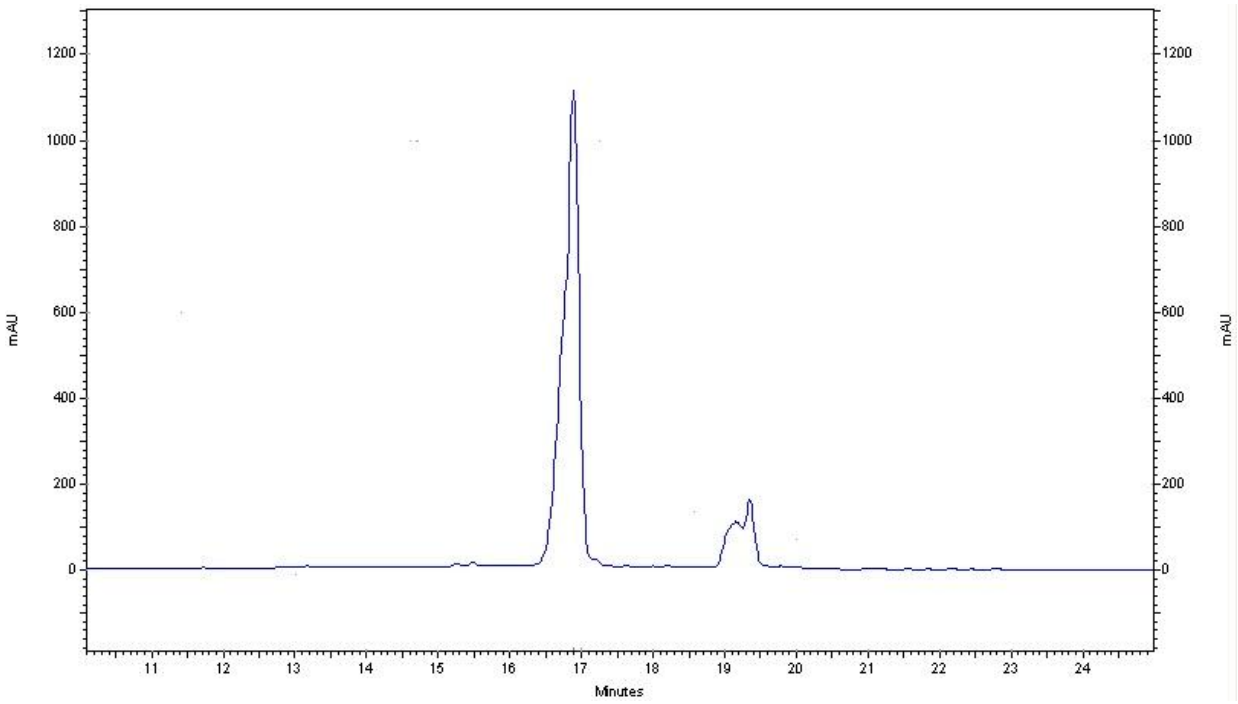

Figure 11. HPLC chromatogram of the crude peptide **9** as obtained by solid-phase peptide synthesis.

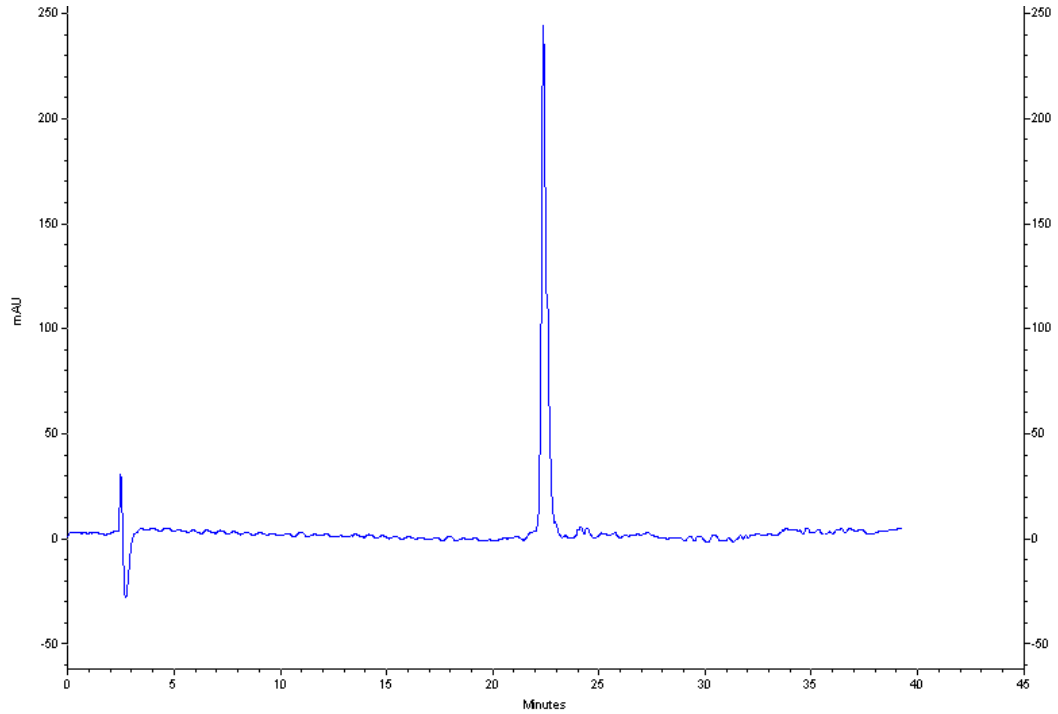

Figure 12. HPLC chromatogram of the pure macrocyclic peptide **3**.

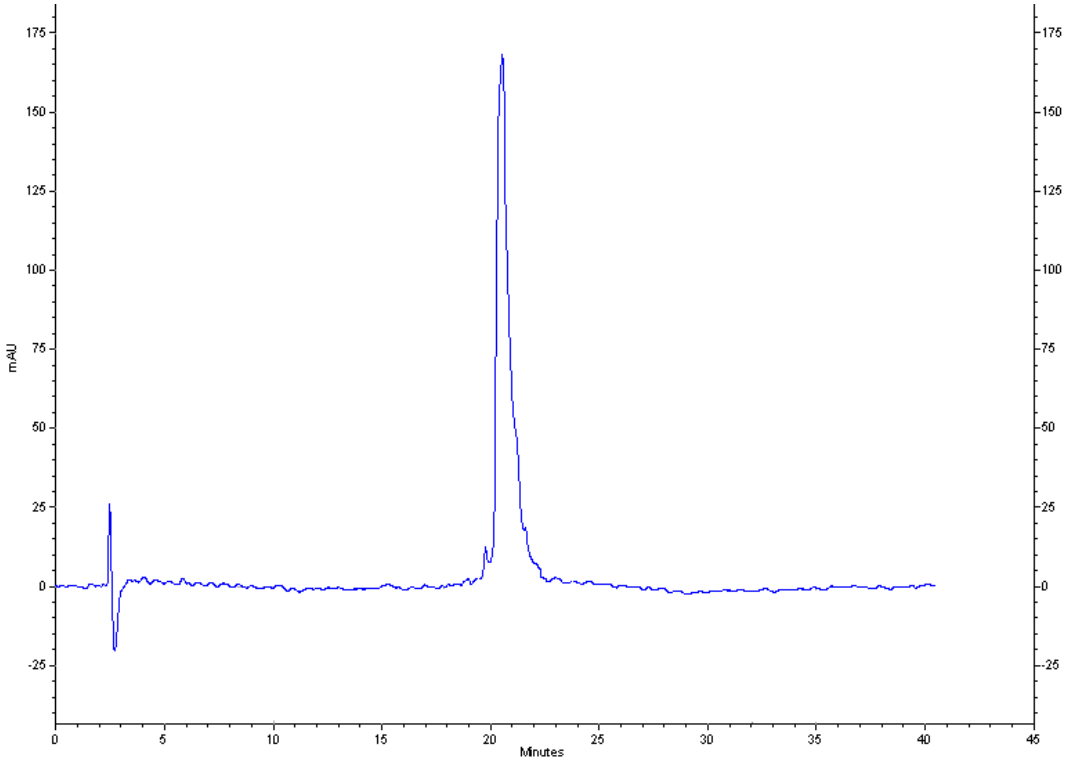

Figure 13. HPLC chromatogram of the pure macrocyclic peptide **5**.

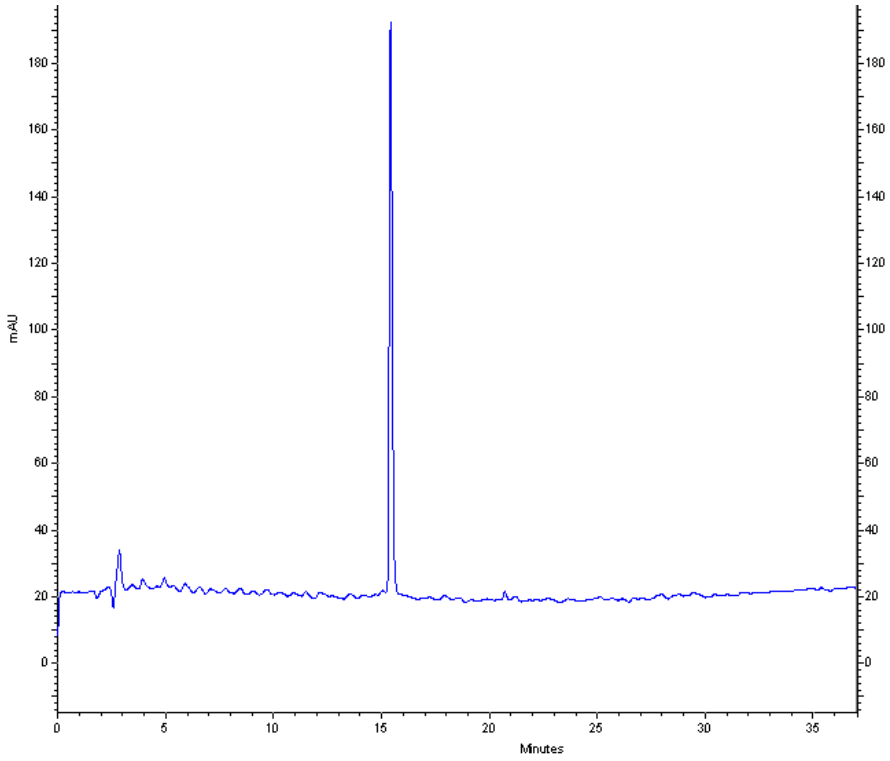

Figure 14. HPLC chromatogram of the pure macrocyclic peptide **8**.

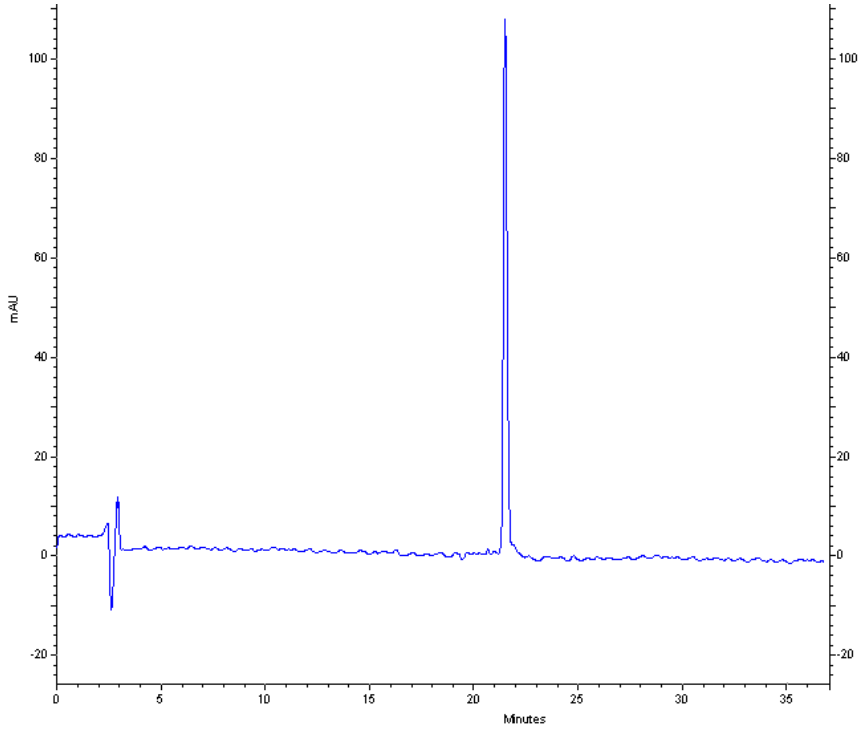

Figure 15. HPLC chromatogram of the pure macrocyclic peptide **10**.

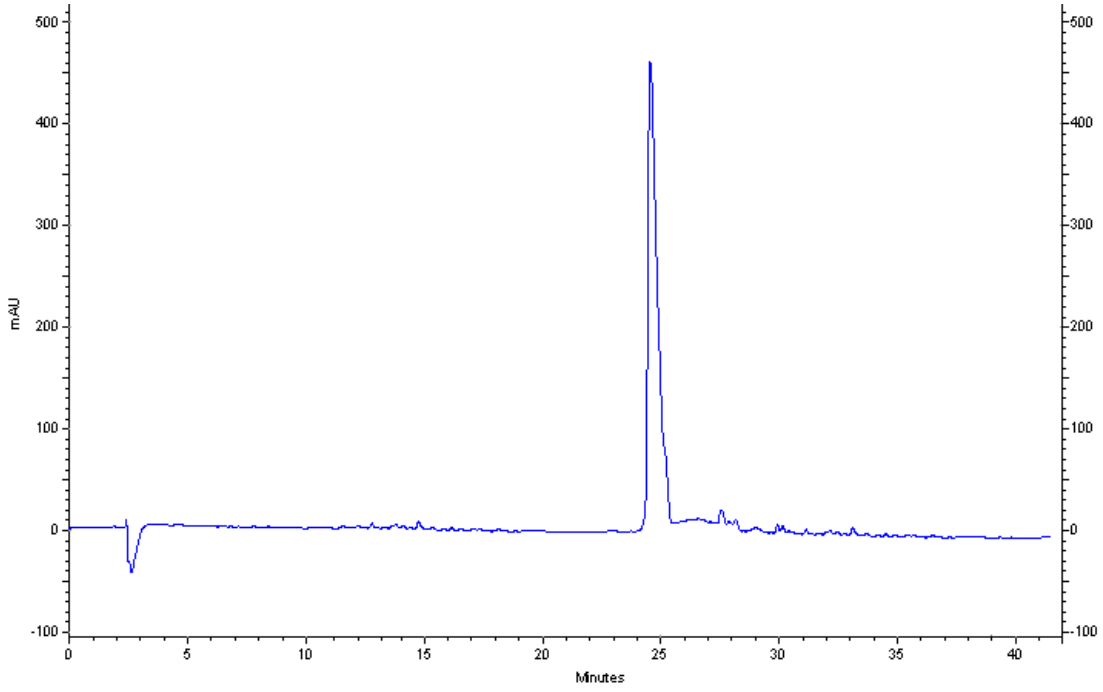

Figure 16. HPLC chromatogram of the pure macrocyclic peptide **12**.

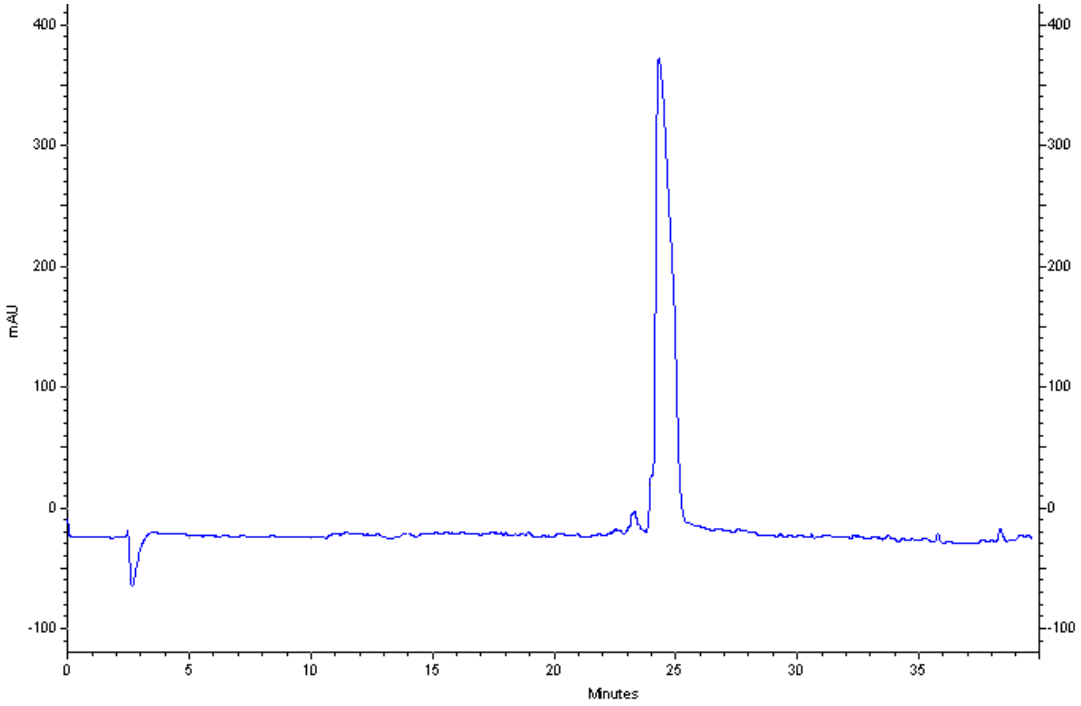

Figure 17. HPLC chromatogram of the pure macrocyclic peptide **14**.

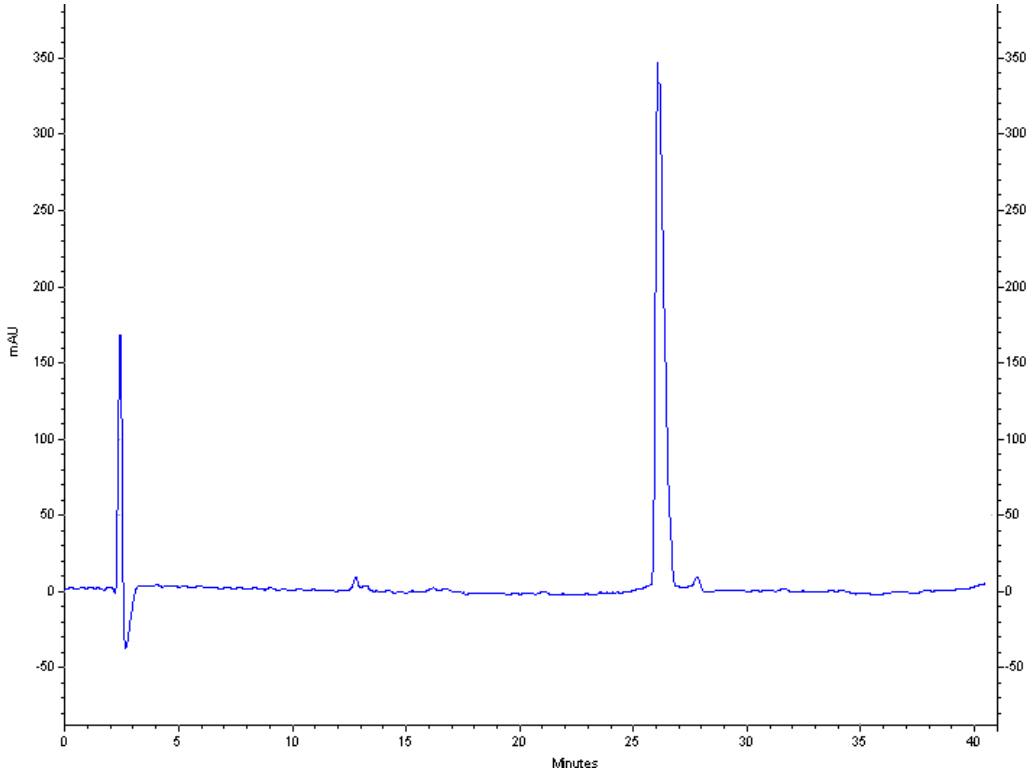

Figure 18. HPLC chromatogram of the pure macrocyclic peptide **16**.

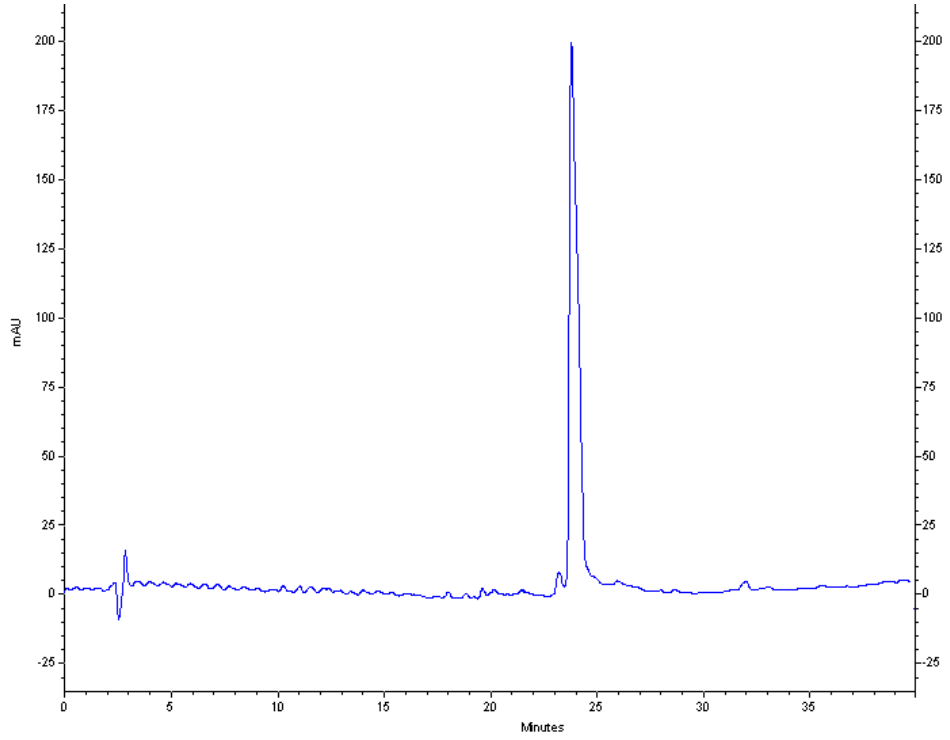

Figure 19. HPLC chromatogram of the pure macrocyclic peptide **18**.

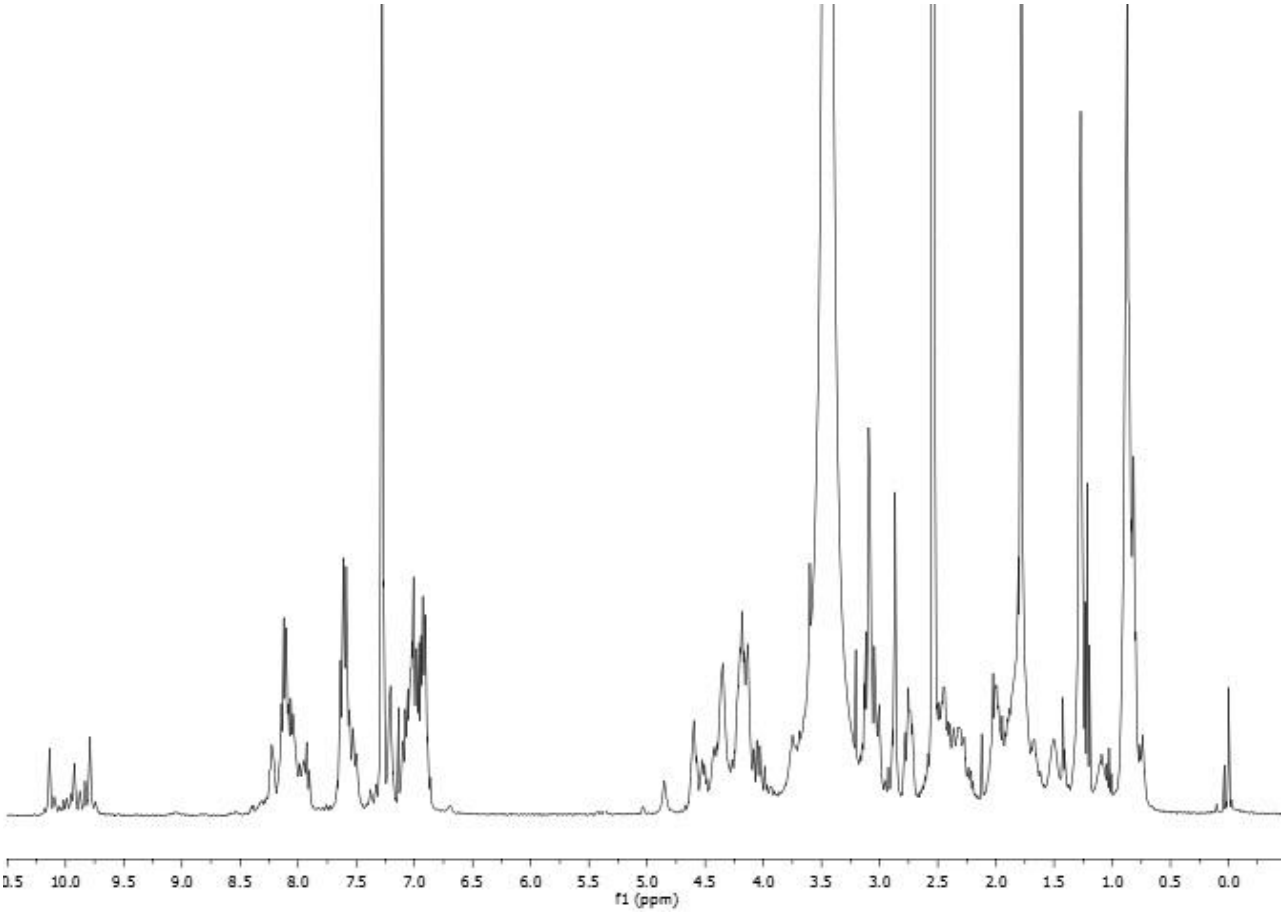

Figure 20. 400 MHz ^{1}H NMR spectrum in $CDCl_3$ of macrocycle **5.**

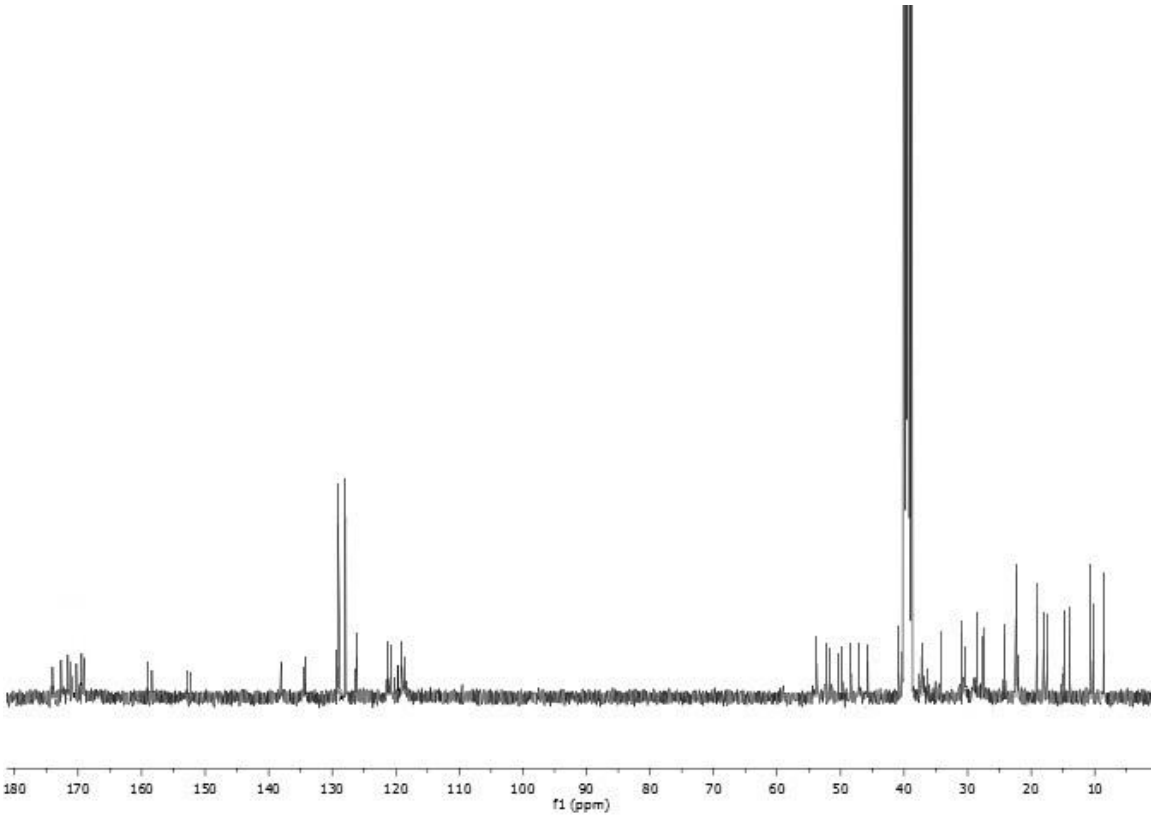

Figure 21. 100 MHz ^{13}C NMR spectrum in $CDCl_3$ of macrocycle **5**.

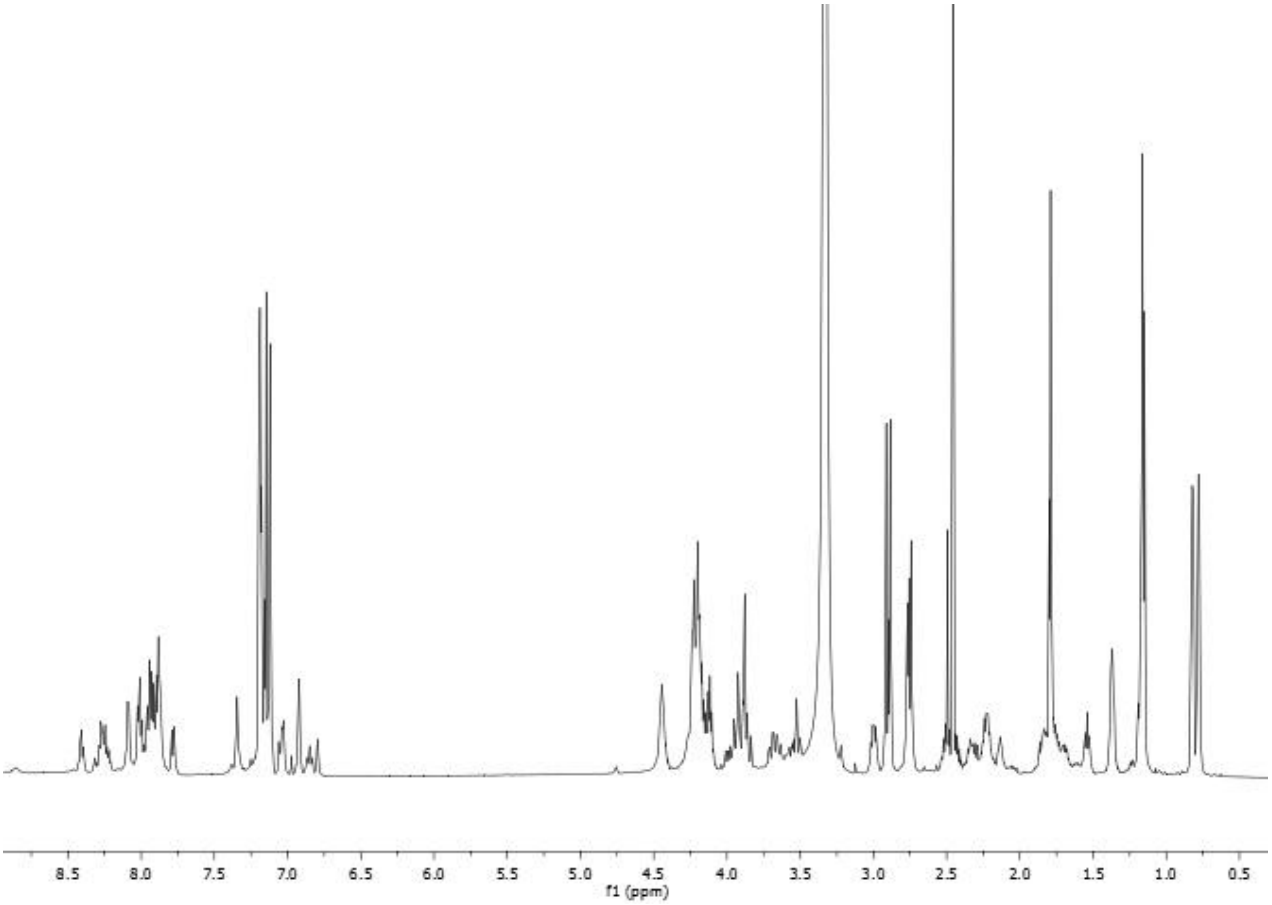

Figure 22. 600 MHz ^{1}H NMR spectrum in $CDCl_3$ of macrocycle **8.**

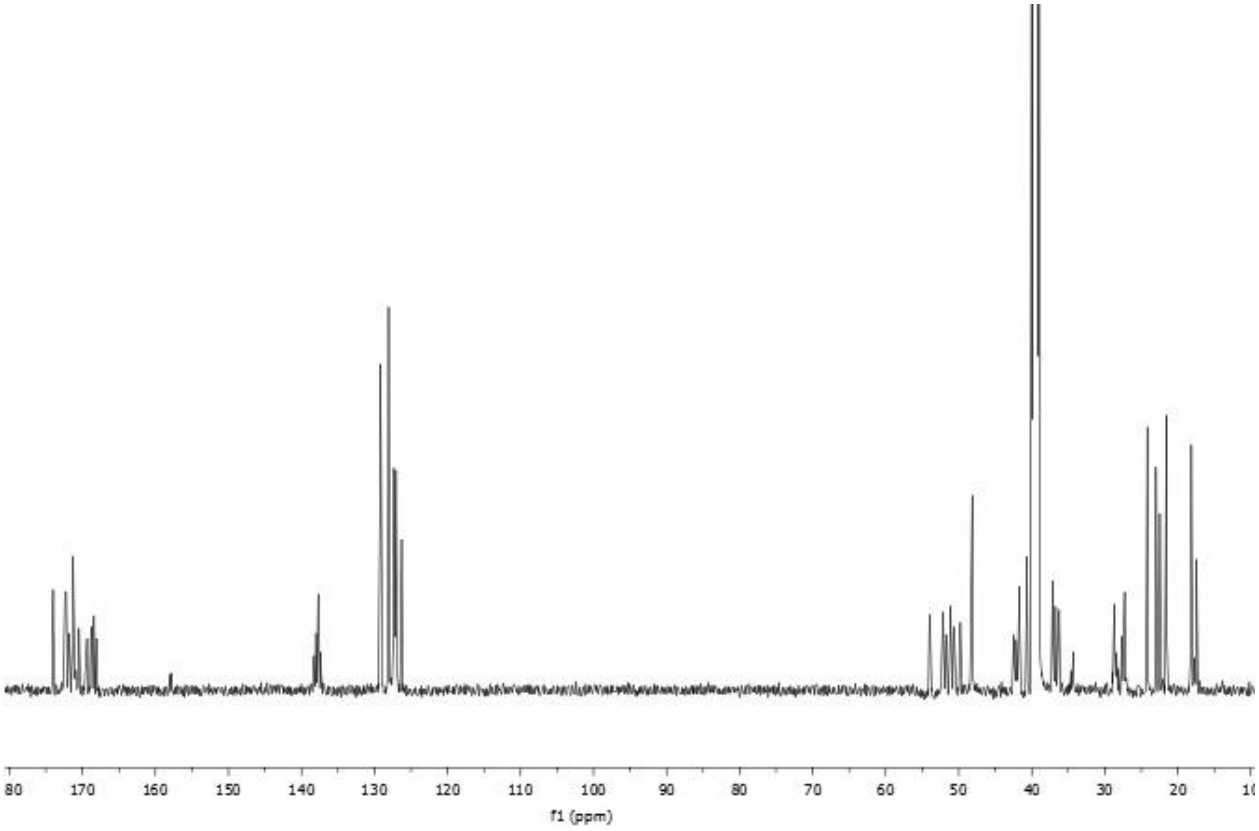

Figure 23. 150 MHz ^{13}C NMR spectrum in $CDCl_3$ of macrocycle **8**.

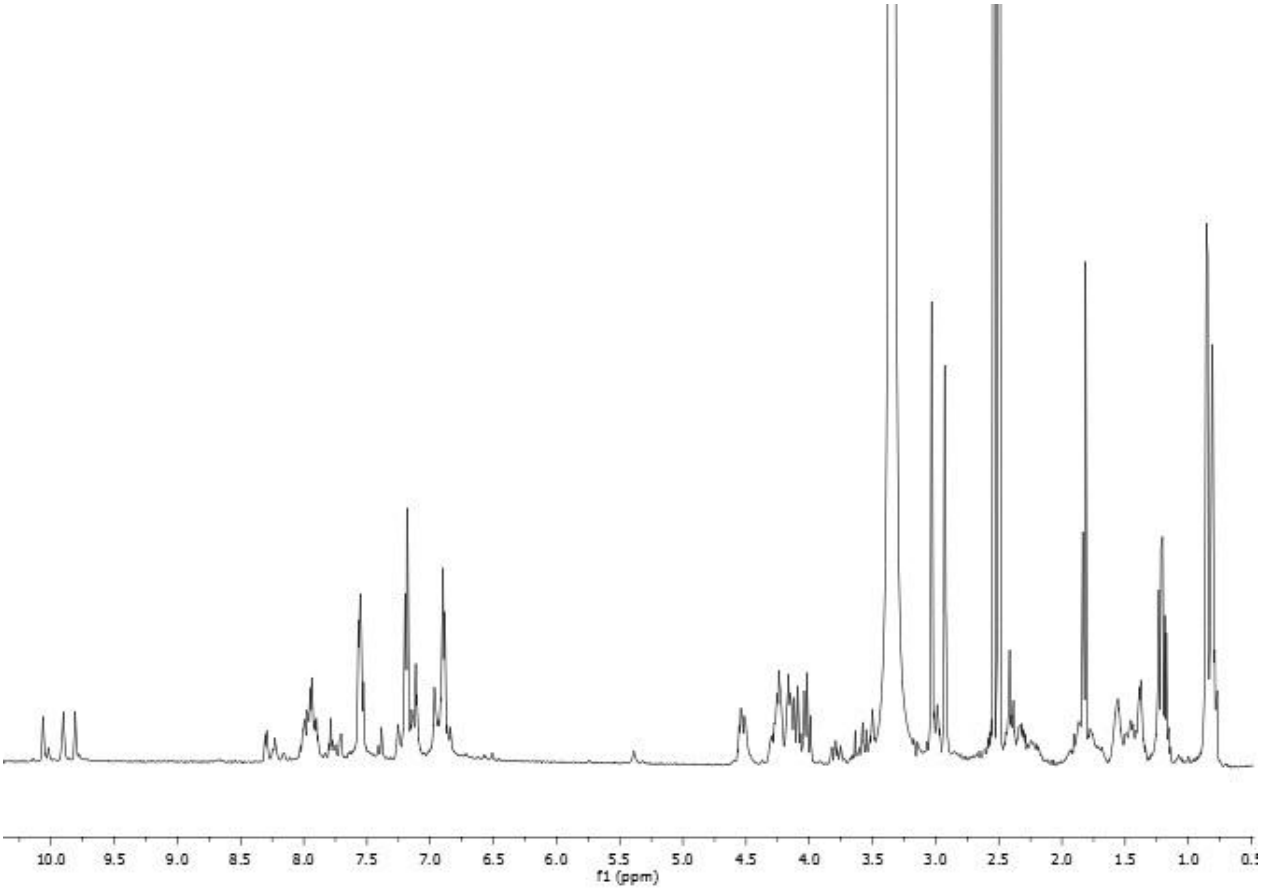

Figure 24. 600 MHz ^{1}H NMR spectrum in $CDCl_3$ of macrocycle **10.**

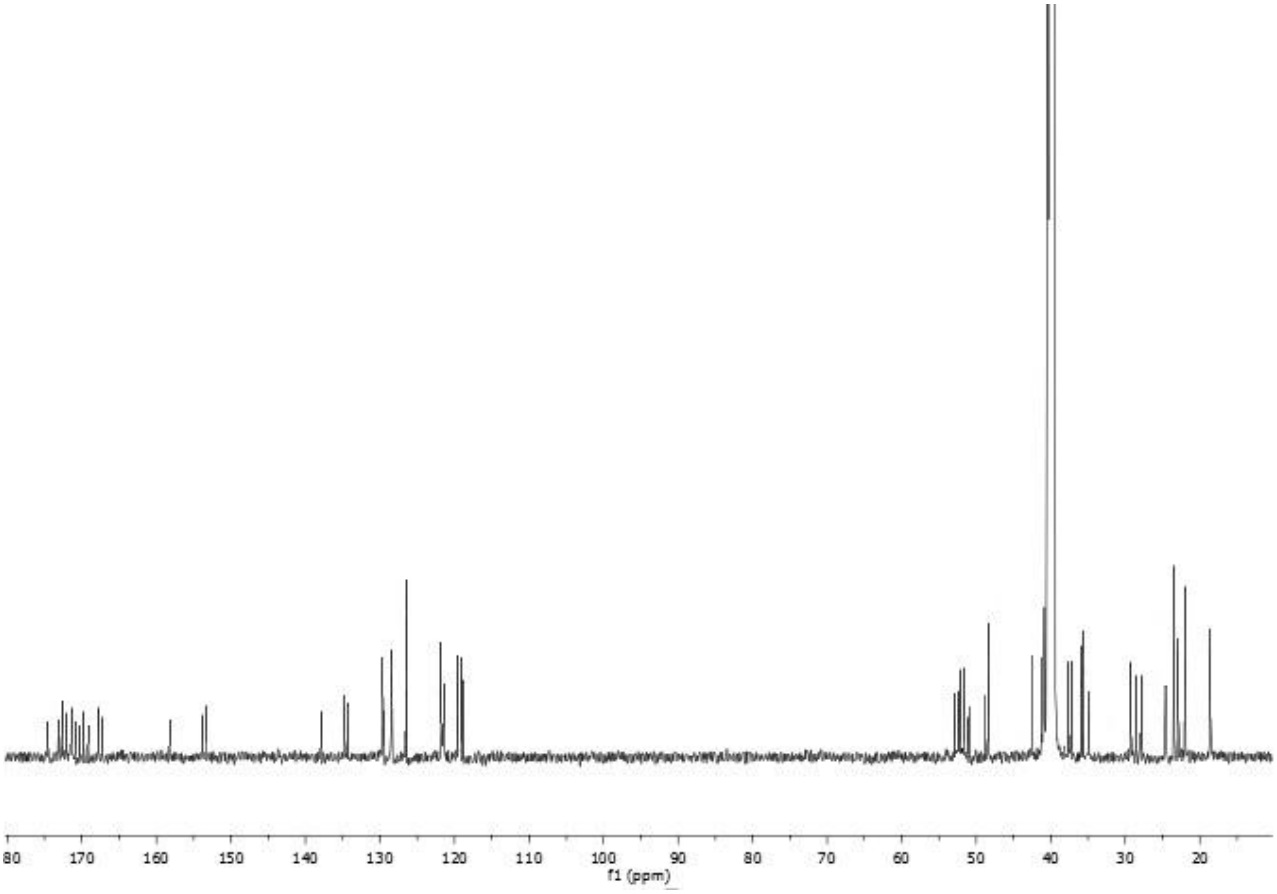

Figure 25. 150 MHz ^{13}C NMR spectrum in $CDCl_3$ of macrocycle **10**.