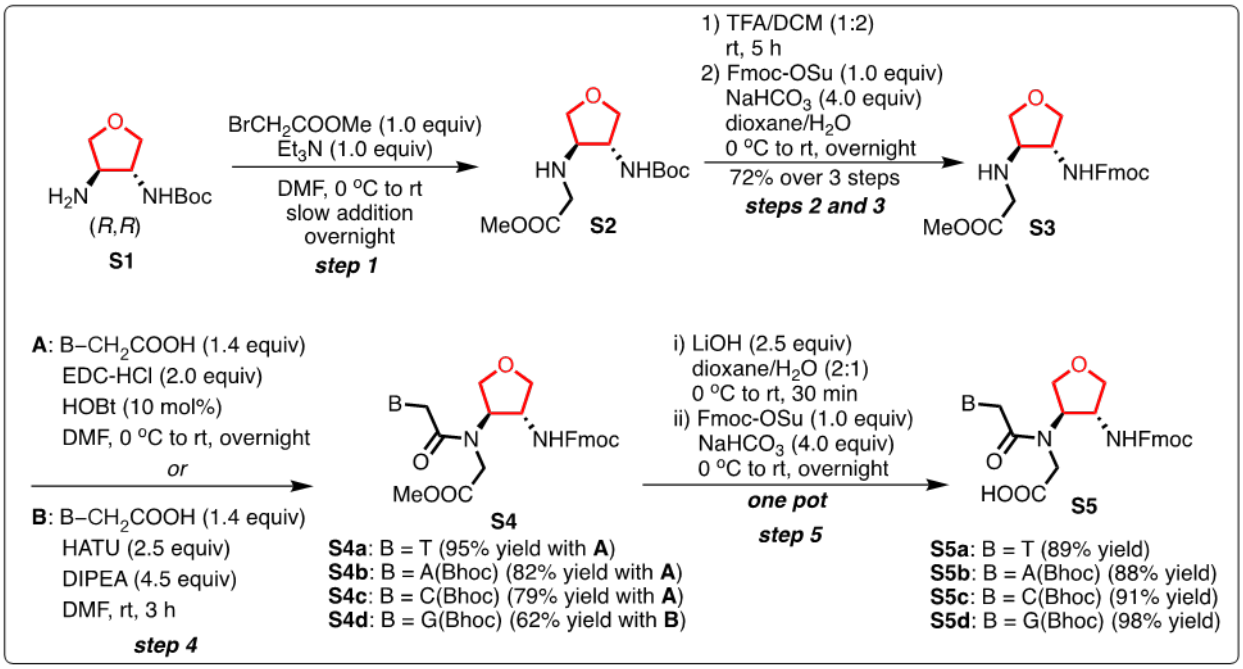
SUPPLEMENTARY INFORMATION

General information for the preparation of thyclotides monomers:

Unless otherwise stated, all reactions were performed under a nitrogen atmosphere using flame-dried glassware and all reagents were reagent grade quality and used as received from Sigma-Aldrich. (*1R*,*2R*)-*trans*-*N*-Boc-4-oxa-1,2-cyclopentanediamine (>95% purity, >99% e.e.) was purchased from EntreChem SL (Spain) and used as received. All nucleobase acetic acids were purchased from PolyOrg, Inc. (MA, USA). Anhydrous 1,4-Dioxane (99.8%), *N*,*N*-Dimethylformamide (99.8%) and Dichloromethane (≥99.8%, contains 40–150 ppm amylene as stabilizer) were purchased from Sigma-Aldrich and used as received. Thin layer chromatography (TLC) was performed on SiliCycle Silica Gel 60 F254 plates and was visualized with UV light and $KMnO_4$ stain. All NMR spectra were recorded on either a Bruker Avance 500 MHz or 400 MHz spectrometer at STP. All deuterated solvents were used as received from Cambridge Isotope Laboratories, Inc. The residual solvent protons (^1H) or the solvent carbons (^{13}C) were used as internal standards. ^1H NMR data are presented as follows: chemical shift in ppm (δ) downfield from tetramethylsilane (multiplicity, coupling constant, integration). The following abbreviations are used in reporting NMR data: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; dd, doublet of doublets; m, multiplet. High-resolution mass spectrometry (HRMS) data were obtained using a Waters Xevo-G2 XS qTOF™ instrument.

Figure S1: Synthetic route for thyclotide monomers S5a-S5d



Step 1: To a solution of *tert*-butyl ((3*R*,4*R*)-4-aminotetrahydrofuran-3-yl)carbamate **S1** (4.04 g, 20.0 mmol) and triethylamine (3.79 mL, 20.0 mmol) in DMF (10 mL) at 0°C was slowly added a solution of methyl 2-bromoacetate (3.06 g, 20.0 mmol) in DMF (10 mL) dropwise. The resulting solution was allowed to warm to room temperature and stirred at room temperature overnight. The resulting mixture was extracted between EtOAc (3 × 30 mL) and a saturated aqueous $NaHCO_3$ solution (30 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure to provide the crude product **S2** as a pale-yellow oil, which was used in next step without any further purification.

Steps 2 and 3: To a solution of crude **S2** (from ***step 1***) in CH_2Cl_2 (20 mL) at room temperature was added trifluoroacetic acid (10 mL). The resulting solution was stirred at room temperature for 5 hours. Upon evaporation of solvent, the crude amine TFA salt was obtained as a yellow oil. To a solution of this freshly prepared crude amine TFA salt and $NaHCO_3$ (6.88 g, 80.0 mmol) in dioxane/H_2O (20 mL/20mL) at 0°C was slowly added a solution of Fmoc-OSu (6.74 g, 20.0 mmol) in dioxane (20 mL). The resulting mixture was allowed to warm to room temperature and stirred at room temperature overnight. The resulting mixture was extracted between EtOAc (3 × 50 mL) and H_2O (30 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by silica gel chromatography (EtOAc:hexanes = 1:1 to 4:1) to give the desired compound **S3** as a white solid (5.72 g, 72% yield over 3 steps). **^{1}H NMR** (400 MHz, $CDCl_3$) *δ* 7.76 (d, *J* = 7.5 Hz, 2H), 7.57 (d, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 5.14 (d, *J* = 6.6 Hz, 1H), 4.43 (d, *J* = 5.8 Hz, 2H), 4.19 (t, *J* = 6.3 Hz, 1H), 4.08–3.94 (m, 3H), 3.72 (s, 3H), 3.63 (d, *J* = 9.2 Hz, 1H), 3.54 (s, 2H), 3.48 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.21–3.16 (m, 1H), 2.00–1.70 (br s, 1H); **^{13}C NMR** (100 MHz, $CDCl_3$) *δ* 173.2, 156.2, 144.2, 141.7, 128.1, 127.4, 125.3, 120.4, 73.4, 72.1, 66.9, 65.6, 57.8, 52.3, 49.2, 47.6; **HRMS** (ESI) for $C_{22}H_{25}N_2O_5$: calcd. 397.1763; found 397.1758.

Step 4 (**Method A**): To a solution of **S3** (1.19 g, 3.0 mmol), C(Bhoc)CH_2COOH (1.59 g, 4.2 mmol) and HOBt (40 mg, 0.3 mmol) in DMF (10 mL) at 0°C was added EDC (1.15 g, 6.0 mmol) in one portion. The resulting mixture was allowed to warm to room temperature and stirred at room temperature overnight. The resulting mixture was extracted between EtOAc (3 × 50 mL) and H_2O (50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by silica gel chromatography (CH_2Cl_2:MeOH = 60:1 to 20:1) to give the desired compound **S4c** as a white solid (1.79 g, 79% yield).

Compound **S4a** and **S4b** were prepared following the same procedure as that described for the preparation of **S4c**.

S4a: **^{1}H NMR** (500 MHz, $CDCl_3$): Major rotamer: *δ* 9.64 (s, 1H), 7.75–7.65 (m, 2H), 7.60–7.50 (m, 2H), 7.40–7.32 (m, 2H), 7.29–7.22 (m, 2H), 6.97 (s, 1H), 5.94 (s, 1H), 4.84–4.30 (m, 5H), 4.27–3.85 (m, 7H), 3.68 (s, 3H), 3.62–3.55 (m, 1H), 1.85 (s, 3H); Minor rotamer: *δ* 9.46 (s, 1H), 7.75–7.65 (m, 2H), 7.60–7.50 (m, 2H), 7.40–7.32 (m, 2H), 7.29–7.22 (m, 2H), 6.97 (s, 1H), 5.57 (s, 1H), 4.84–4.30 (m, 5H), 4.27–3.85 (m, 7H), 3.78 (s, 3H), 3.55–3.48 (m, 1H), 1.91 (s, 3H); **^{13}C NMR** (125 MHz, $CDCl_3$): Major rotamer: *δ* 169.8, 167.5, 164.4, 156.1, 151.5, 143.7, 141.4, 141.2, 127.8, 127.1, 124.9, 120.1, 110.9, 71.1, 69.4, 66.6, 62.6, 58.2, 52.5, 48.1, 47.2, 44.8, 12.4; Minor rotamer: *δ* 170.0, 168.1, 164.4, 156.1, 151.2, 143.5, 141.3, 140.9, 127.8, 127.1, 125.1, 120.1, 111.0, 72.4, 68.5, 66.9, 63.1, 56.2, 53.1, 48.3, 47.1, 46.8, 12.4; **HRMS** (ESI) for $C_{29}H_{31}N_4O_8$: calcd. 563.2142; found 563.2148.

S4b: **^{1}H NMR** (500 MHz, $CDCl_3$): Major rotamer: *δ* 8.66 (s, 1H), 8.06 (s, 1H), 7.70–7.60 (m, 2H), 7.51–7.44 (m, 2H), 7.40–7.16 (m, 15H), 6.95 (s, 1H), 5.86 (s, 1H), 5.50–5.40 (m, 1H), 4.97–4.89 (m, 1H), 4.53–3.85 (m, 9H), 3.80–3.45 (m, 5H); Minor rotamer: *δ* 8.66 (s, 1H), 8.06 (s, 1H), 7.70–7.60 (m, 2H), 7.51–7.44 (m, 2H), 7.40–7.16 (m, 15H), 6.95 (s, 1H), 5.50 (s, 1H), 5.20–5.10 (m, 1H), 4.62–4.56 (m, 1H), 4.53–3.85 (m, 9H), 3.80–3.45 (m, 5H); **^{13}C NMR** (125 MHz, $CDCl_3$): Major rotamer: *δ* 169.7, 166.6, 156.2, 152.7, 151.5, 150.3, 149.1, 144.2, 143.6, 141.3, 139.6, 128.6, 128.1, 127.8, 127.7, 127.3, 127.0, 124.8, 120.0, 78.8, 71.2, 69.2, 66.5, 63.0, 58.4, 52.5, 47.2, 45.0, 44.3; Minor rotamer: *δ* 167.7, 167.0, 156.2, 152.8, 151.4, 150.3, 149.2, 144.0, 143.4, 141.4, 141.3, 128.6, 128.1, 127.8, 127.7, 127.3, 127.0, 125.0, 120.9, 78.8, 72.3, 68.4, 66.8, 63.6, 56.1, 53.2, 47.1, 45.0, 44.3; **HRMS** (ESI) for $C_{43}H_{40}N_7O_8$: calcd. 782.2938; found 782.2937.

S4c: **^{1}H NMR** (500 MHz, $CDCl_3$): Major rotamer: *δ* 8.28 (br s, 1H), 7.82–7.65 (m, 2H), 7.60–7.07 (m, 18H), 6.80–6.73 (m, 1H), 5.37–5.20 (m, 1H), 5.00–3.50 (m, 16H); Minor rotamer: *δ* 8.28 (br s, 1H), 7.82–7.65 (m, 2H), 7.60–7.07 (m, 18H), 6.70–6.63 (m, 1H), 5.73–5.57 (m, 1H), 5.00–3.50 (m, 16H); **^{13}C NMR** (125 MHz, $CDCl_3$): Major rotamer: *δ* 169.7, 167.3, 163.1, 156.3, 155.7, 151.7, 143.74, 143.66,

141.3, 139.4, 128.6, 128.2, 127.8, 127.1, 126.9, 125.1, 120.0, 95.3, 79.0, 71.4, 69.4, 66.8, 62.5, 57.5, 52.4, 50.0, 47.1, 44.8; Minor rotamer: *δ* 169.4, 167.1, 163.1, 157.4, 156.2, 150.1, 143.74, 143.66, 141.3, 139.4, 128.6, 128.2, 128.0, 127.3, 126.9, 124.9, 120.0, 95.3, 78.8, 72.4, 69.9, 67.2, 61.0, 57.1, 53.0, 50.2, 46.7, 44.8; **HRMS** (ESI) for $C_{42}H_{40}N_5O_9$: calcd. 758.2826; found 758.2833.

Step 4 (**Method B**): A suspension of **S3** (1.00 g, 2.52 mmol), G(Bhoc)CH_2COOH (1.48 g, 3.53 mmol), and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) (2.42 g, 6.36 mmol) in toluene (10 mL) was evaporated under reduced pressure to remove trace amounts of water. Then the resulting mixture was dissolved in DMF (freshly dried over 4Å molecular sieves, 5 mL) and *N*,*N*-diisopropylethylamine (2.0 ml, 11.30 mmol) was added in one portion. The resulting solution was stirred at room temperature for 3 hours. The resulting mixture was extracted between EtOAc (3 × 50 mL) and H_2O (50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by Biotage® Selekt column chromatography system using Biotage® SNAP KP-Sil 100g silica gel column (EtOAc:hexanes = 0:100 to 100:0, then EtOAc/MeOH = 100:0 to 3:1) to give the desired compound **S4d** as a white solid (1.25 g, 62% yield). **^{1}H NMR** (500 MHz, DMSO-d_6 with one drop of $CDCl_3$): Major rotamer: *δ* 11.70 (s, 1H), 11.27 (s, 1H), 7.90–7.60 (m, 6H), 7.50–7.20 (m, 14H), 6.85 (s, 1H), 5.23 (s, 2H), 4.60–4.17 (m, 5H), 4.10–3.74 (m, 5H), 3.72–3.46 (m, 4H); Minor rotamer: *δ* 11.76 (s, 1H), 11.27 (s, 1H), 7.90–7.60 (m, 6H), 7.50–7.20 (m, 14H), 6.85 (s, 1H), 4.98 (s, 2H), 4.60–4.17 (m, 5H), 4.10–3.74 (m, 5H), 3.72–3.46 (m, 4H); **^{13}C NMR** (125 MHz, DMSO-d_6 with one drop of $CDCl_3$): Major rotamer: *δ* 170.0, 167.2, 156.6, 155.5, 154.3, 150.0, 147.6, 144.2, 141.3, 140.6, 140.2, 128.9, 128.4, 128.0, 127.4, 127.0, 125.5, 120.4, 119.7, 78.6, 71.9, 69.1, 66.0, 62.3, 57.1, 52.3, 47.3, 45.0, 44.8; Minor rotamer: *δ* 170.6, 167.8, 156.3, 155.5, 154.3, 150.0, 147.6, 144.2, 141.3, 140.7, 140.2, 128.9, 128.4, 128.0, 127.4, 127.0, 125.4, 120.4, 119.6, 78.6, 72.5, 68.8, 66.0, 62.8, 55.2, 52.7, 47.2, 44.9, 44.8; **HRMS** (ESI) for $C_{43}H_{40}N_7O_9$: calcd. 798.2888; found 798.2883.

Step 5: To a solution of **S4c (**1.42 g, 1.87 mmol) in dioxane/H_2O (20 mL/10mL) at 0°C was added LiOH·H_2O (196 mg, 4.68 mmol). The resulting solution was allowed to warm to room temperature and stirred at room temperature for 30 minutes. Then 20% citric acid was added to adjust the reaction mixture to pH 7 and $NaHCO_3$ was subsequently added to adjust the reaction mixture to pH 8. The resulting mixture was cooled to 0°C and Fmoc-OSu (630 mg, 1.87 mmol) was added. The resulting solution was allowed to warm to room temperature and stirred at room temperature overnight. The reaction mixture was washed with Et_2O (3 × 30 mL). The aqueous layer was acidified with 20% citric acid to pH 3 and then extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was saturated with Et_2O (30 mL) to provide a precipitate which was filtered and washed with Et_2O (3 × 20 mL) to give the desired compound **S5c** as a white solid (1.26 g, 91% yield).

Compound **S5a**, **S5b**, and **S5d** were prepared following the same procedure as that described for the preparation of **5c**.

S5a: **^{1}H NMR** (500 MHz, DMSO–d_6): Major rotamer: *δ* 12.66 (br s, 1H), 11.33 (s, 1H), 7.95–7.85 (m, 2H), 7.75–7.65 (m, 2H), 7.50–7.31 (m, 5H), 7.20 (s, 1H), 4.83–4.70 (m, 1H), 4.60–4.10 (m, 6H), 4.09–3.77 (m, 4H), 3.50–3.45 (m, 1H), 3.43–3.35 (m, 1H), 1.72 (s, 3H); Minor rotamer: *δ* 12.66 (br s, 1H), 11.33 (s, 1H), 7.95–7.85 (m, 2H), 7.75–7.65 (m, 2H), 7.50–7.31 (m, 5H), 7.30 (s, 1H), 4.83–4.70 (m, 1H), 4.60–4.10 (m, 6H), 4.09–3.77 (m, 4H), 3.75–3.67 (m, 1H), 3.43–3.35 (m, 1H), 1.75 (s, 3H);**^{13}C NMR** (125 MHz, DMSO–d_6): Major rotamer: *δ* 171.1, 167.8, 164.9, 156.5, 151.5, 144.3, 142.3, 141.2, 128.2, 127.6, 125.6, 120.6, 108.8, 71.6, 68.8, 66.0, 62.0, 56.4, 48.5, 47.2, 45.0, 12.4; Minor rotamer: *δ* 171.6, 168.4, 164.9, 156.3, 151.5, 144.4, 142.6, 141.2, 128.2, 127.6, 125.6, 120.6, 108.7, 72.8, 68.7, 65.9, 62.4, 55.4, 48.8, 47.2, 45.0, 12.4; **HRMS** (ESI) for $C_{28}H_{29}N_4O_8$: calcd. 549.1985; found 549.1980.

S5b: **^{1}H NMR** (500 MHz, DMSO–d_6): Major rotamer: *δ* 12.68 (br s, 1H), 10.96 (s, 1H), 8.53 (s, 1H), 8.31 (s, 1H), 7.96–7.65 (m, 5H), 7.60–7.50 (m, 4H), 7.45–7.25 (m, 10H), 6.84 (s, 1H), 5.54–5.40 (m, 1H), 5.26–5.15 (m, 1H), 4.64–4.42 (m, 2H), 4.37–3.80 (m, 7H), 3.75–3.35 (m, 2H); Minor rotamer: *δ* 12.68

(br s, 1H), 10.96 (s, 1H), 8.60 (s, 1H), 8.37 (s, 1H), 7.96–7.65 (m, 5H), 7.60–7.50 (m, 4H), 7.45–7.25 (m, 10H), 6.84 (s, 1H), 5.54–5.40 (m, 1H), 5.26–5.15 (m, 1H), 4.64–4.42 (m, 2H), 4.37–3.80 (m, 7H), 3.75–3.35 (m, 2H); **^{13}C NMR** (125 MHz, DMSO–d_6): Major rotamer: δ 171.0, 167.1, 156.6, 152.8, 152.0, 151.6, 149.8, 145.6, 144.4, 144.2, 141.4, 129.0, 128.2, 128.1, 127.5, 127.0, 125.6, 123.1, 120.6, 77.7, 71.6, 68.8, 66.0, 62.1, 56.4, 47.2, 45.1, 44.8; Minor rotamer: δ 171.7, 167.8, 156.3, 152.8, 152.0, 151.6, 149.8, 145.8, 144.4, 144.2, 141.2, 129.8, 128.2, 128.1, 127.6, 127.0, 125.6, 123.1, 120.6, 77.7, 72.7, 68.7, 65.9, 62.3, 55.3, 47.2, 45.1, 44.8; **HRMS** (ESI) for $C_{42}H_{38}N_7O_8$: calcd. 768.2782; found 768.2789.

S5c: **^{1}H NMR** (500 MHz, DMSO–d_6): Major rotamer: δ 12.67 (br s, 1H), 11.04 (br s, 1H), 7.92–7.65 (m, 6H), 7.50–7.25 (m, 14H), 6.97–6.93 (m, 1H), 6.82 (s, 1H), 4.95 (s, 1H), 4.67–4.20 (m, 6H), 4.17–3.80 (m, 4H), 3.52–3.45 (m, 1H), 3.42–3.36 (m, 1H); Minor rotamer: δ 12.67 (br s, 1H), 11.04 (br s, 1H), 7.92–7.65 (m, 6H), 7.50–7.25 (m, 14H), 6.97–6.93 (m, 1H), 6.82 (s, 1H), 4.95 (s, 1H), 4.67–4.20 (m, 6H), 4.17–3.80 (m, 4H), 3.75–3.67 (m, 1H), 3.42–3.36 (m, 1H); **^{13}C NMR** (125 MHz, DMSO–d_6): Major rotamer: δ 171.0, 167.7, 163.6, 156.5, 155.4, 151.3, 144.4, 144.3, 141.2, 140.9, 129.1, 128.4, 128.1, 127.6, 126.9, 125.6, 120.6, 94.4, 77.9, 71.8, 68.8, 66.0, 61.8, 56.4, 50.3, 47.2, 45.0; Minor rotamer: δ 171.6, 168.3, 163.6, 156.3, 152.9, 151.5, 144.4, 144.3, 141.2, 140.9, 129.1, 128.4, 128.1, 127.6, 126.9, 125.6, 120.6, 94.3, 77.9, 72.8, 68.8, 65.9, 62.3, 55.5, 50.6, 47.2, 45.0; **HRMS** (ESI) for $C_{41}H_{37}N_5O_9Na$: calcd. 766.2489; found 766.2489.

S5d: **^{1}H NMR** (500 MHz, DMSO–d_6): Major rotamer: δ 13.27 (br s, 1H), 12.71 (br s, 1H), 11.72 (s, 1H), 11.28 (s, 1H), 7.92–7.67 (m, 5H), 7.50–7.25 (m, 14H), 6.88 (s, 1H), 5.30–5.17 (m, 1H), 4.98 (s, 1H), 4.60–4.14 (m, 6H), 4.12–3.80 (m, 3H), 3.55–3.50 (m, 1H), 3.42–3.37 (m, 1H); Minor rotamer: 13.27 (br s, 1H), 12.71 (br s, 1H), 11.78 (s, 1H), 11.27 (s, 1H), 7.92–7.67 (m, 5H), 7.50–7.25 (m, 14H), 6.88 (s, 1H), 5.30–5.17 (m, 1H), 4.98 (s, 1H), 4.60–4.14 (m, 6H), 4.12–3.80 (m, 3H), 3.75–3.68 (m, 1H), 3.42–3.37 (m, 1H); **^{13}C NMR** (125 MHz, DMSO–d_6): Major rotamer: δ 171.1, 167.1, 156.6, 155.6, 154.3, 150.0, 147.5, 144.3, 141.3, 140.8, 140.6, 129.1, 128.5, 128.1, 127.5, 126.9, 125.6, 120.6, 119.9, 78.5, 72.0, 69.0, 66.0, 62.2, 56.9, 47.3, 45.2, 44.8; Minor rotamer: δ 171.7, 167.8, 156.3, 155.6, 154.3, 150.0, 147.5, 144.2, 141.2, 141.0, 140.6, 129.1, 128.5, 128.1, 127.6, 126.9, 125.6, 120.6, 119.8, 78.5, 72.8, 68.8, 66.0, 62.5, 55.4, 47.2, 45.2, 45.0; **HRMS** (ESI) for $C_{42}H_{38}N_7O_9$: calcd. 784.2731; found 784.2728.

Figure S2: ^{1}H-NMR spectrum of S3 in $CDCl_3$ at 25 °C

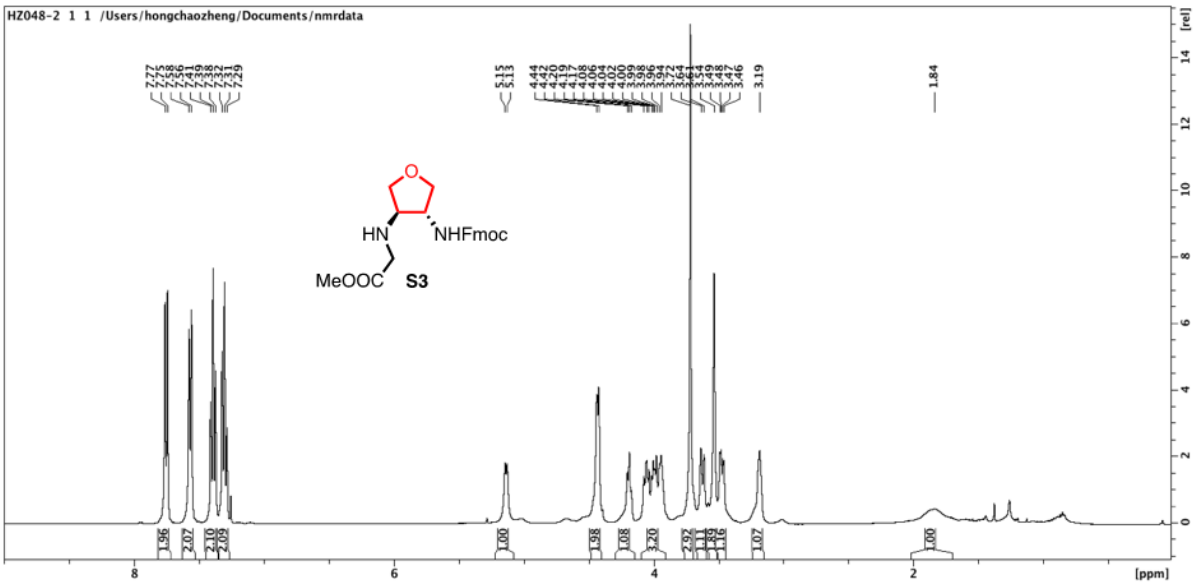


Figure S3: ^{13}C--NMR spectrum of S3 in $CDCl_3$ at 25 °C

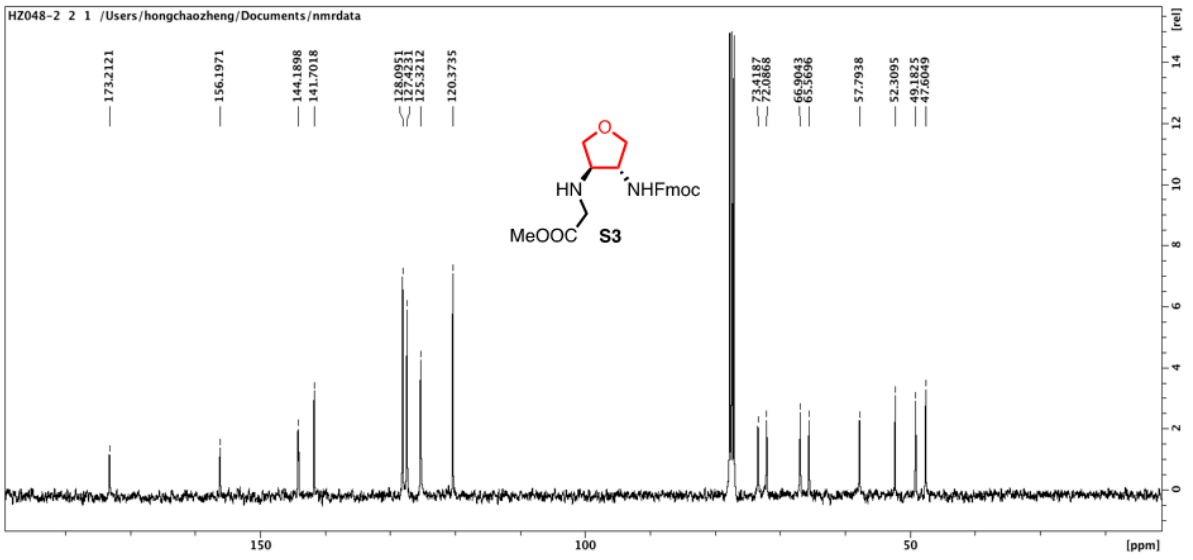


Figure S4. ^{1}H-NMR spectrum of S4a in $CDCl_3$ at 25 °C

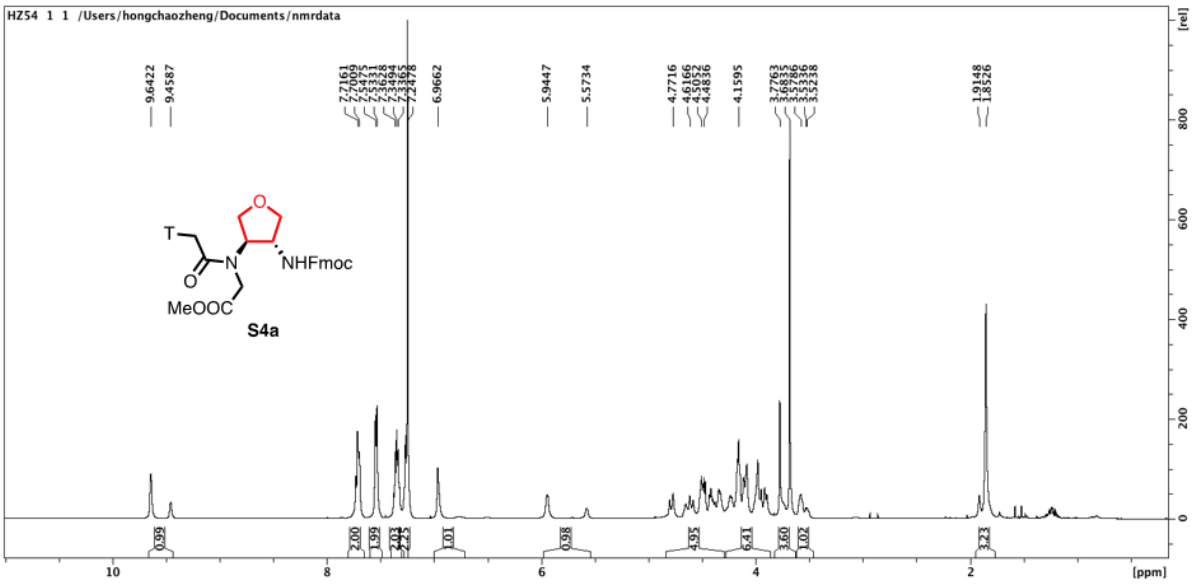


Figure S5. ^{13}C-NMR spectrum of S4a in $CDCl_3$ at 25 °C

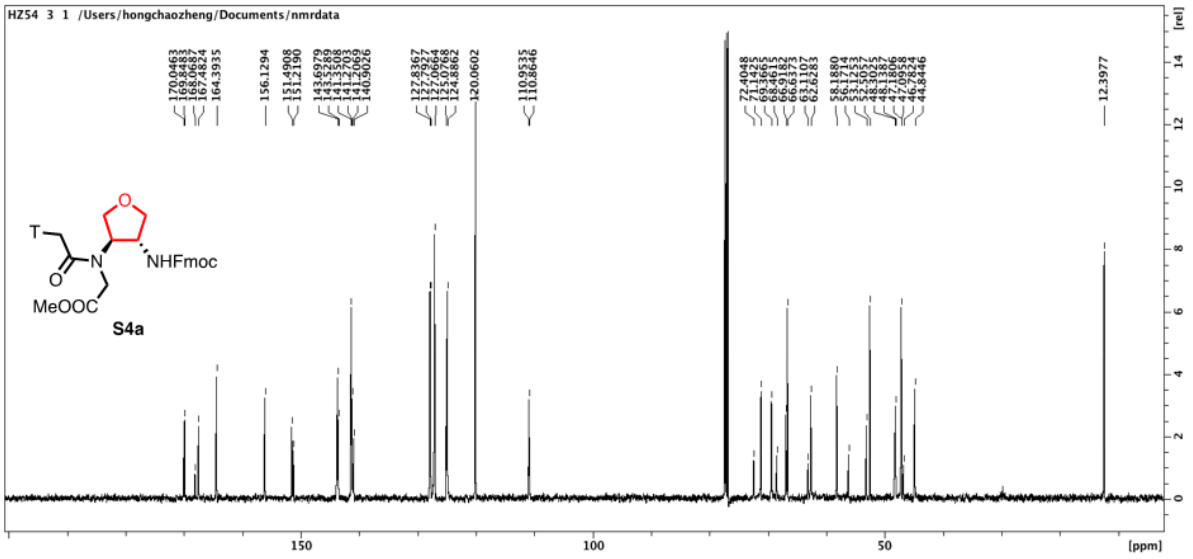


Figure S6. HSQC spectrum of S4a in $CDCl_3$ at 25 °C

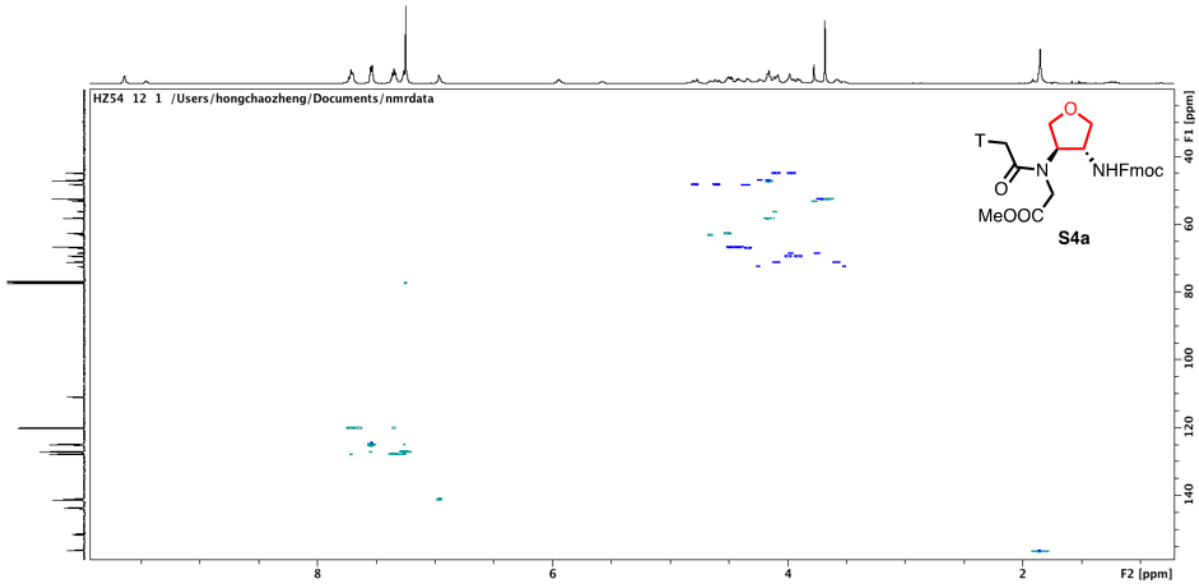


Figure S7. ^{1}H-NMR spectrum of S4b in $CDCl_3$ at 25 °C

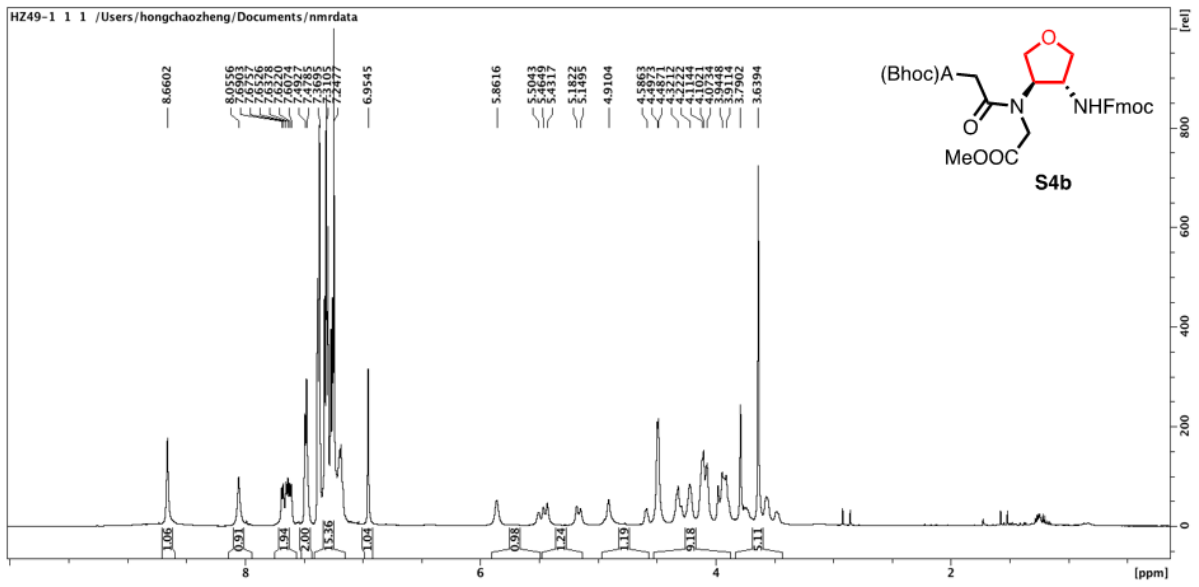


Figure S8. ^{13}C-NMR spectrum of S4b in $CDCl_3$ at 25 °C

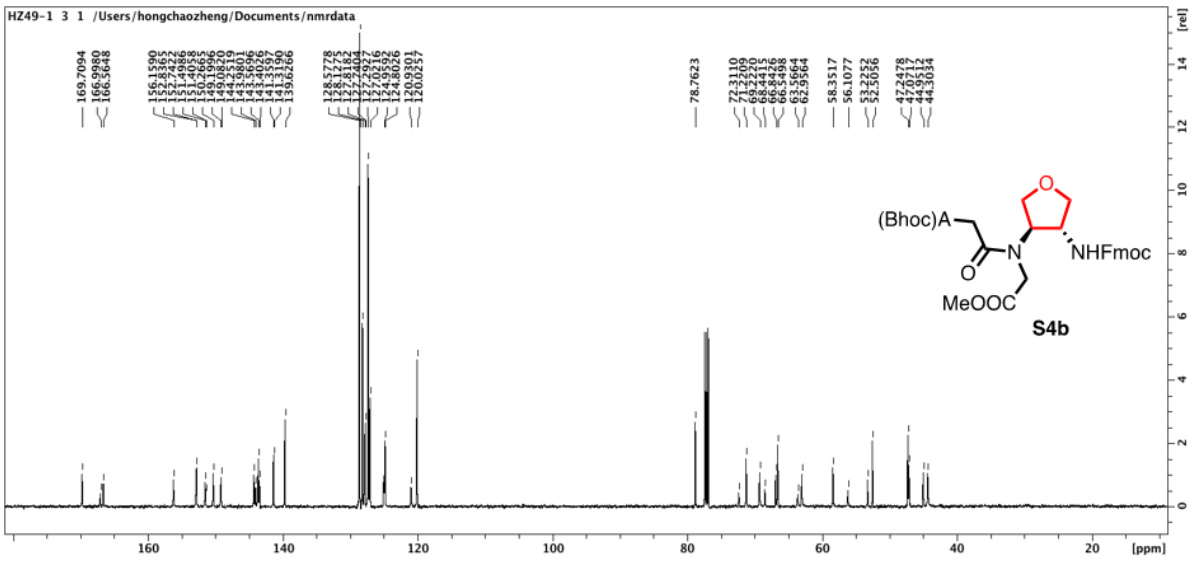


Figure S9. HSQC spectrum of S4b in $CDCl_3$ at 25 °C

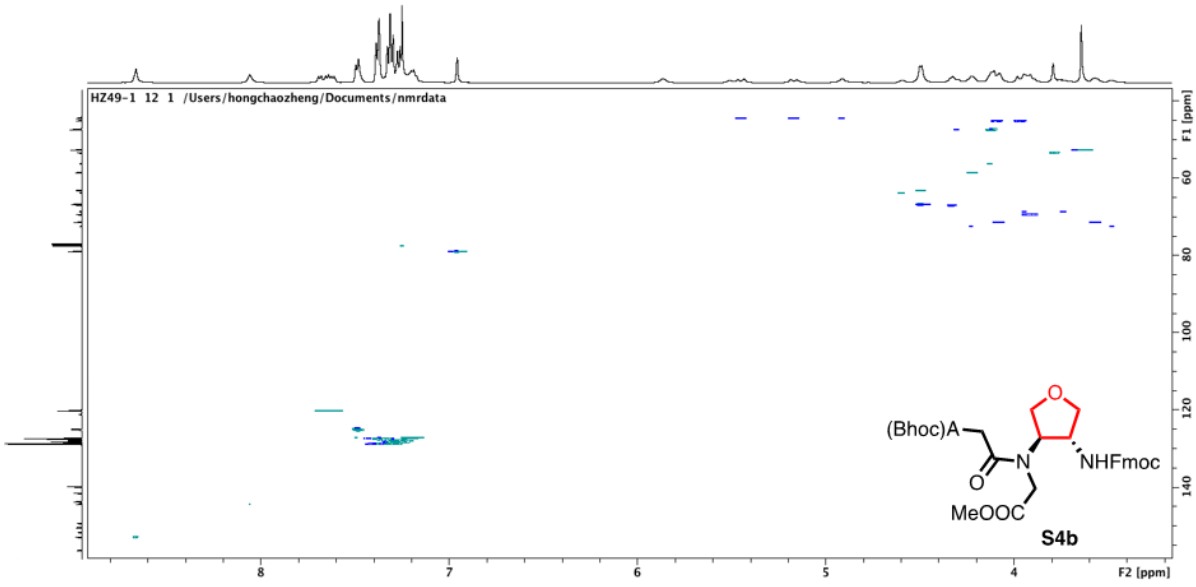


Figure S10. ^{1}H-NMR spectrum of S4c in $CDCl_3$ at 25 °C

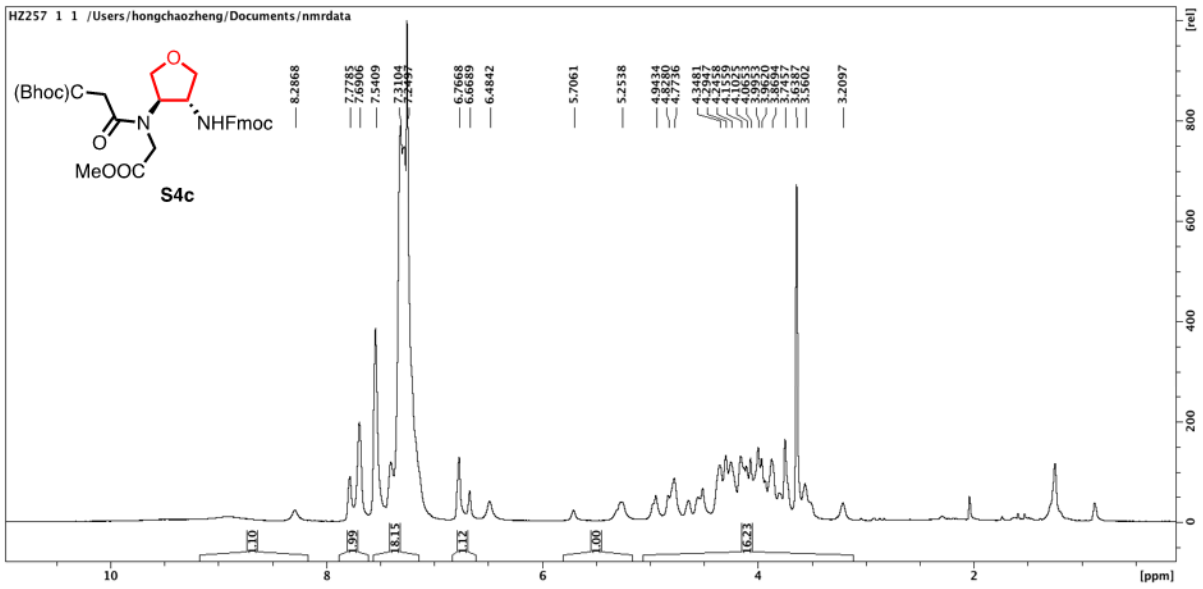


Figure S11. ^{13}C-NMR spectrum of S4c in $CDCl_3$ at 25 °C

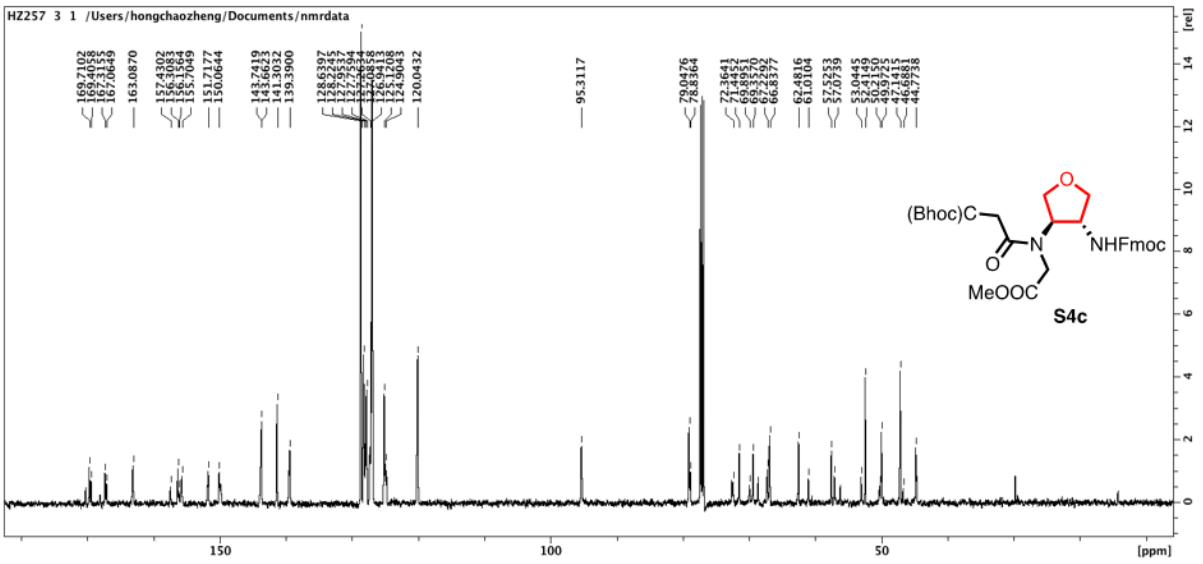


Figure S12. HSQC spectrum of S4c in $CDCl_3$ at 25 °C

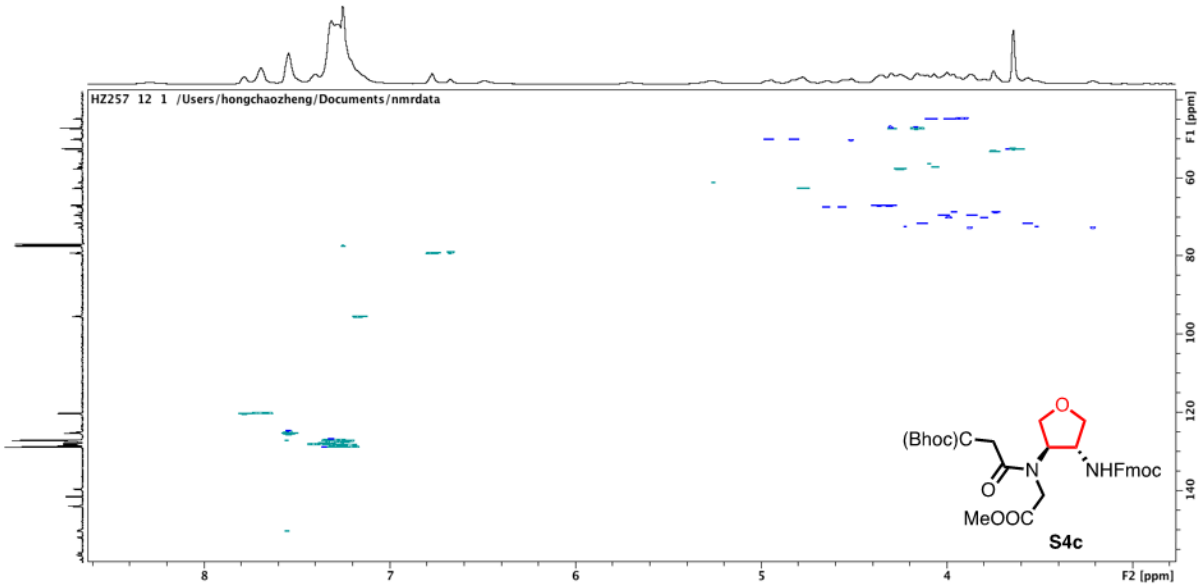


Figure S13. ^{1}H-NMR spectrum of S4d in $CDCl_3$ at 25 °C

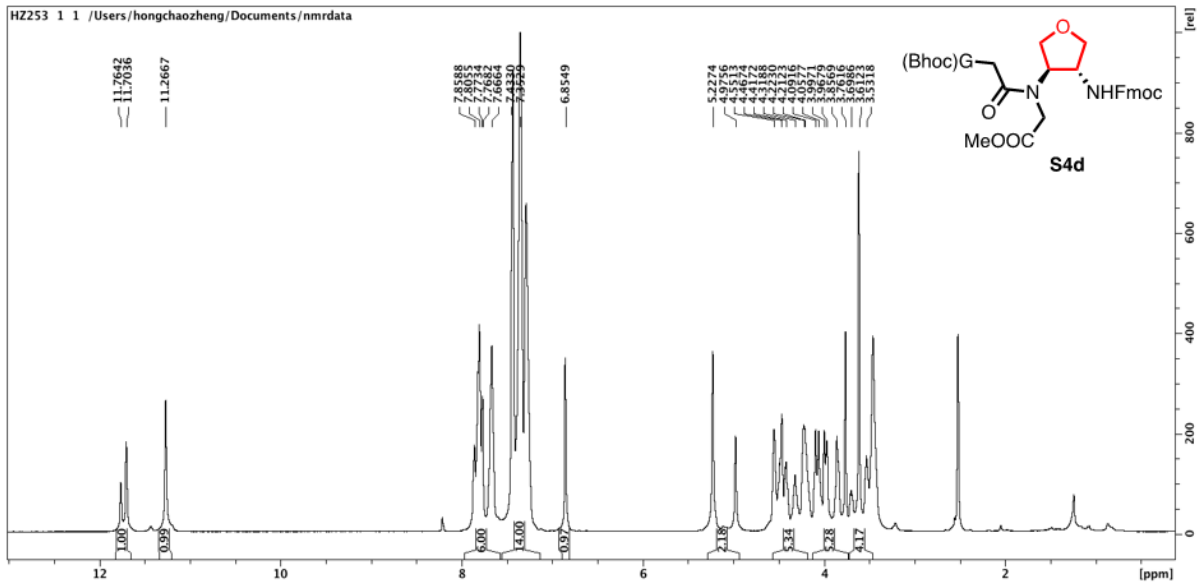


Figure S14. ^{13}C-NMR spectrum of S4d in $CDCl_3$ at 25 °C

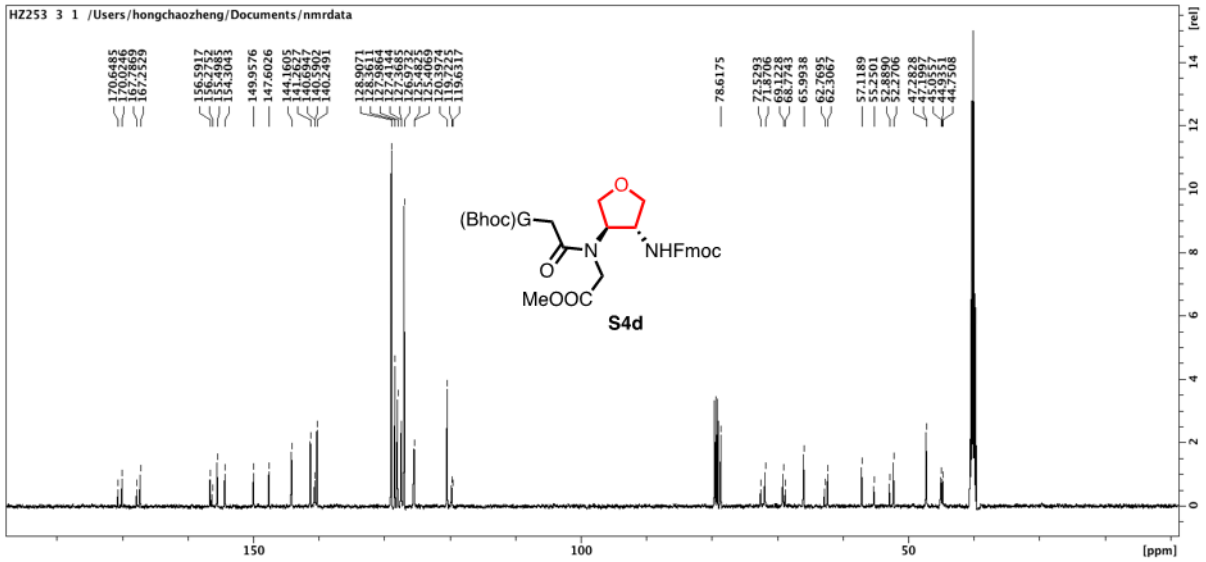


Figure S15. HSQC spectrum of S4d in $CDCl_3$ at 25 °C

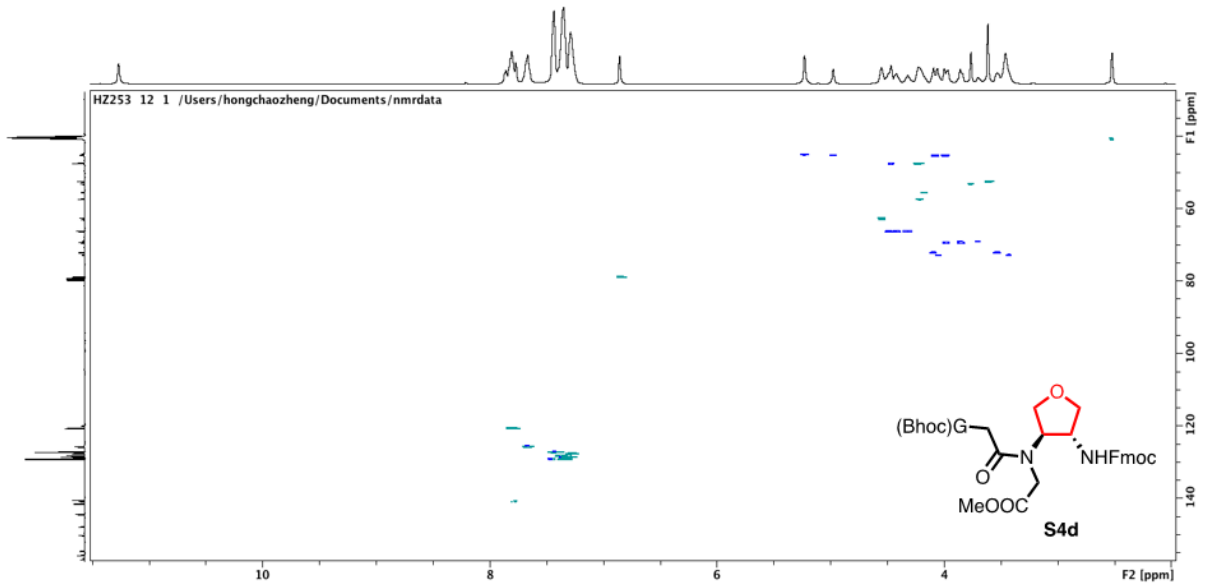


Figure S16. ^{1}H-NMR spectrum of S5a in DMSO-d_6 at 25 °C

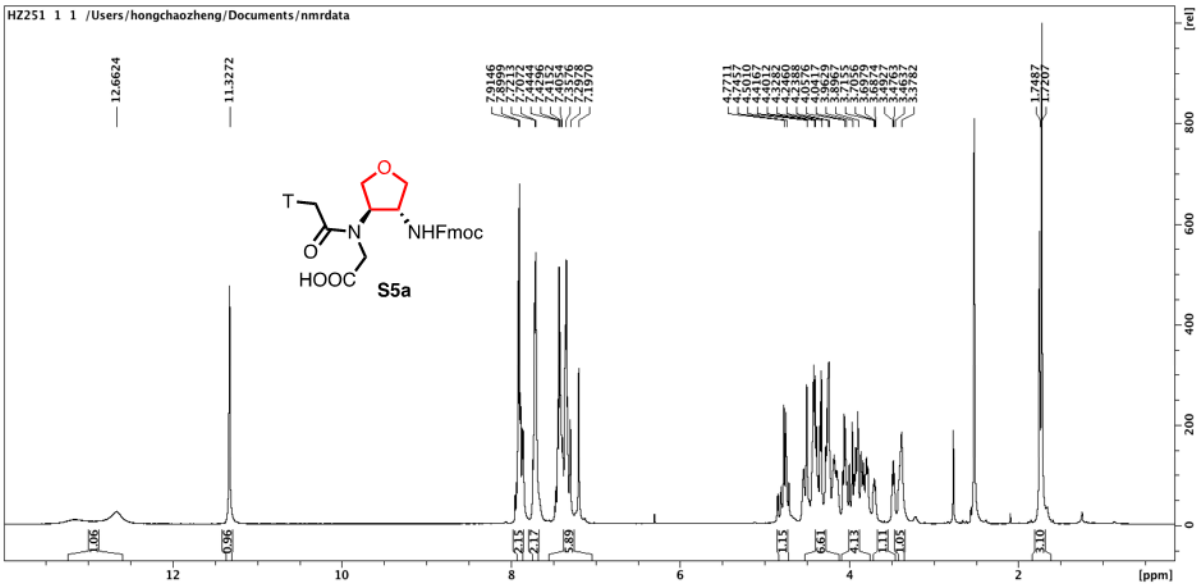


Figure S17. ^{13}C-NMR spectrum of S5a in DMSO-d_6 at 25 °C

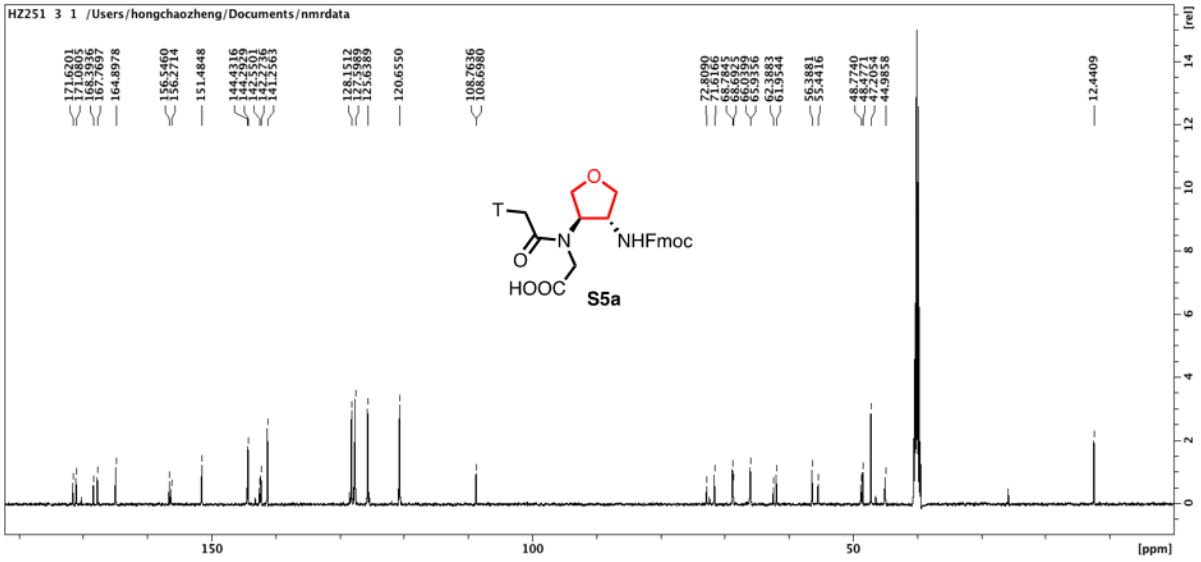


Figure S18. HSQC spectrum of S5a in DMSO-d_6 at 25 °C

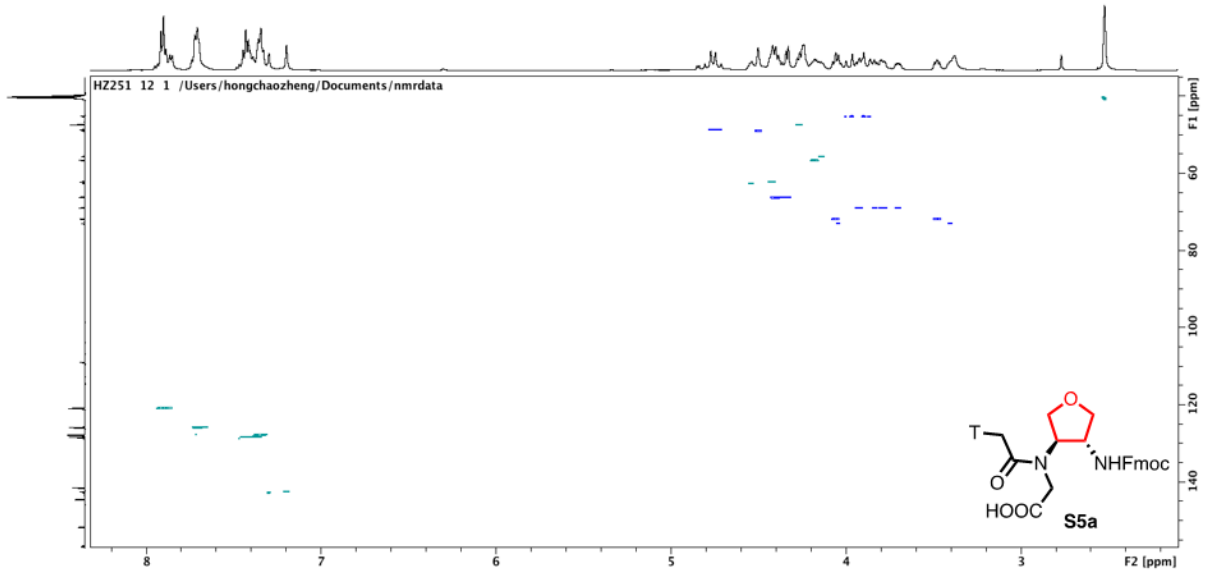


Figure S19. ^{1}H-NMR spectrum of S5b in DMSO-d_6 at 25 °C

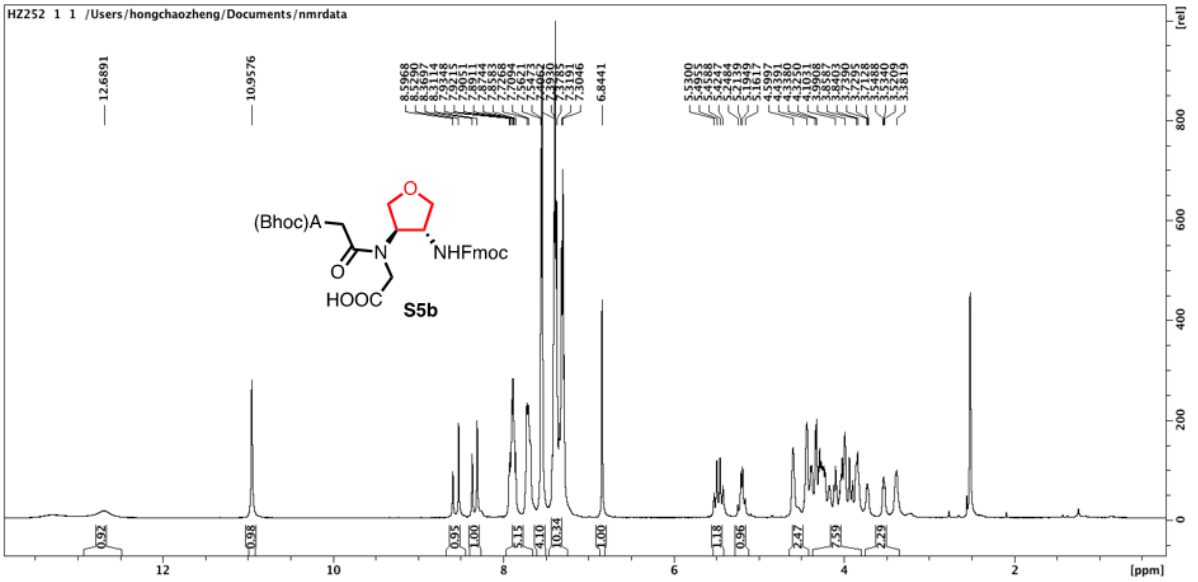


Figure S20. ^{13}C-NMR spectrum of S5b in DMSO-d_6 at 25 °C

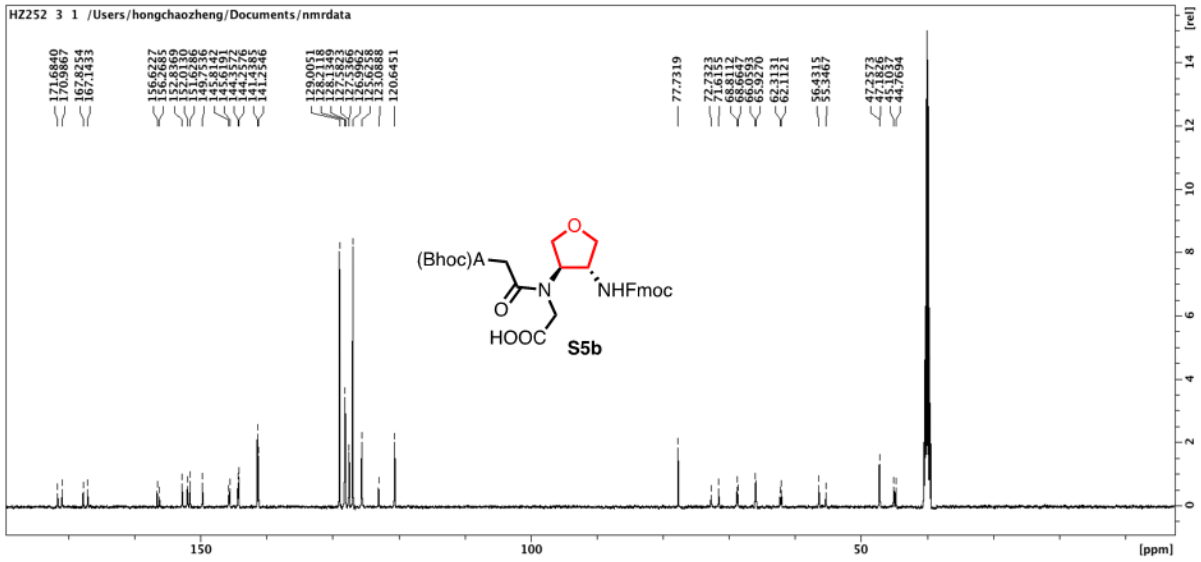


Figure S21. HSQC spectrum of S5b in DMSO-d_6 at 25 °C

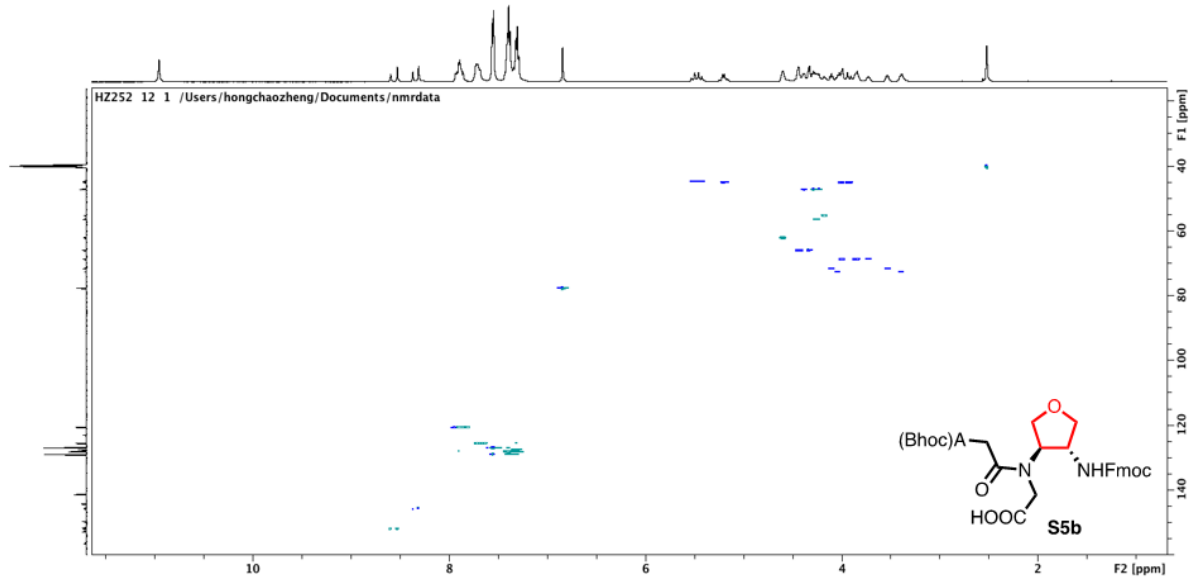


Figure S22. ^{1}H-NMR spectrum of S5c in DMSO-d_6 at 25 °C

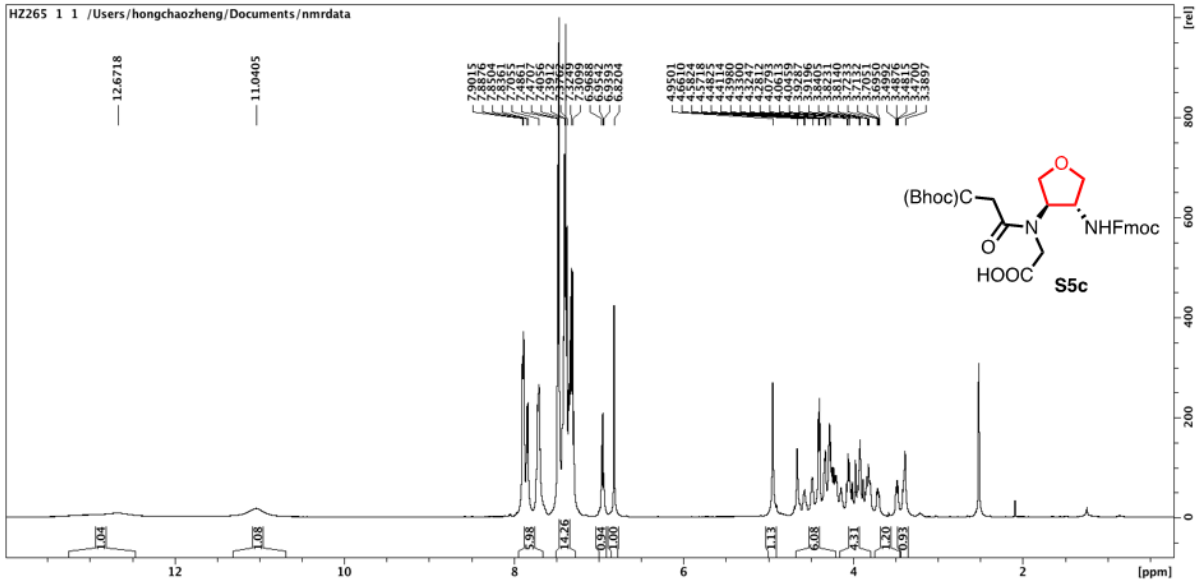


Figure S23. ^{13}C-NMR spectrum of S5c in DMSO-d_6 at 25 °C

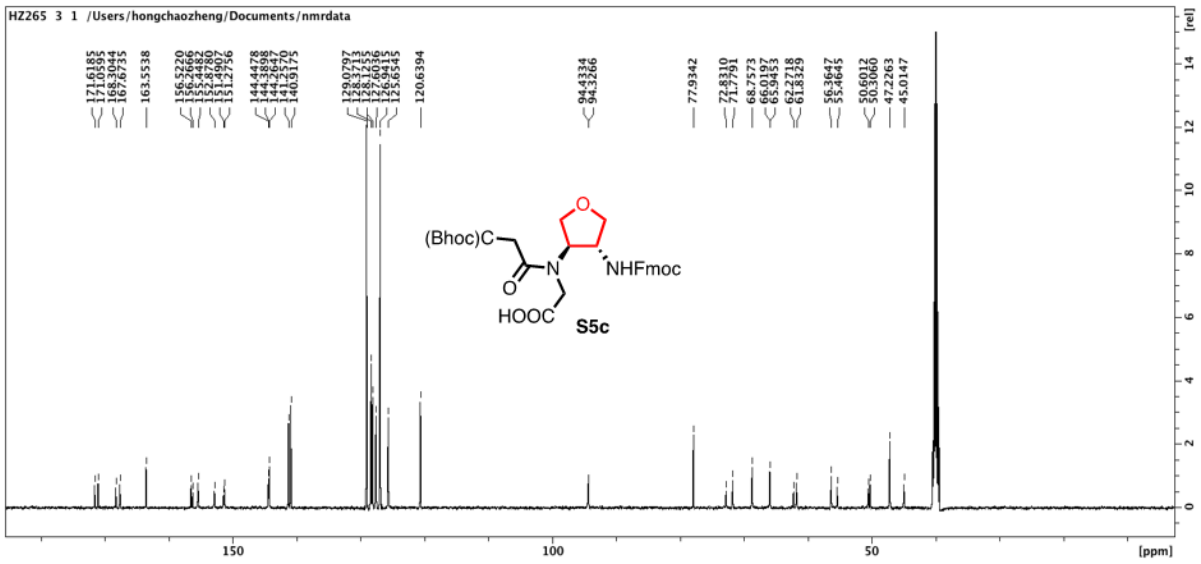


Figure S24. HSQC spectrum of S5c in DMSO-d_6 at 25 °C

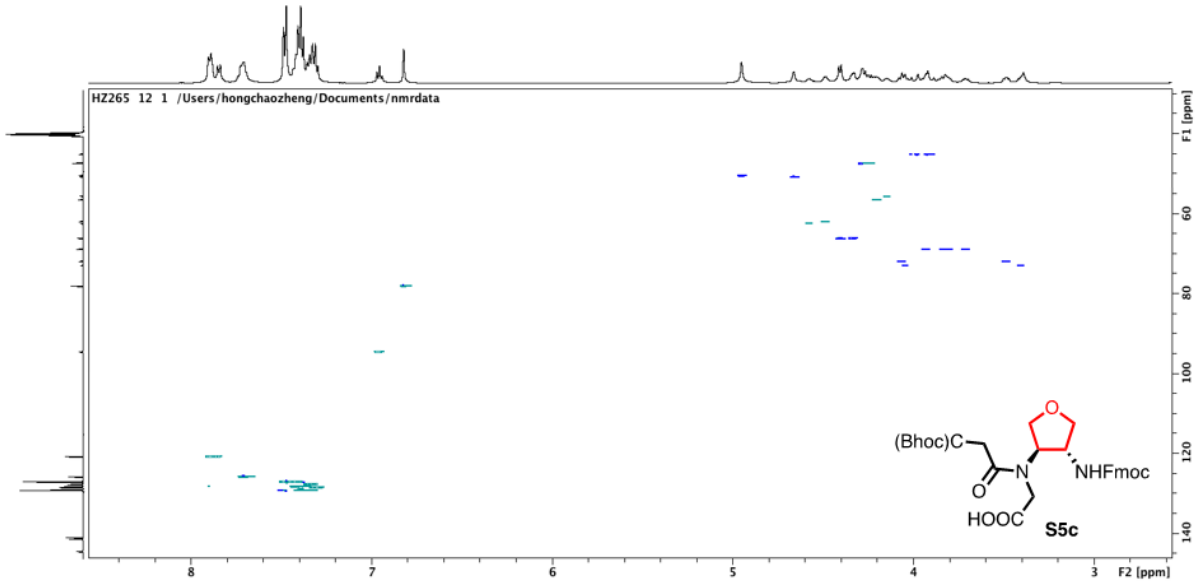


Figure S25. ^{1}H-NMR spectrum of S5d in DMSO-d_6 at 25 °C

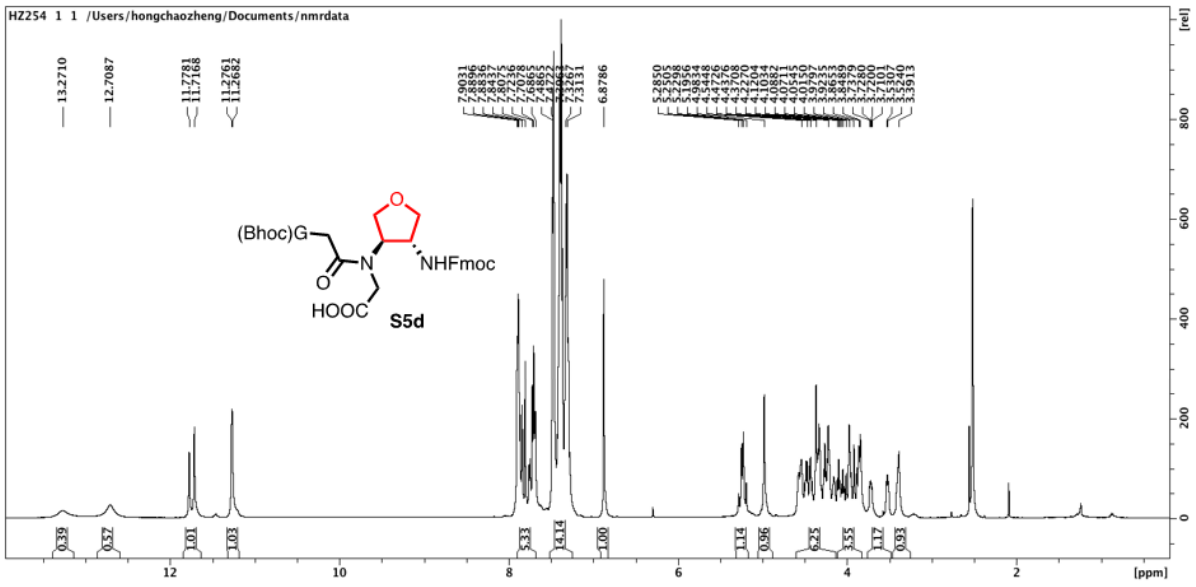


Figure S26. ^{13}C-NMR spectrum of S5d in DMSO-d_6 at 25 °C

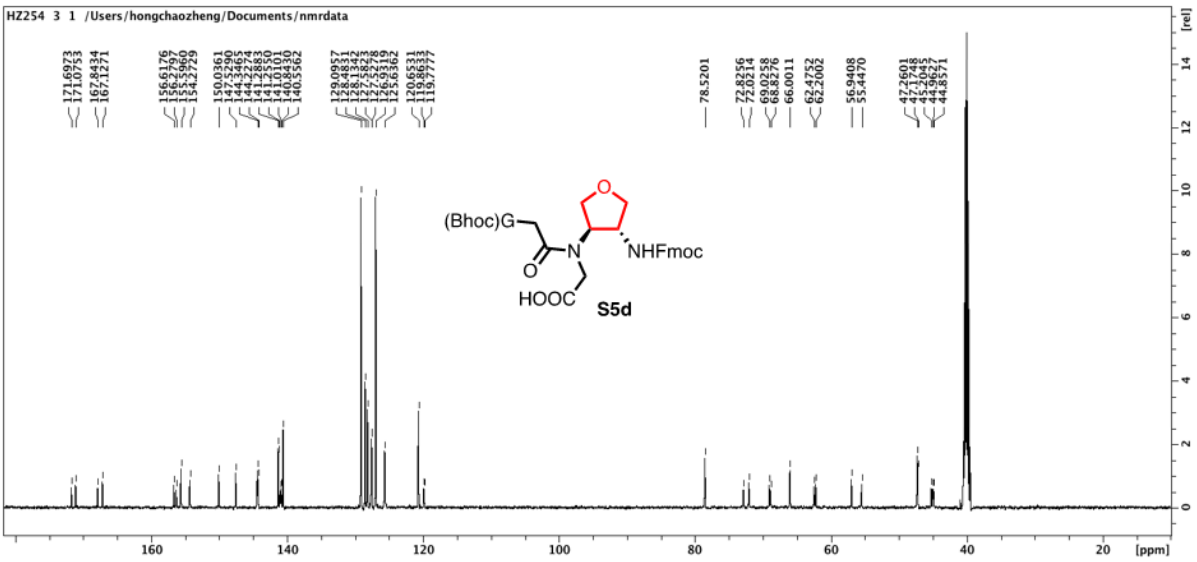


Figure S27. HSQC spectrum of S5d in DMSO-d_6 at 25 °C

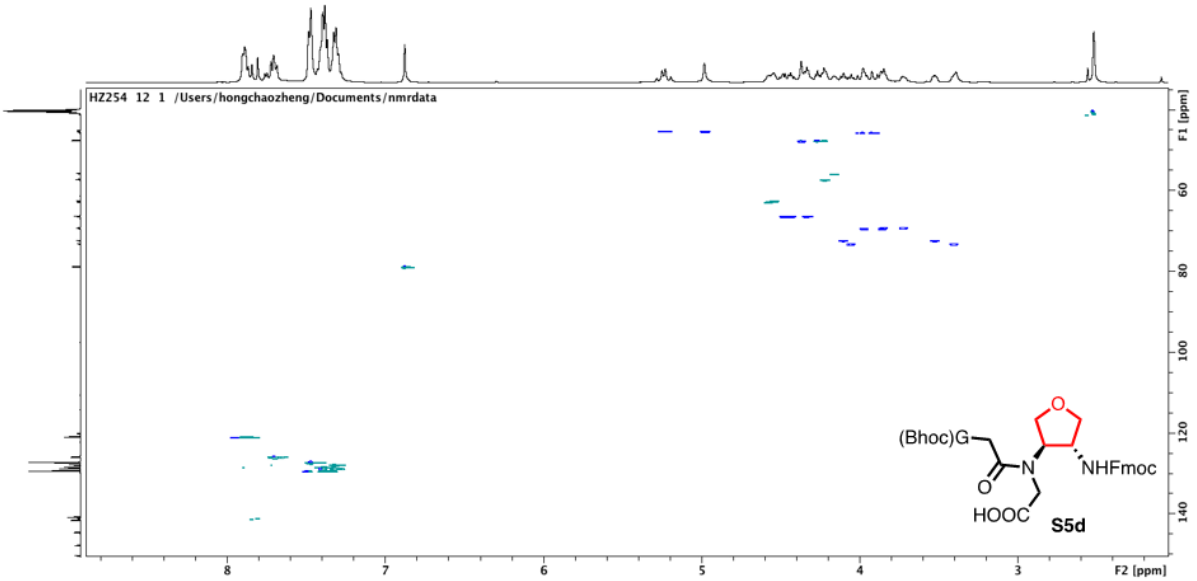


Figure S28: HPLC chromatogram of antimiR-21 14-nucleobase PNA 1

NH_2-AGTCTGATAAGCTA-AEEA-$CONH_2$

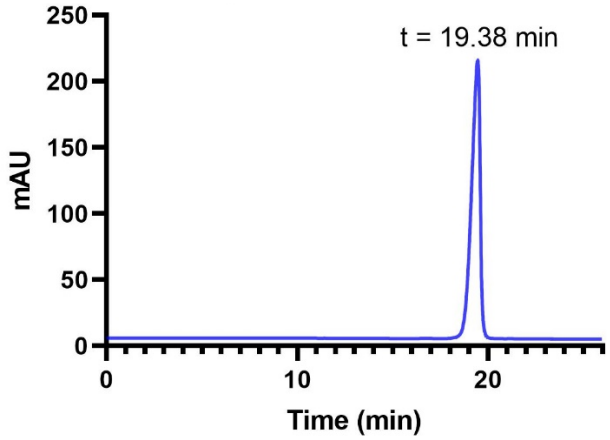


Figure S29: Mass spectrum of antimiR-21 14-nucleobase PNA 1

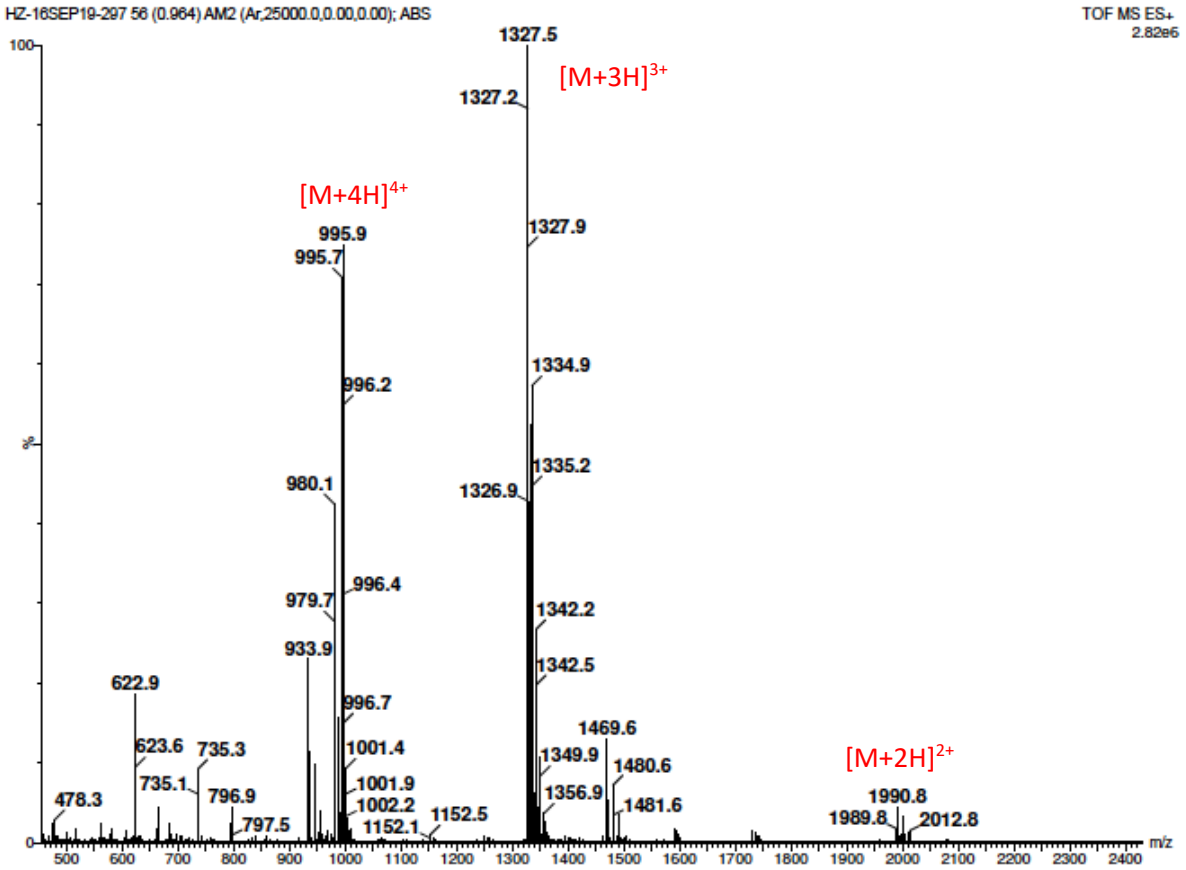


Figure S30: HPLC chromatogram of antimiR-21 14-nucleobase thyclotide 2

NH_2-$A_{thf}G_{thf}T_{thf}C_{thf}T_{thf}G_{thf}A_{thf}T_{thf}A_{thf}A_{thf}G_{thf}C_{thf}T_{thf}A_{thf}$-AEEA-$CONH_2$

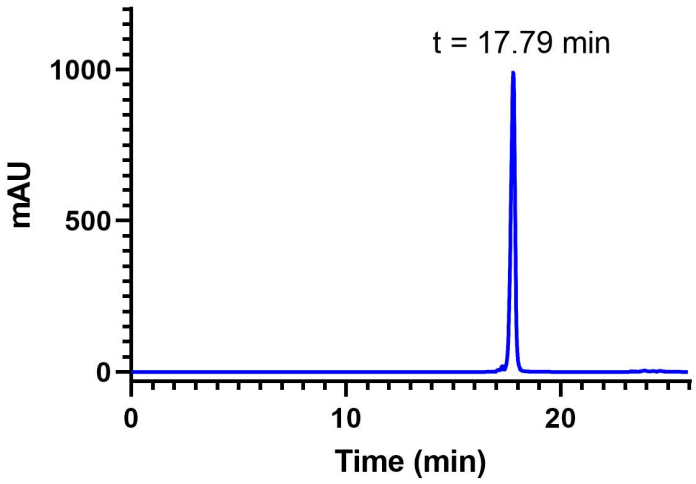


Figure S31: Mass spectrum of antimiR-21 14-nucleobase thyclotide 2

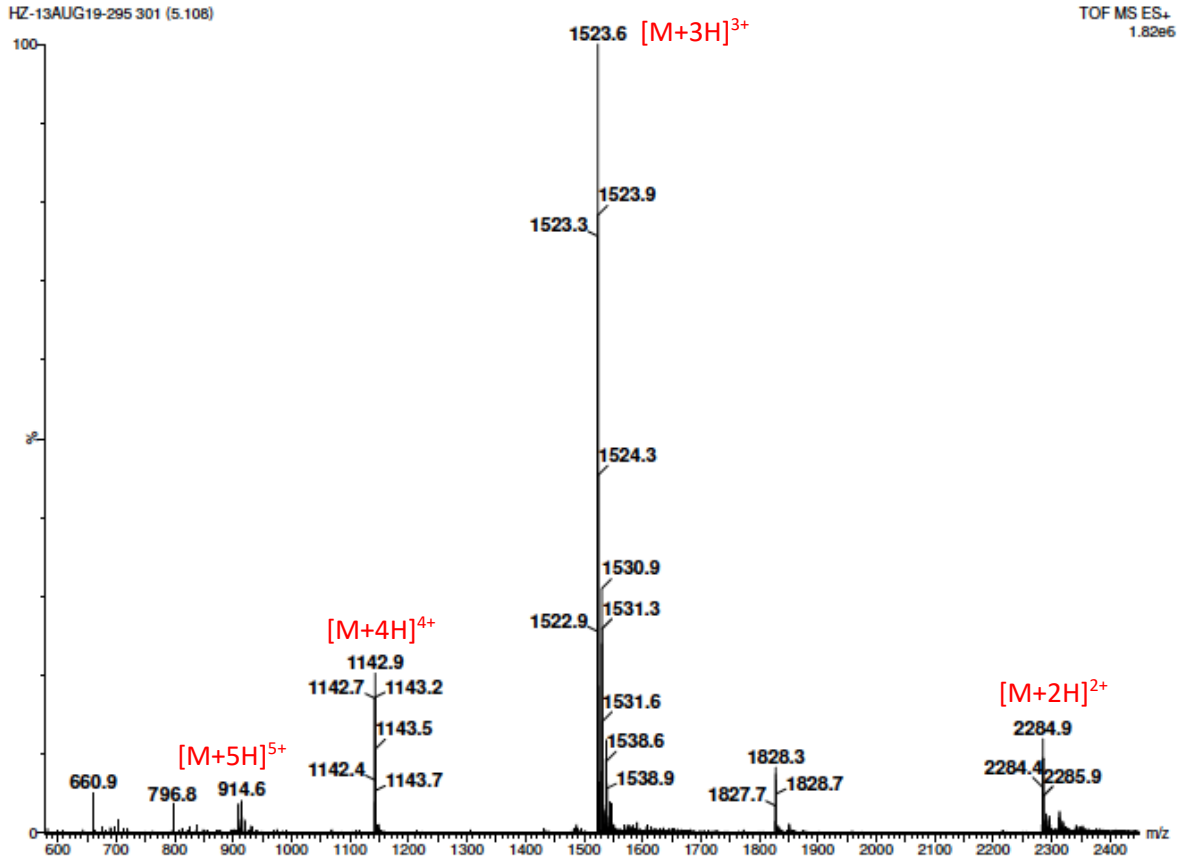


Figure S32: HPLC chromatogram of antimiR-21 14-nucleobase partial thyclotide 3

NH2-AGTCTGA$_{thf}$T$_{thf}$AAGCTA-AEEA-CONH2

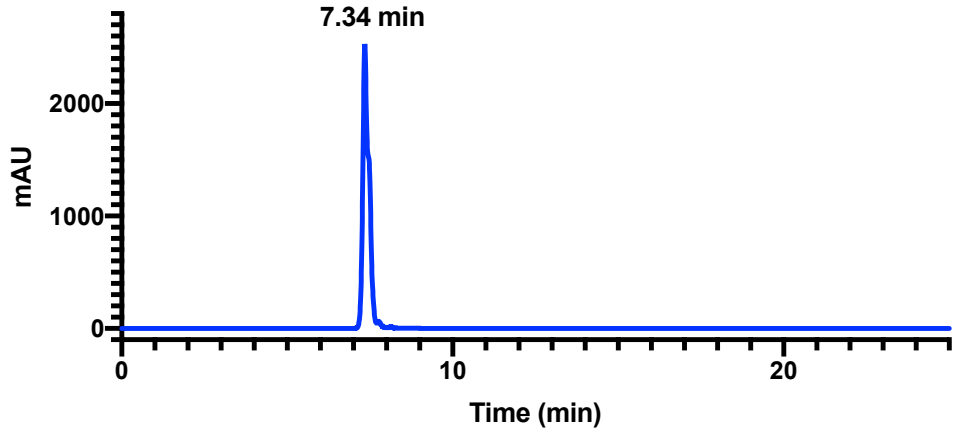


Figure S33: Mass spectrum of antimiR-21 14-nucleobase partial thyclotide 3

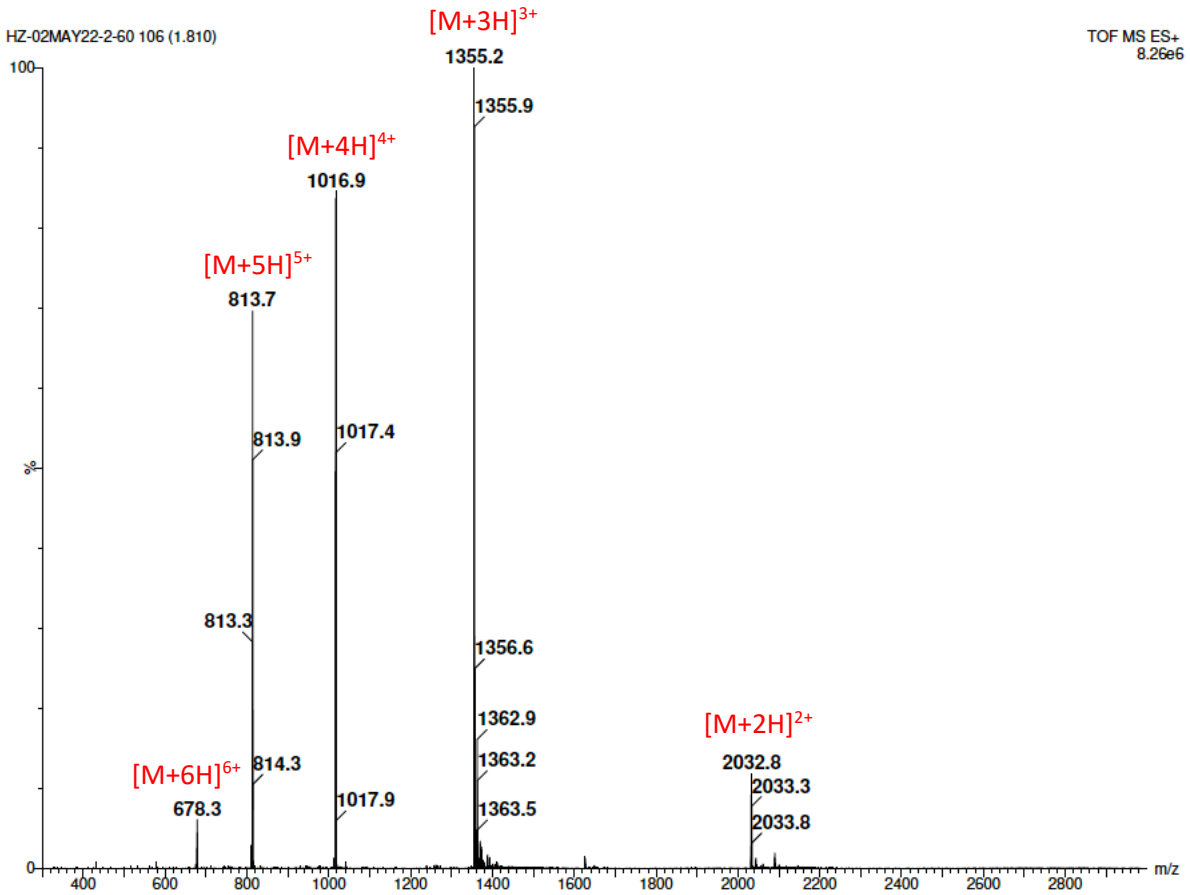


Figure S34: HPLC chromatogram of FAM-antimiR-21 14-nucleobase PNA 4

FAM-(6)5-AGTCTGATAAGCTA-AEEA-CONH2

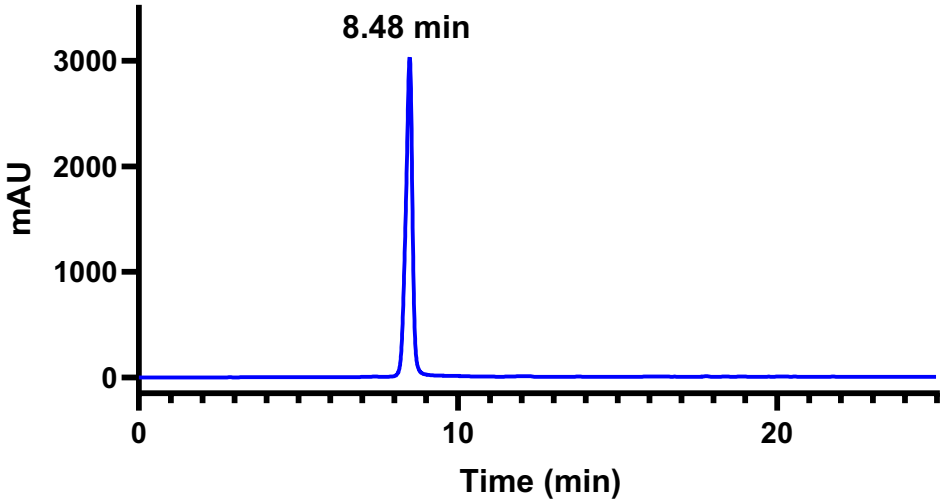


Figure S35: Mass spectrum of FAM-antimiR-21 14-nucleobase PNA 4

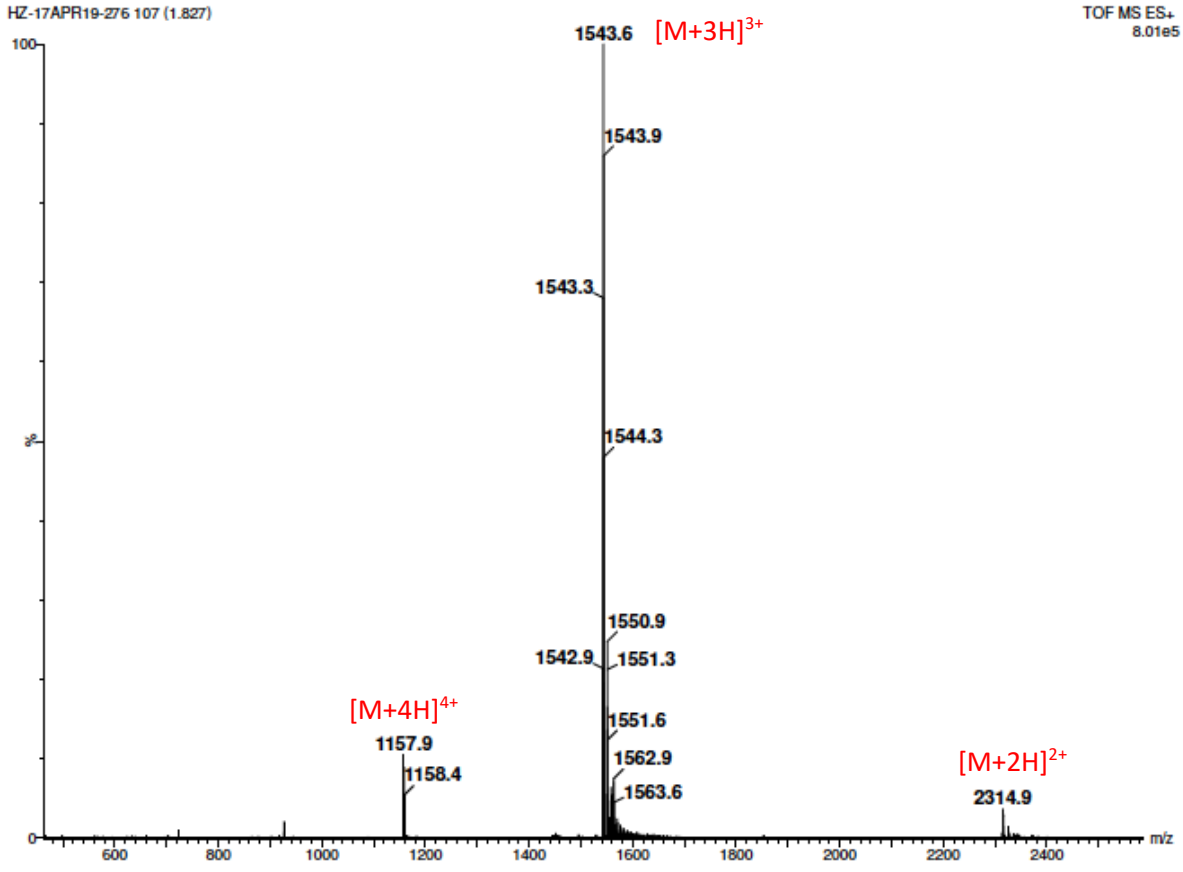


Figure S36: HPLC chromatogram of FAM-antimiR-21 14-nucleobase thyclotide 5

FAM-(6)5-$A_{thf}G_{thf}T_{thf}C_{thf}T_{thf}G_{thf}A_{thf}T_{thf}A_{thf}A_{thf}G_{thf}C_{thf}T_{thf}A_{thf}$-AEEA-CONH2

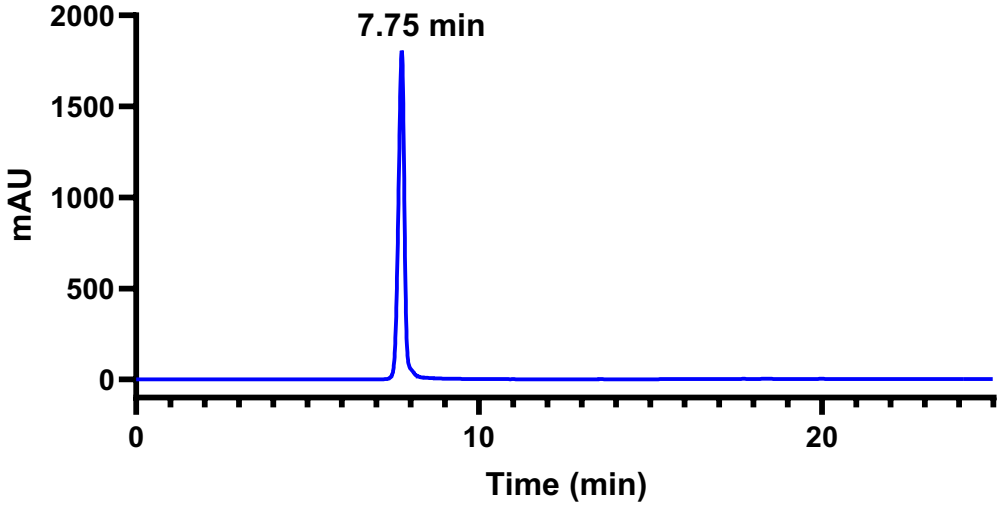


Figure S37: Mass spectrum of FAM-antimiR-21 14-nucleobase thyclotide 5

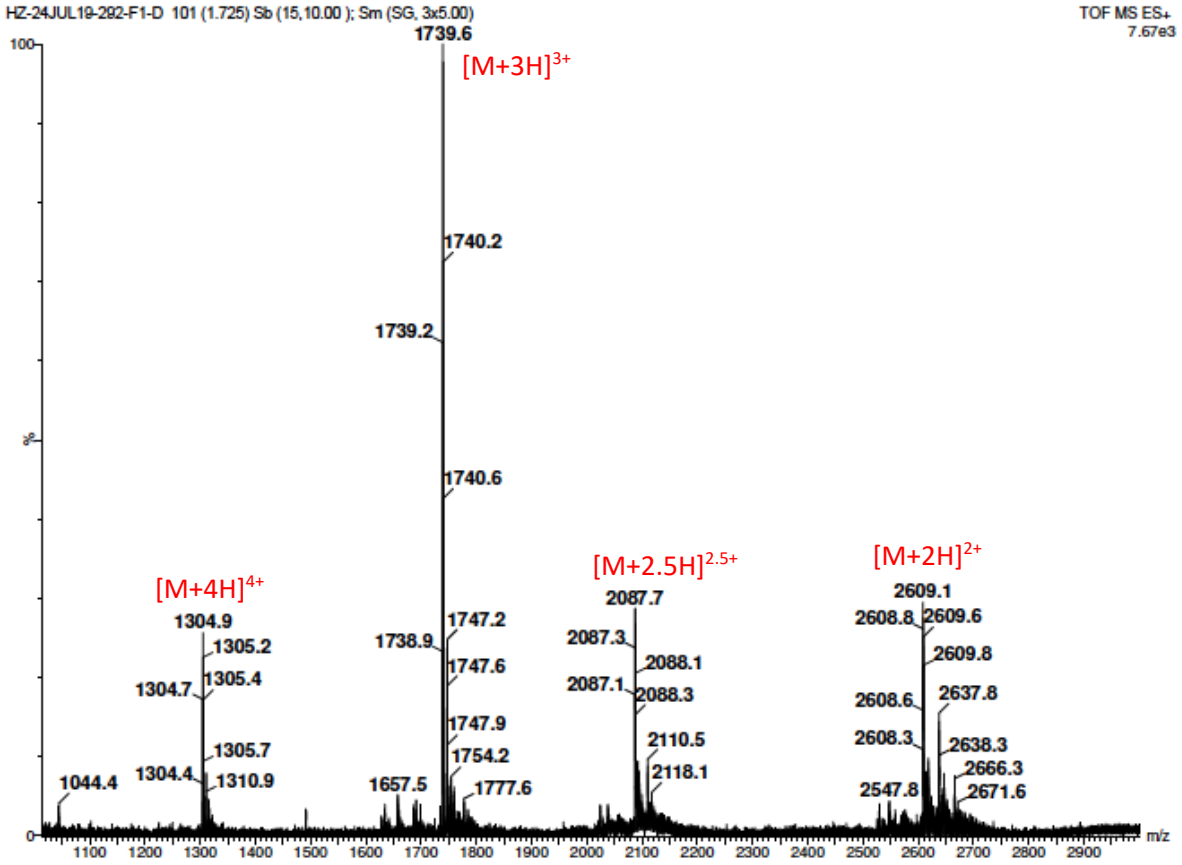


Figure S38: HPLC chromatogram of FAM-antimiR-21 20-nucleobase PNA 6

FAM-AEEA-AEEA-AACATCAGTCTGATAAGCTA-AEEA-$CONH_2$

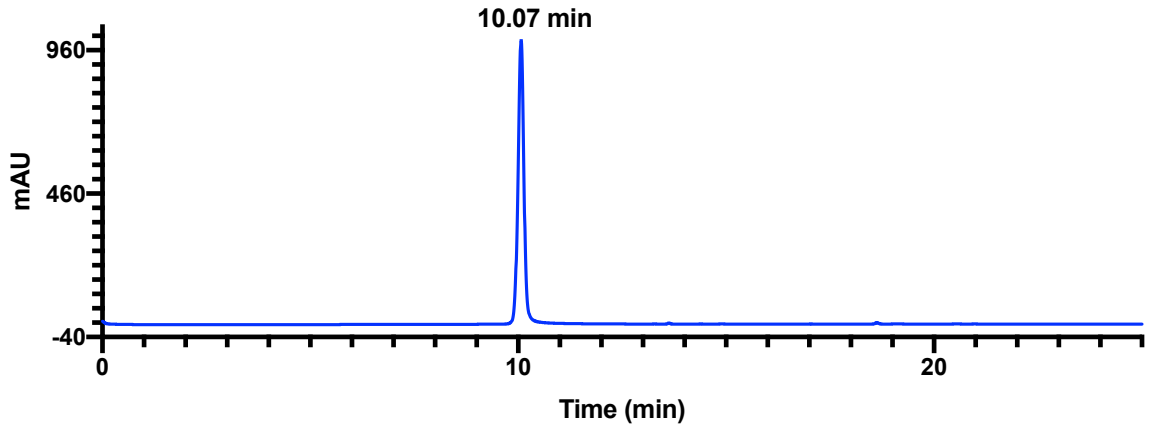


Figure S39: Mass spectrum of FAM-antimiR-21 20-nucleobase PNA 6

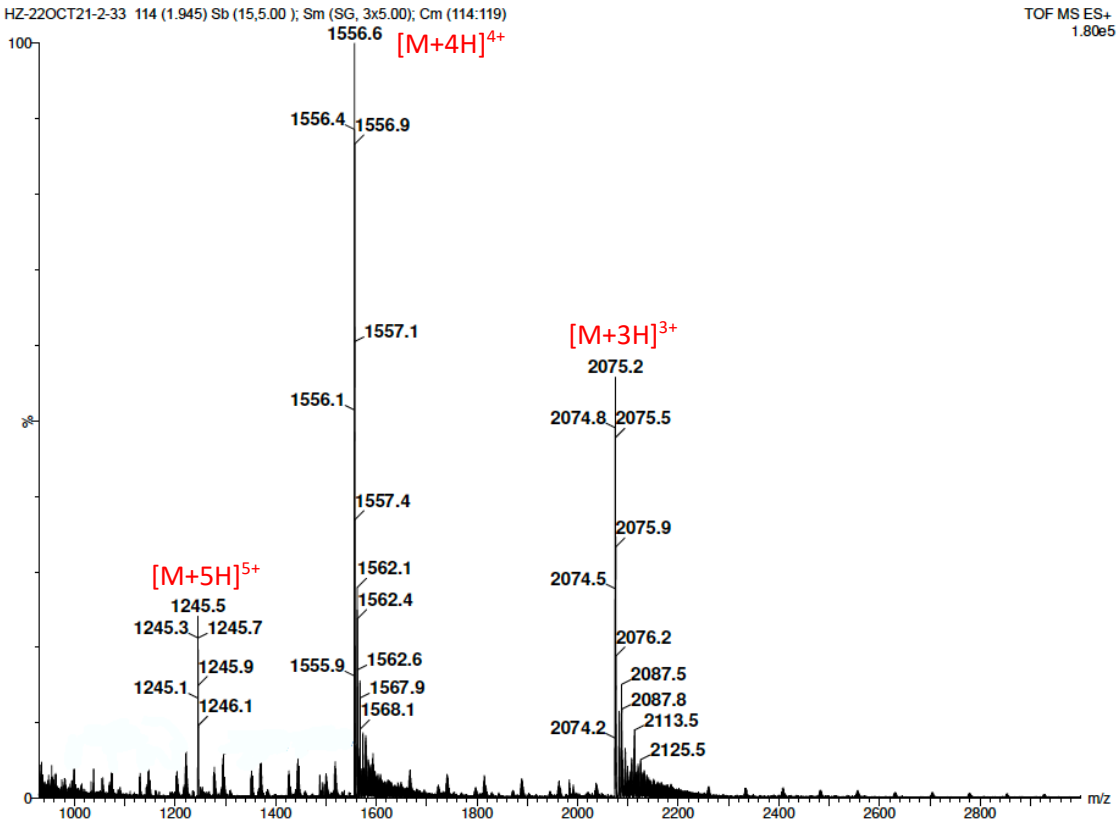


Figure S40: HPLC chromatogram of FAM-antimiR-21 20-nucleobase thyclotide 7

FAM-AEEA-AEEA-$A_{thf}A_{thf}C_{thf}A_{thf}T_{thf}C_{thf}A_{thf}G_{thf}T_{thf}C_{thf}T_{thf}G_{thf}A_{thf}T_{thf}A_{thf}A_{thf}G_{thf}C_{thf}T_{thf}A_{thf}$-AEEA-$CONH_2$

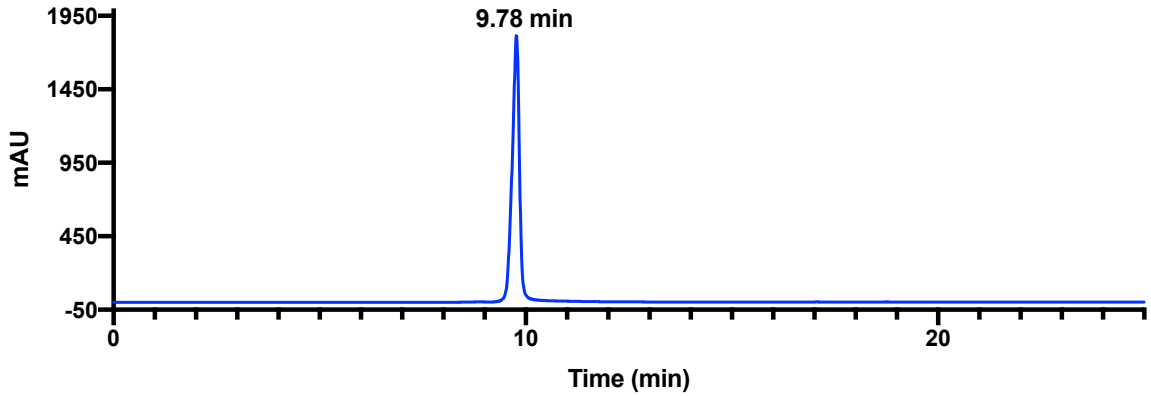


Figure S41: Mass spectrum of FAM-antimiR-21 20-mer thyclotide 7

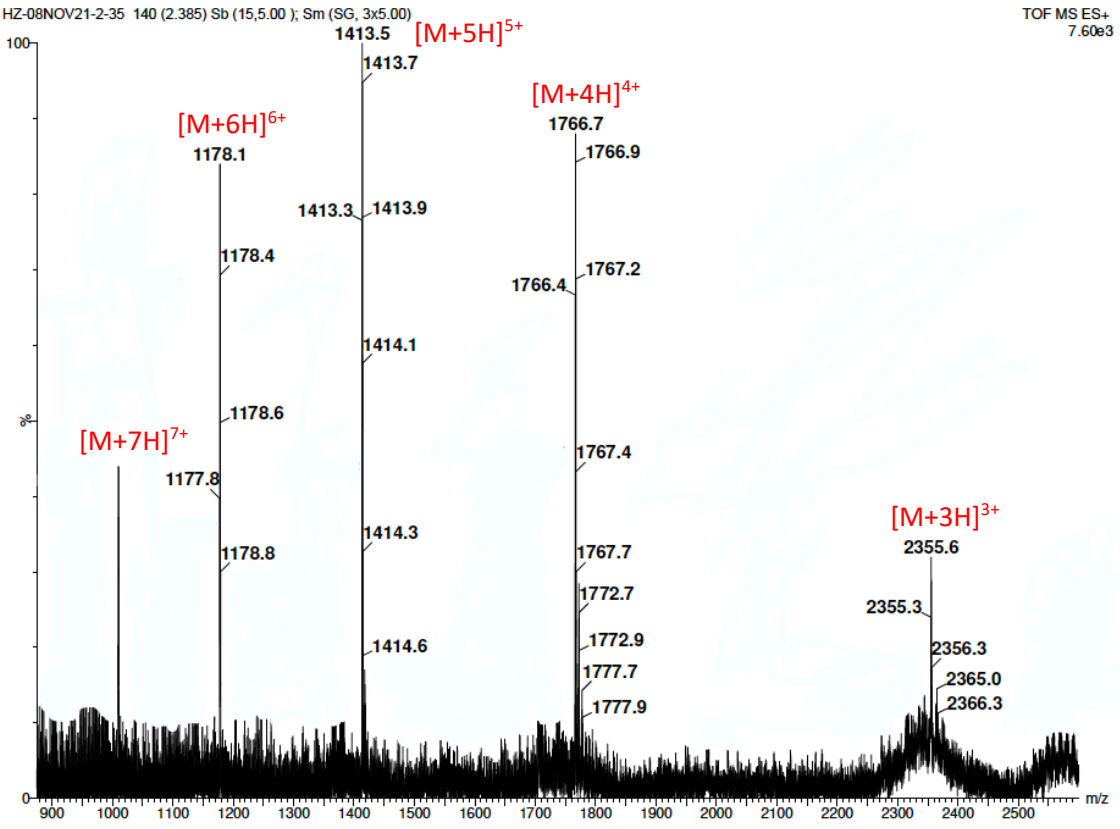


Figure S42: HPLC chromatogram of scrambled 20-nucleobase thyclotide 8

NH_2-$G_{thf}T_{thf}A_{thf}C_{thf}A_{thf}T_{thf}T_{thf}A_{thf}C_{thf}G_{thf}A_{thf}C_{thf}G_{thf}A_{thf}T_{thf}A_{thf}C_{thf}A_{thf}A_{thf}T_{thf}$-AEEA-$CONH_2$

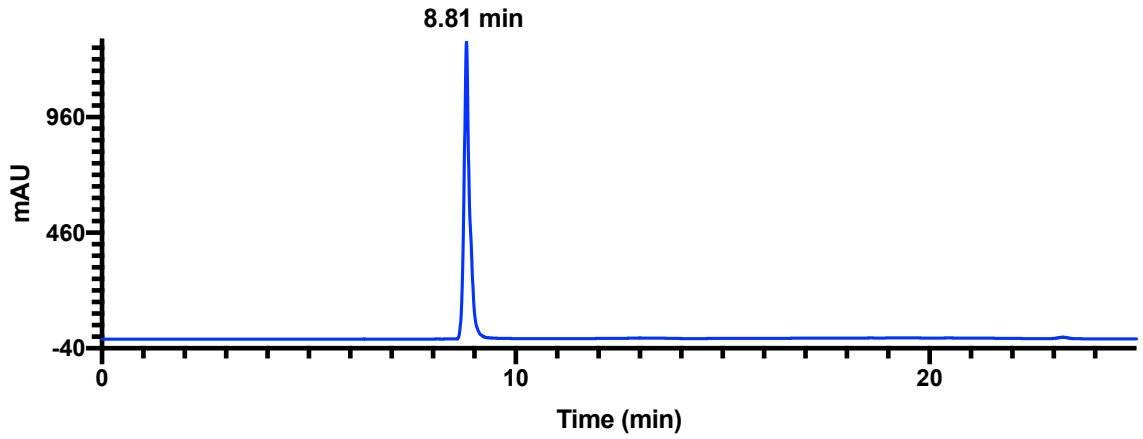


Figure S43: Mass spectrum of scrambled 20-nucleobase thyclotide 8

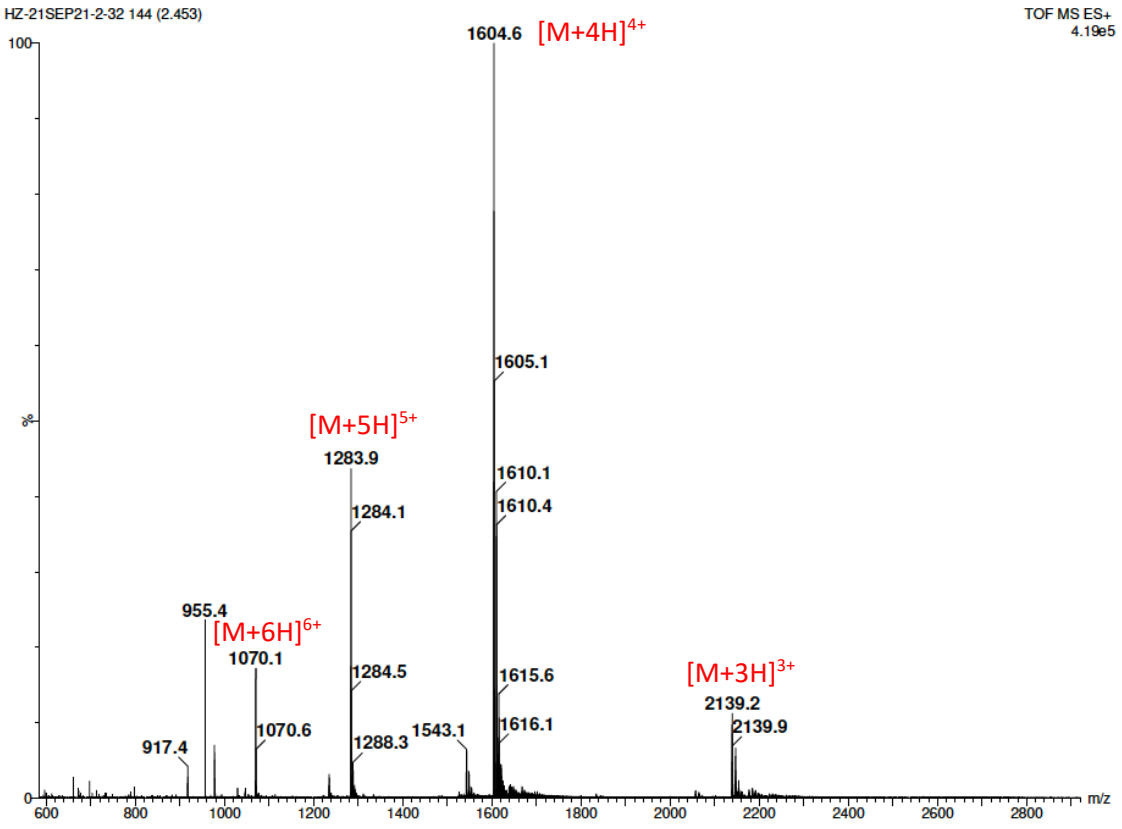


Figure S44: HPLC chromatogram of antimiR-21 20-nucleobase PNA 9

NH_2-AACATCAGTCTGATAAGCTA-AEEA-$CONH_2$

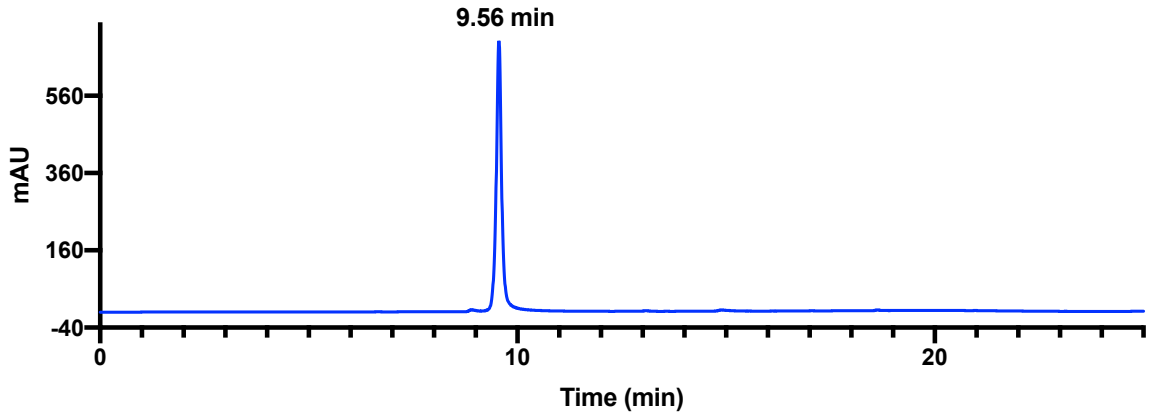


Figure S45: Mass spectrum of antimiR-21 20-nucleobase PNA 9

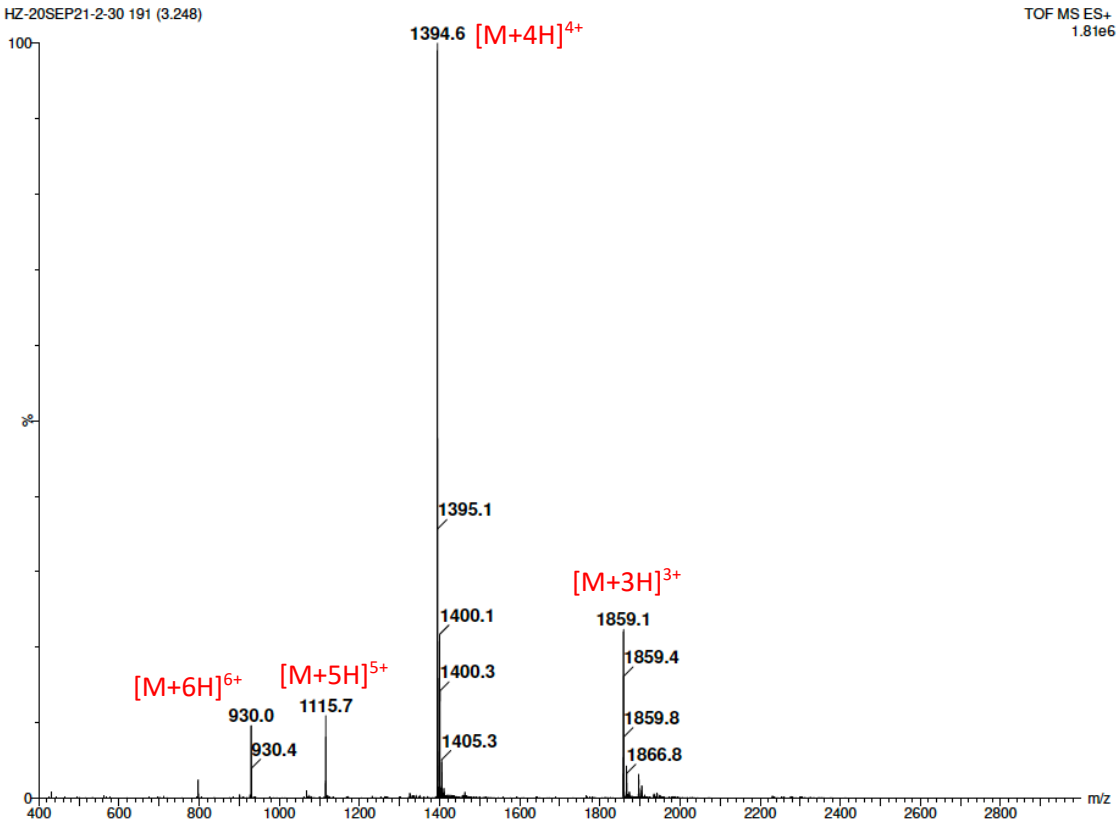


Figure S46: HPLC chromatogram of antimiR-21 20-nucleobase thyclotide 10

NH_2-$A_{thf}A_{thf}C_{thf}A_{thf}T_{thf}C_{thf}A_{thf}G_{thf}T_{thf}C_{thf}T_{thf}G_{thf}A_{thf}T_{thf}A_{thf}A_{thf}G_{thf}C_{thf}T_{thf}A_{thf}$-AEEA-$CONH_2$

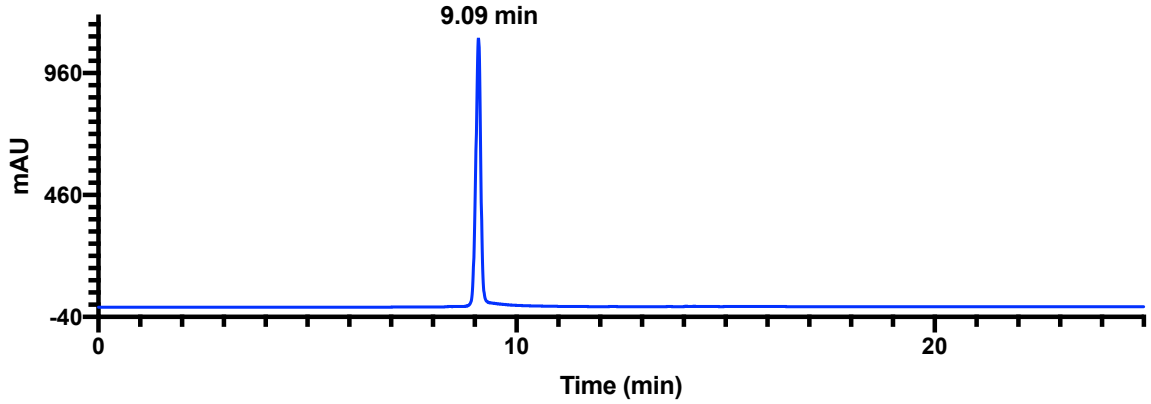


Figure S47: Mass spectrum of antimiR-21 20-nucleobase thyclotide 10

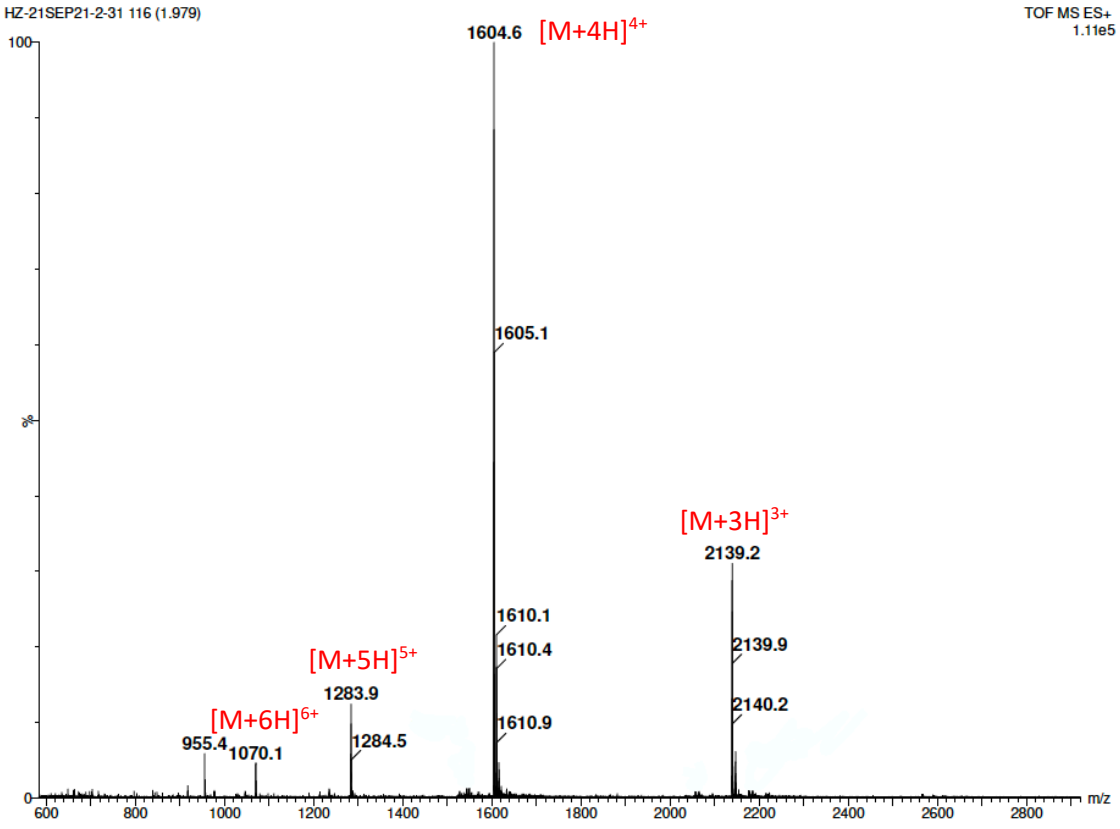


Figure S48: HPLC chromatogram of antimiR-21 20-nucleobase Lys-PNA 11

NH2-Lys-AACATCAGTCTGATAAGCTA-Lys-CONH2

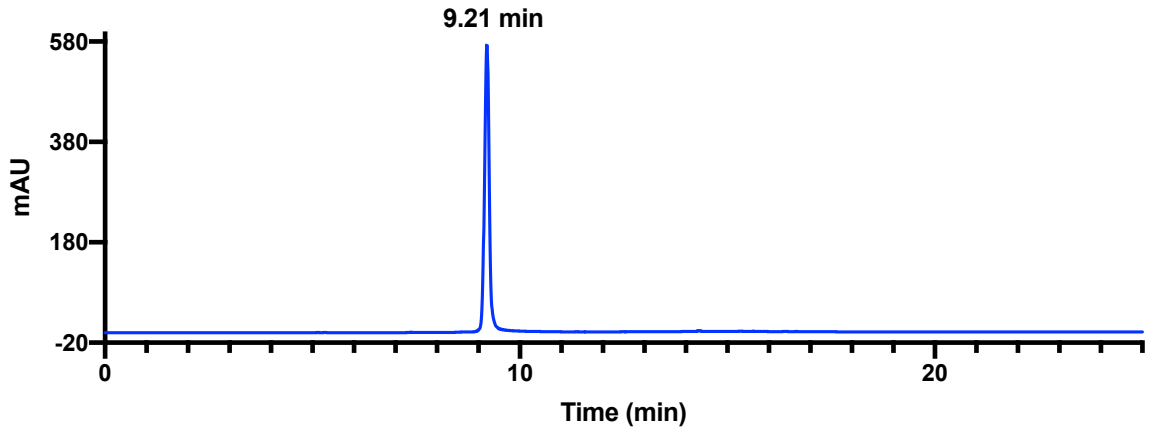


Figure S49: Mass spectrum of antimiR-21 20-nucleobase Lys-PNA 11

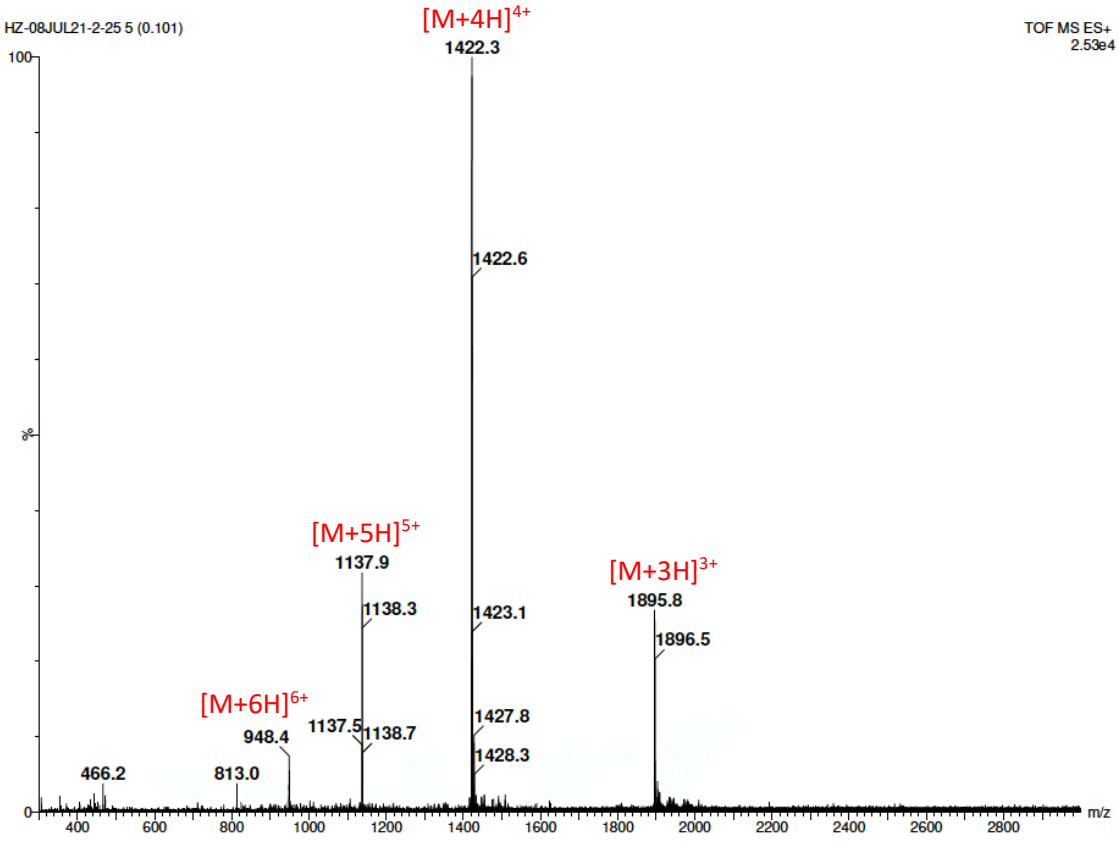


Figure S50: HPLC chromatogram of antimiR-21 20-nucleobase Lys-thyclotide 12

NH_2-Lys-$A_{thf}A_{thf}C_{thf}A_{thf}T_{thf}C_{thf}A_{thf}G_{thf}T_{thf}C_{thf}T_{thf}G_{thf}A_{thf}T_{thf}A_{thf}A_{thf}G_{thf}C_{thf}T_{thf}A_{thf}$-Lys-$CONH_2$

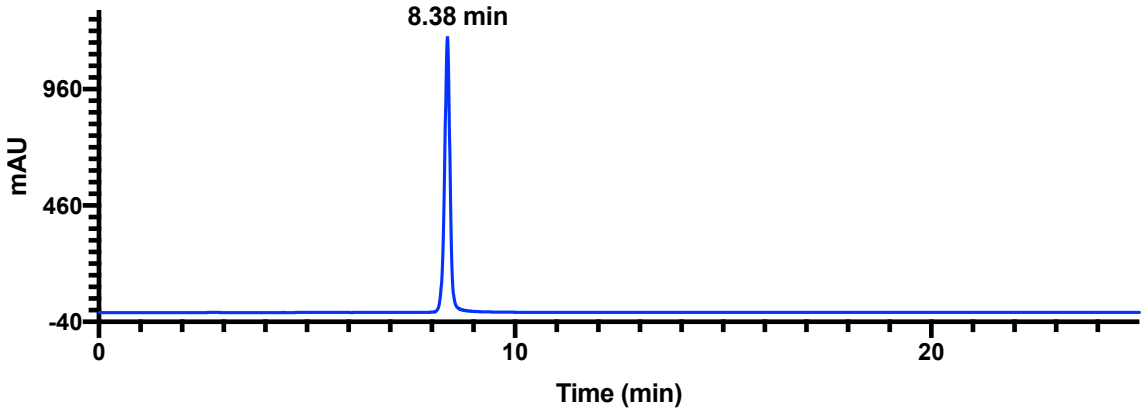


Figure S51: Mass spectrum of antimiR-21 20-nucleobase Lys-thyclotide 12

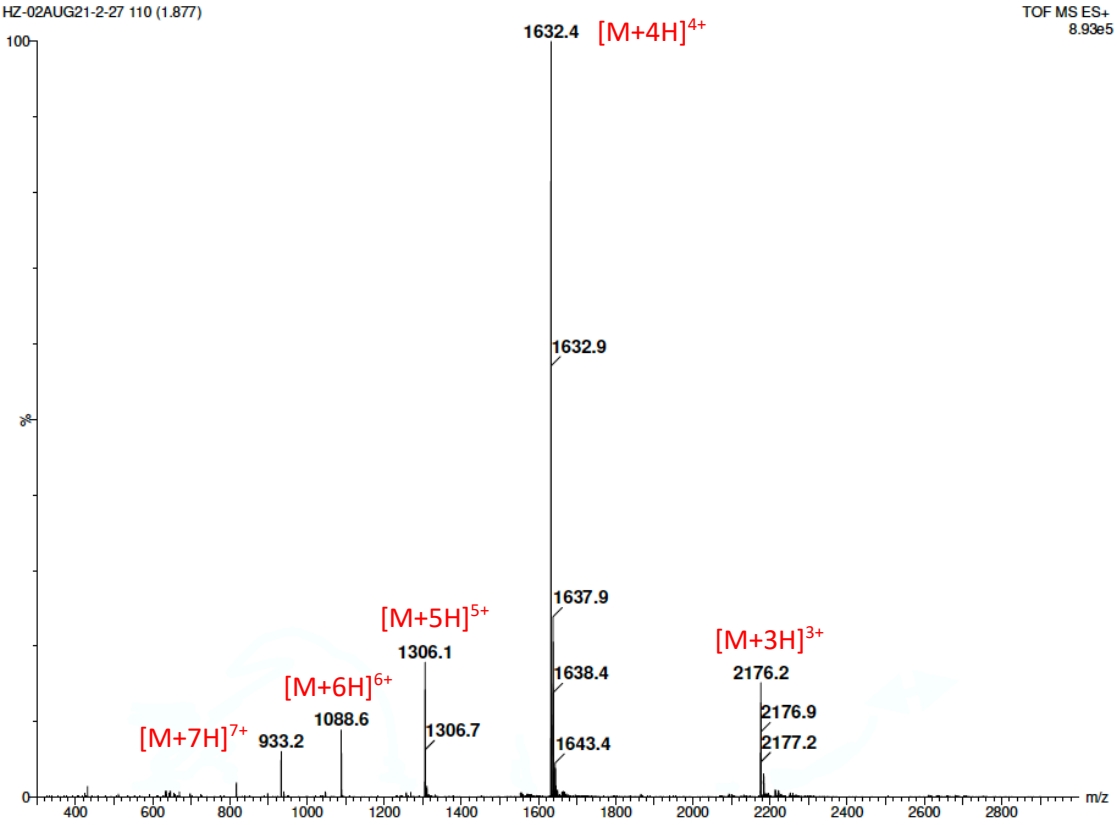


Figure S52: HPLC chromatogram of scrambled 20-nucleobase Lys-thyclotide 13

NH_2-Lys-$G_{thf}T_{thf}A_{thf}C_{thf}A_{thf}T_{thf}T_{thf}A_{thf}C_{thf}G_{thf}A_{thf}C_{thf}G_{thf}A_{thf}T_{thf}A_{thf}C_{thf}A_{thf}A_{thf}T_{thf}$-Lys-$CONH_2$

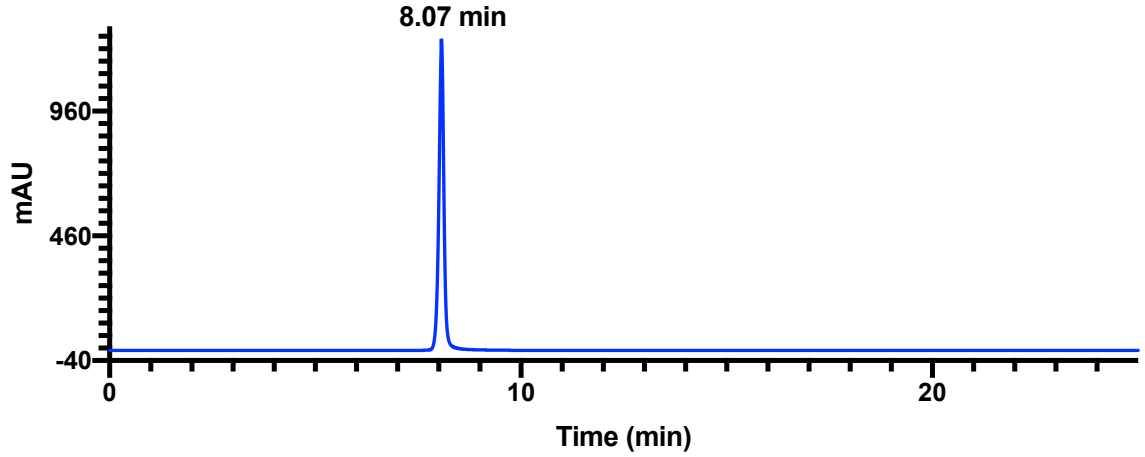


Figure S53: Mass spectrum of scrambled 20-nucleobase Lys-thyclotide 13

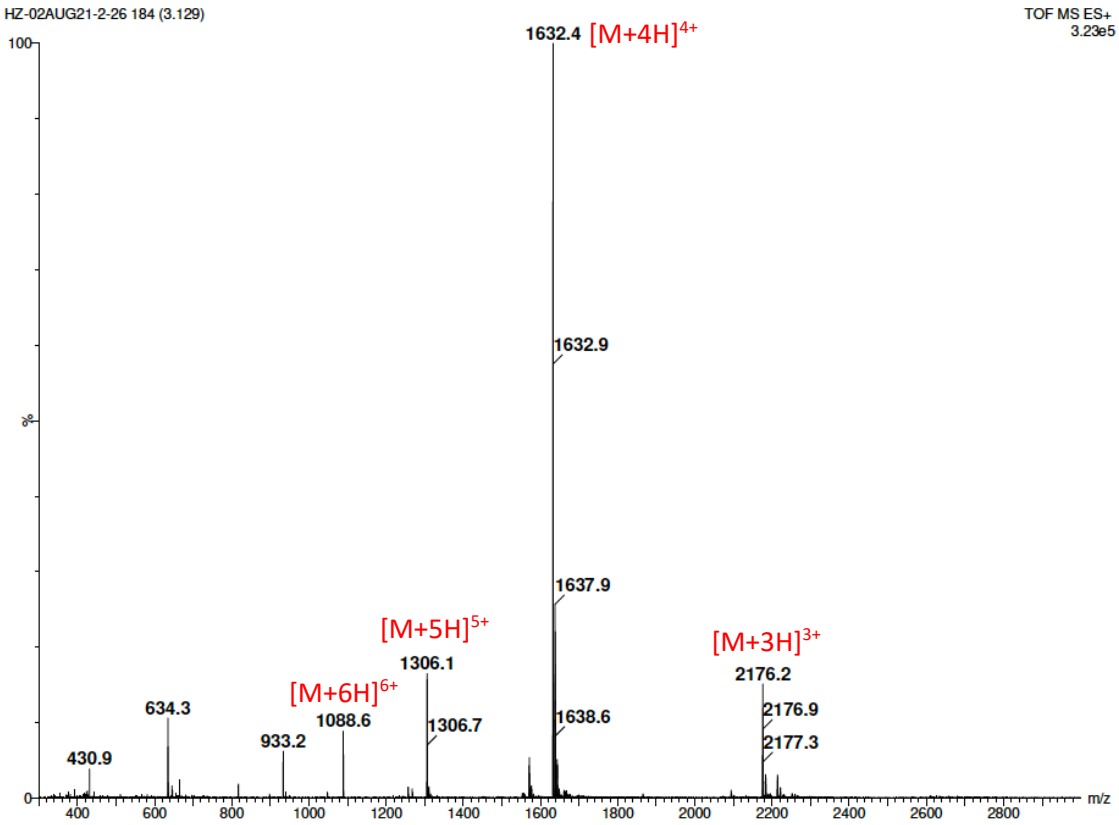


Figure S54: HPLC chromatogram of FAM-antimiR-21 20-nucleobase Lys-PNA 14

FAM-AEEA-AEEA-Lys-AACATCAGTCTGATAAGCTA-Lys-$CONH_2$

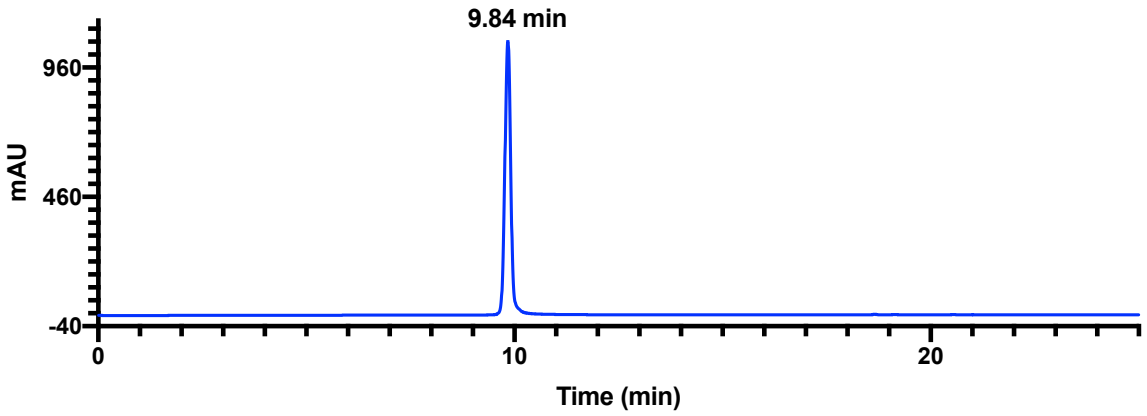


Figure S55: Mass spectrum of FAM-antimiR-21 20-nucleobase Lys-PNA 14

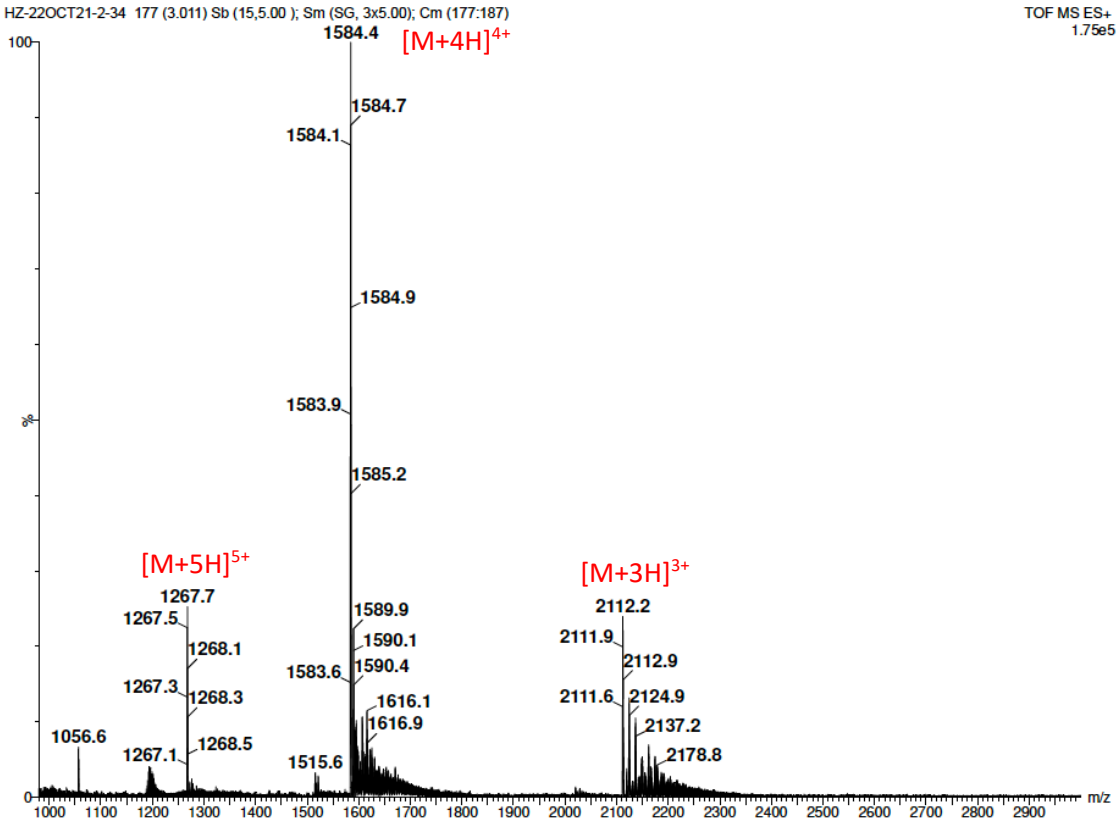


Figure S56: HPLC chromatogram of FAM-antimiR-21 20-nucleobase Lys-thyclotide 15

FAM-AEEA-AEEA-Lys-$A_{thf}A_{thf}C_{thf}A_{thf}T_{thf}C_{thf}A_{thf}G_{thf}T_{thf}C_{thf}T_{thf}G_{thf}A_{thf}T_{thf}A_{thf}A_{thf}G_{thf}C_{thf}T_{thf}A_{thf}$-Lys-$CONH_2$

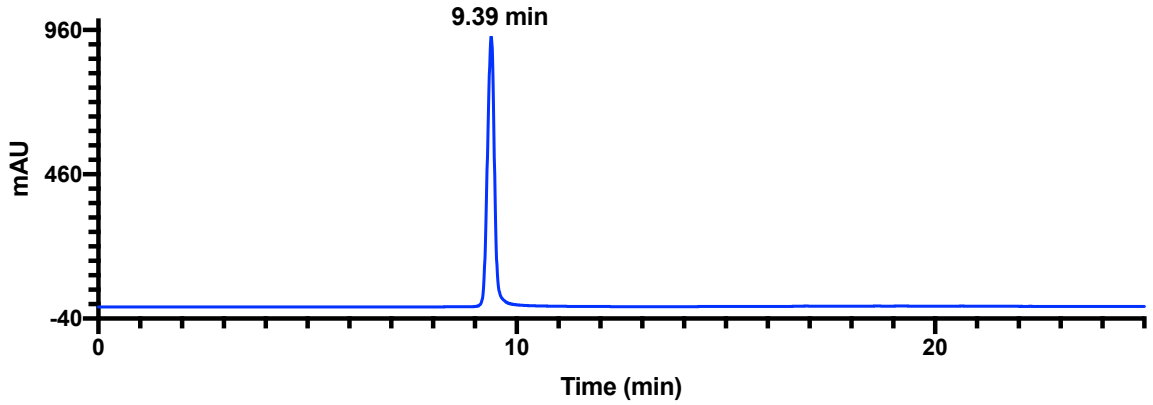


Figure S57: Mass spectrum of FAM-antimiR-21 20-nucleobase Lys-thyclotide 15

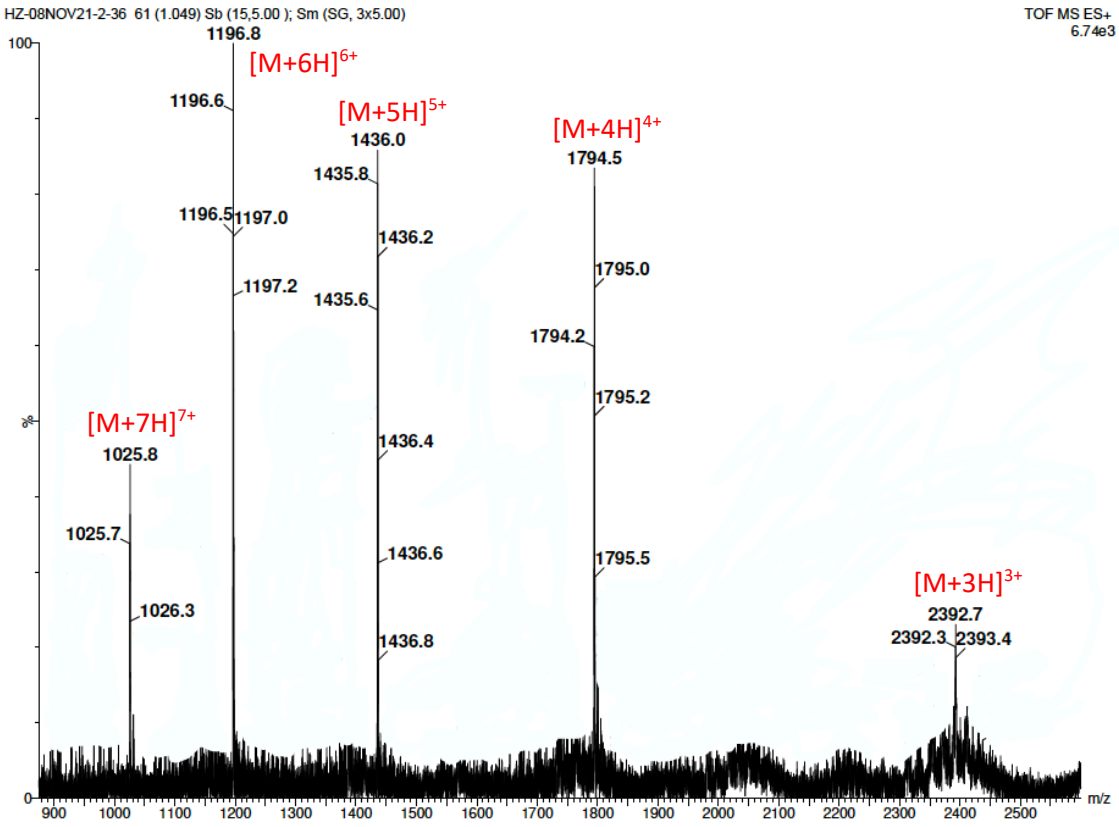


Figure S58: HPLC chromatogram of FAM-scrambled 20-nucleobase Lys-thyclotide 16

FAM-AEEA-AEEA-Lys-$G_{thf}T_{thf}A_{thf}C_{thf}A_{thf}T_{thf}T_{thf}A_{thf}C_{thf}G_{thf}A_{thf}C_{thf}G_{thf}A_{thf}T_{thf}A_{thf}C_{thf}A_{thf}A_{thf}T_{thf}$-Lys-$CONH_2$

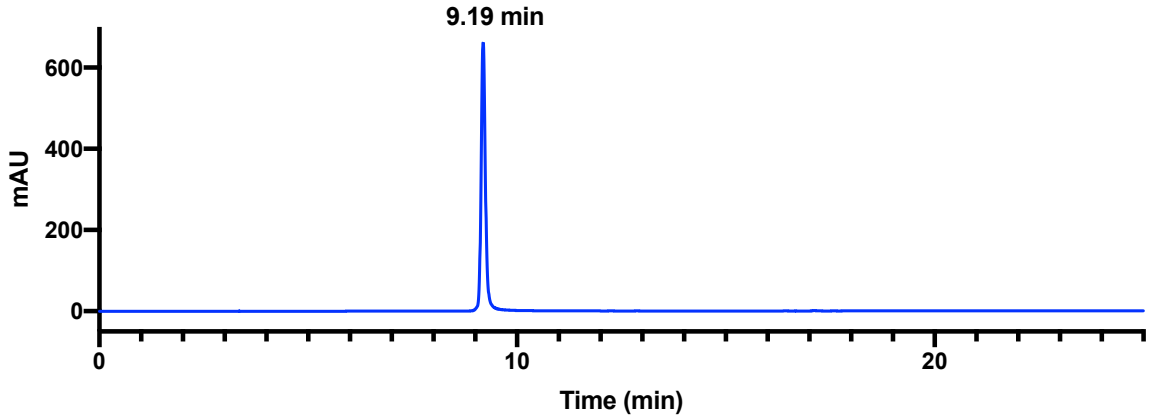


Figure S59: Mass spectrum of FAM-scrambled 20-nucleobase Lys-thyclotide 16

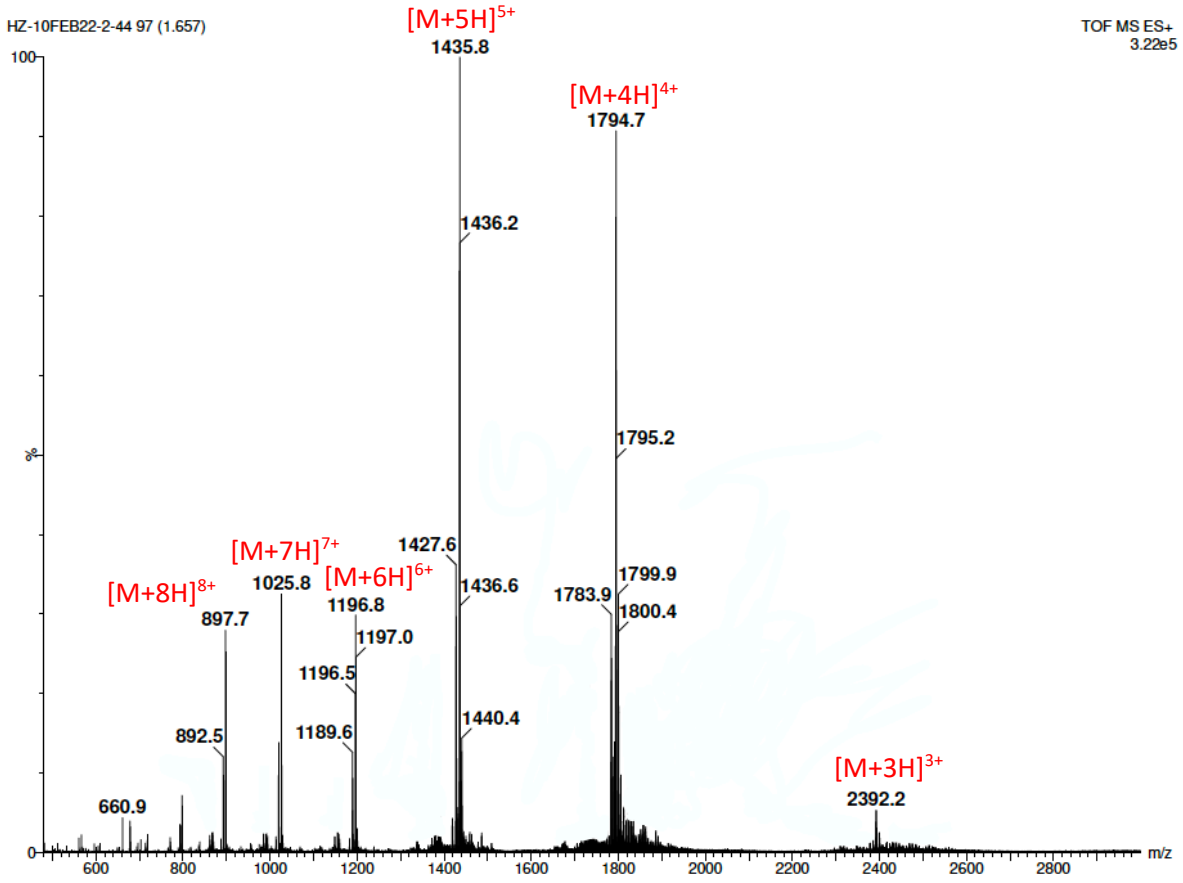


Figure S60: Full structure of antimiR-21 Lys-thyclotide 12

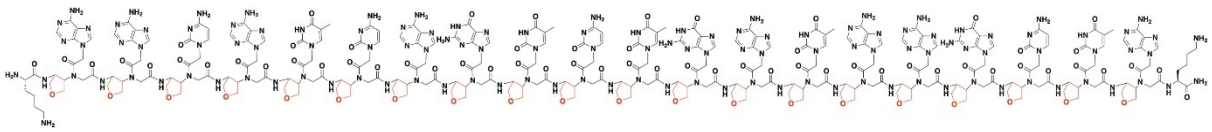


Figure S61: Thyclotide 12 increases mRNA and protein levels of miR-21 downstream targets KRIT1 and Cdc25a. (**A**) RT-qPCR of Cdc25a and KRIT1 mRNA. Cells were treated with 25 nM of scrambled thyclotide **13** or antimiR-21 thyclotide **12**. Total RNA was isolated and Cdc25a and KRIT1 mRNA levels were assessed and normalized to β-actin control expression. (**B, C**) Western Blot for detection of Cdc25a and KRIT1 proteins. Samples were treated with either 25 nM of scrambled thyclotide **13** or 25 nM of antimiR-21 thyclotide **12**. For both blots, densitometry analysis was performed with the software ImageJ. In the figure, 'scr' refers to scrambled thyclotide **13** and 'antimiR' to antimiR-21 thyclotide **12**.

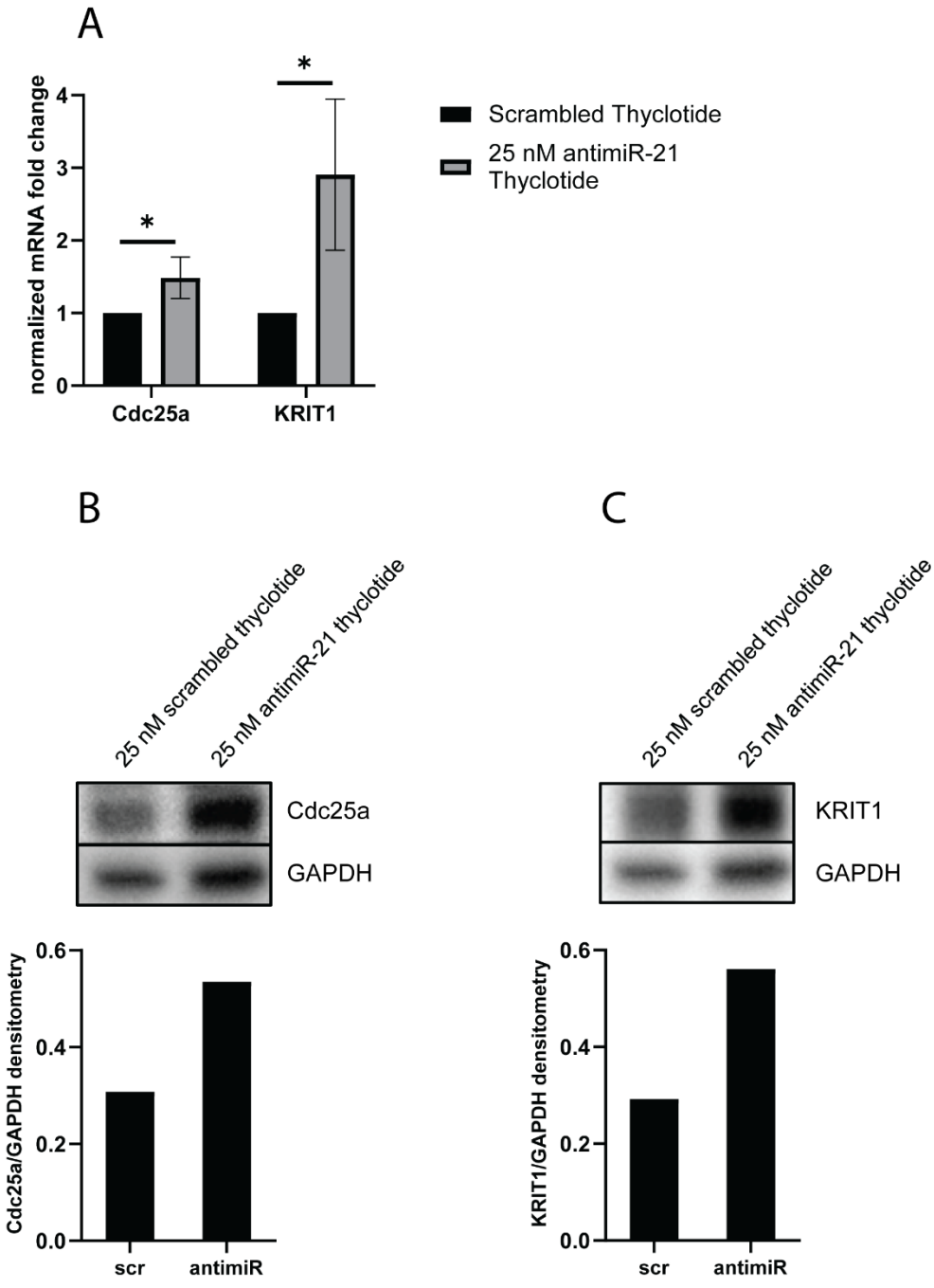


Figure S62: FACS of SKHEP1 cells either non-treated (blue) or treated for 3 hours with scrambled 20-mer thyclotide **14** (red) conjugated to fluorescein.

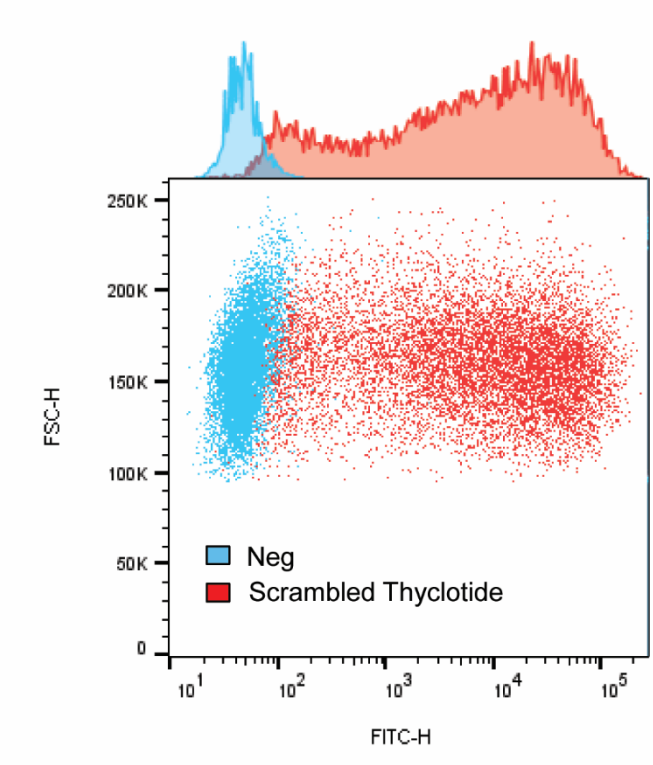


Table S1: List and mass characterization data of splicing thyclotides and *aeg*PNA oligomers. Tetrahydrofuran residues are represented by the symbol * in the sequences. Tetrahydrofuran stereochemistry is (R,R). The data for 15-mer PNAs or thyclotides in this table correspond to the triply charged ion $[M+3H]^{3+}$. FAM = 5/6-fluorescein, AEEA = 2-(2-aminoethoxy)ethoxyacetyl group.

Entry	PNA or Thyclotide	Calculated	Observed
17 MDM2 splicing FAM-*aeg*PNA	FAM-AEEA-AEEA-TGCACATTTGCCTAC-AEEA-$CONH_2$	1603.0	1603.0
18 MDM2 splicing FAM-thyclotide	FAM-AEEA-AEEA-T*G*C*A*C*A*T*T*T*G*C*C*T*A*C*-AEEA-$CONH_2$	1813.0	1813.0
19 MDM2 splicing *aeg*PNA	NH_2-TGCACATTTGCCTAC-AEEA-$CONH_2$	1386.6	1386.9
20 Scrambled splicing thyclotide	NH_2-G*A*G*T*C*T*T*A*T*C*T*T*A*T*C*-AEEA-$CONH_2$	1606.9	1607.0
21 MDM2 splicing thyclotide	NH_2-T*G*C*A*C*A*T*T*T*G*C*C*T*A*C*-AEEA-$CONH_2$	1596.9	1597.0

Figure S63: HPLC chromatogram of MDM2 splicing FAM-*aeg*PNA 17

FAM-AEEA-AEEA-TGCACATTTGCCTAC-AEEA-$CONH_2$

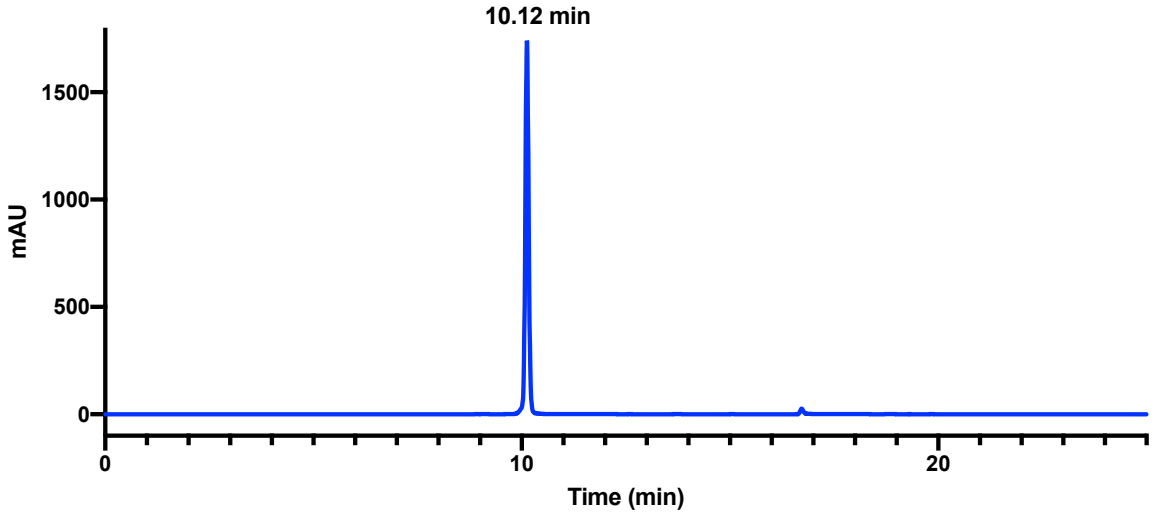


Figure S64: Mass spectrum of MDM2 splicing FAM-*aeg*PNA 17

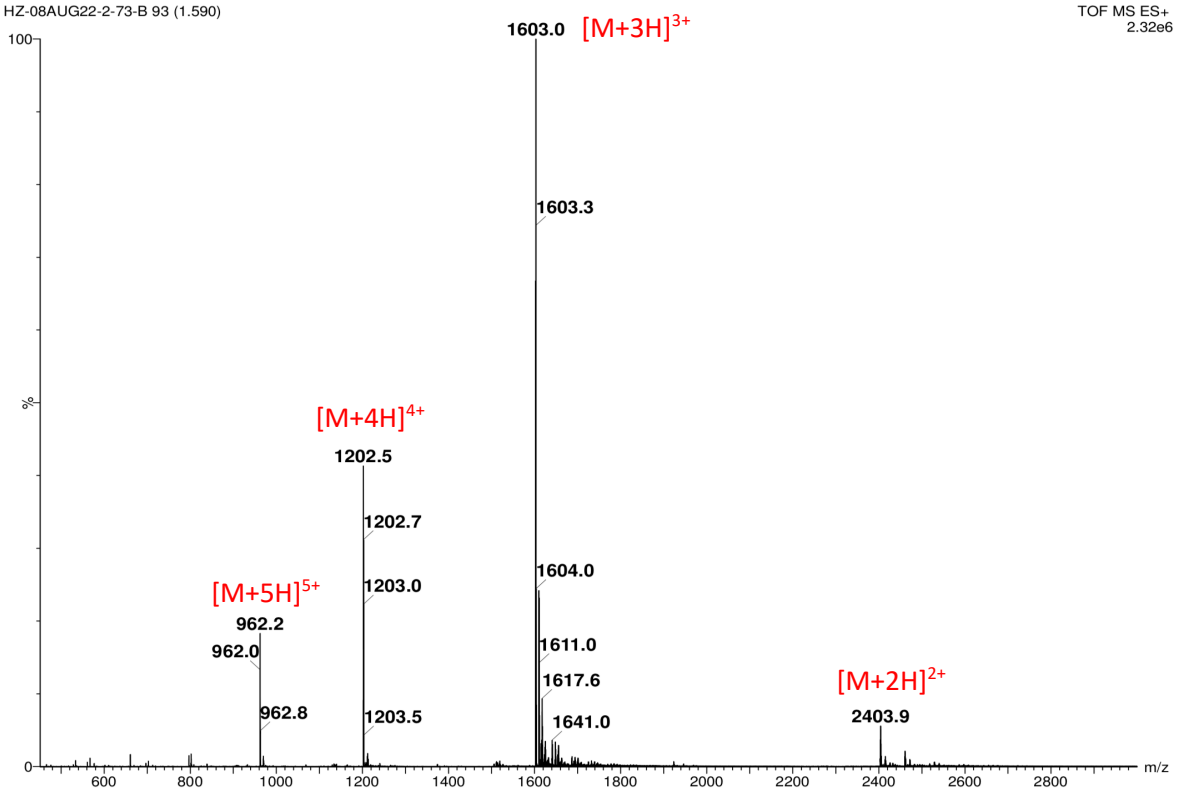


Figure S65: HPLC chromatogram of MDM2 splicing FAM-thyclotide 18

FAM-AEEA-AEEA-$T_{thf}G_{thf}C_{thf}A_{thf}C_{thf}A_{thf}T_{thf}T_{thf}T_{thf}G_{thf}C_{thf}C_{thf}T_{thf}A_{thf}C_{thf}$-AEEA-$CONH_2$

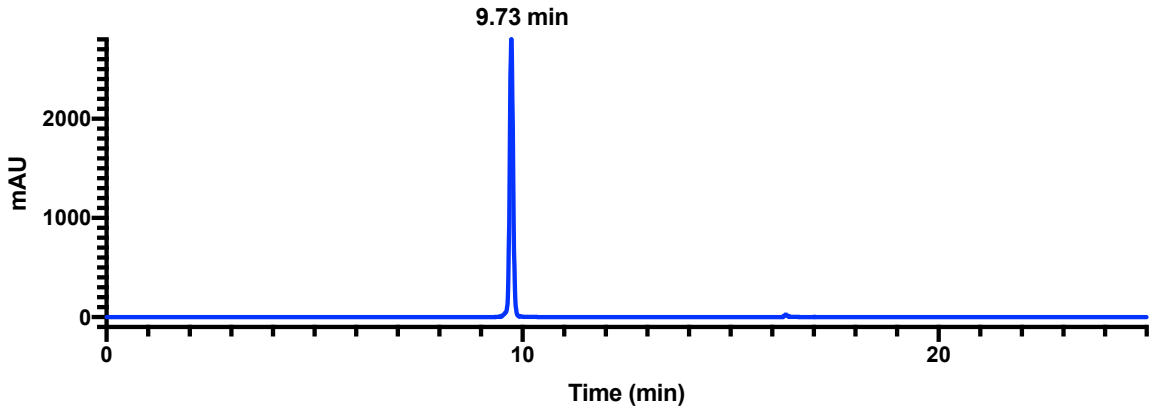


Figure S66: Mass spectrum of MDM2 splicing FAM-thyclotide 18

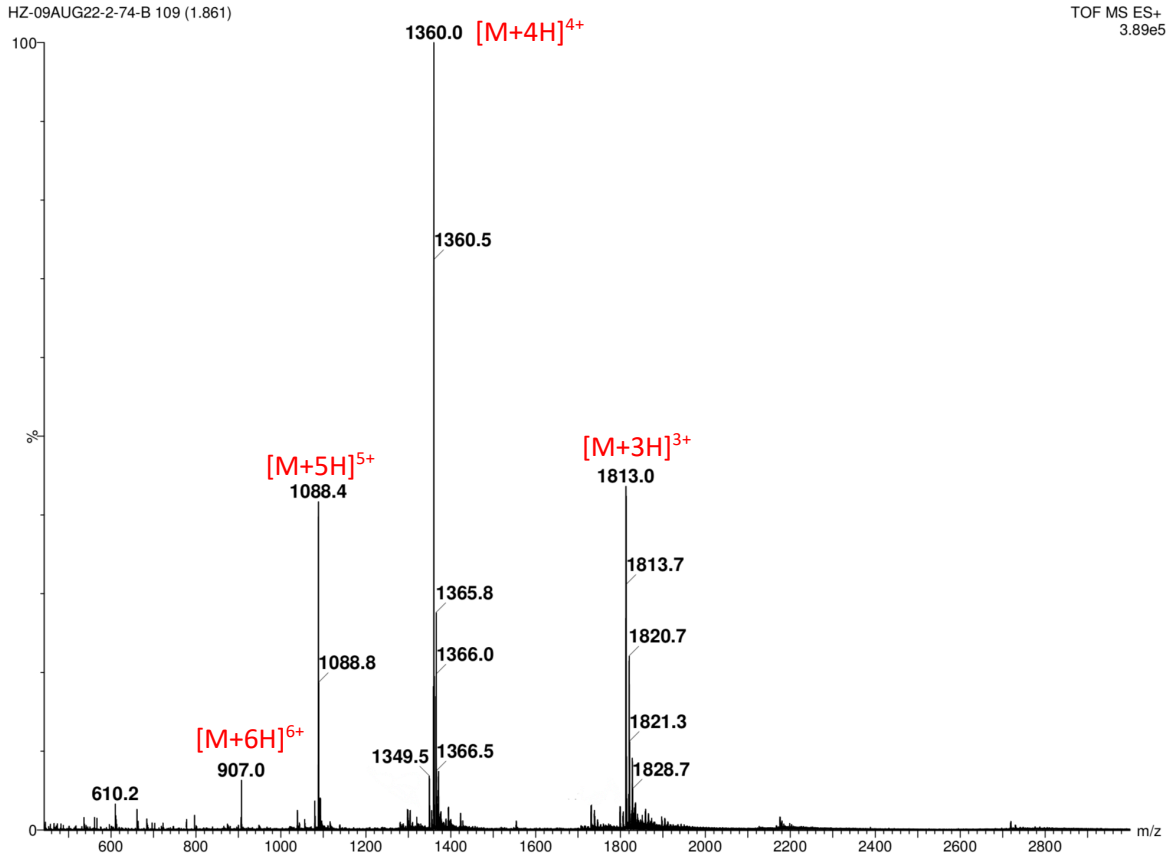


Figure S67: HPLC chromatogram of MDM2 splicing *aeg*PNA 19

NH_2-TGCACATTTGCCTAC-AEEA-$CONH_2$

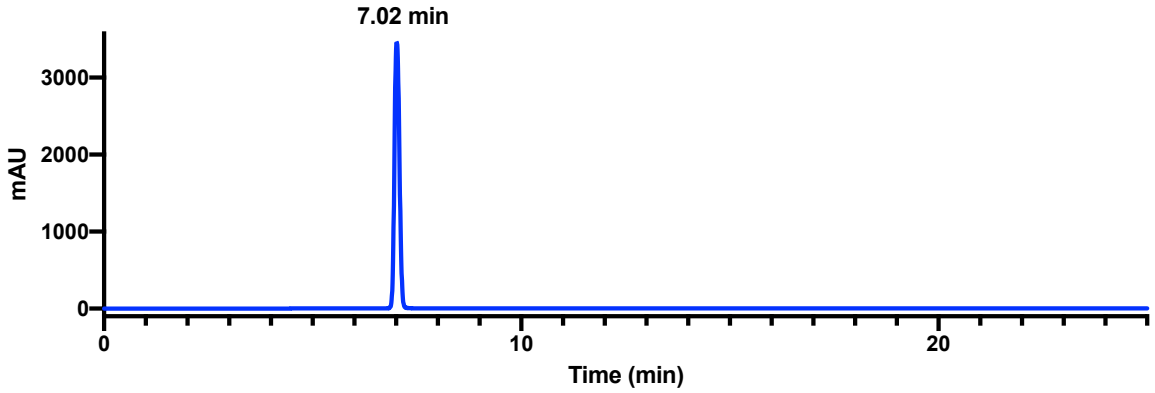


Figure S68: Mass spectrum of MDM2 splicing *aeg*PNA 19

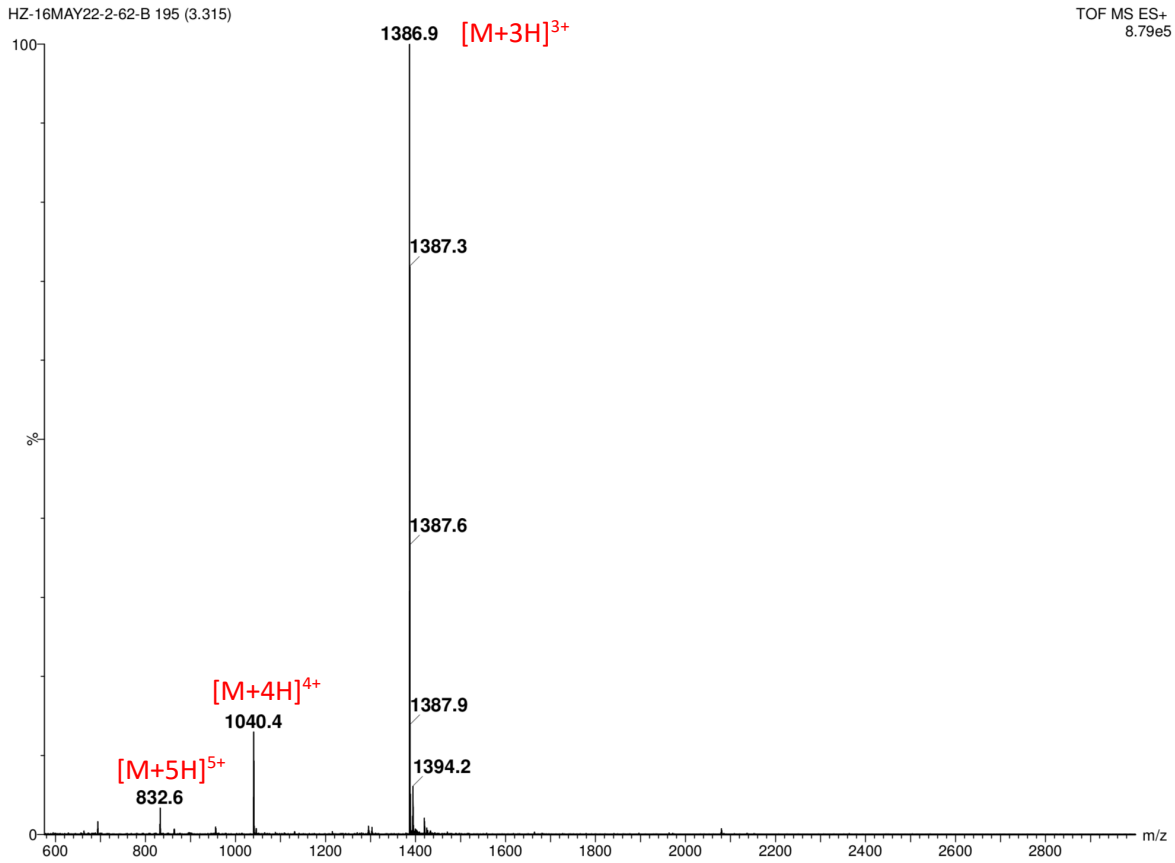


Figure S69: HPLC chromatogram of scrambled splicing thyclotide 20

NH_2-$G_{thf}A_{thf}G_{thf}T_{thf}C_{thf}T_{thf}T_{thf}A_{thf}T_{thf}C_{thf}T_{thf}T_{thf}A_{thf}T_{thf}C_{thf}$-AEEA-$CONH_2$

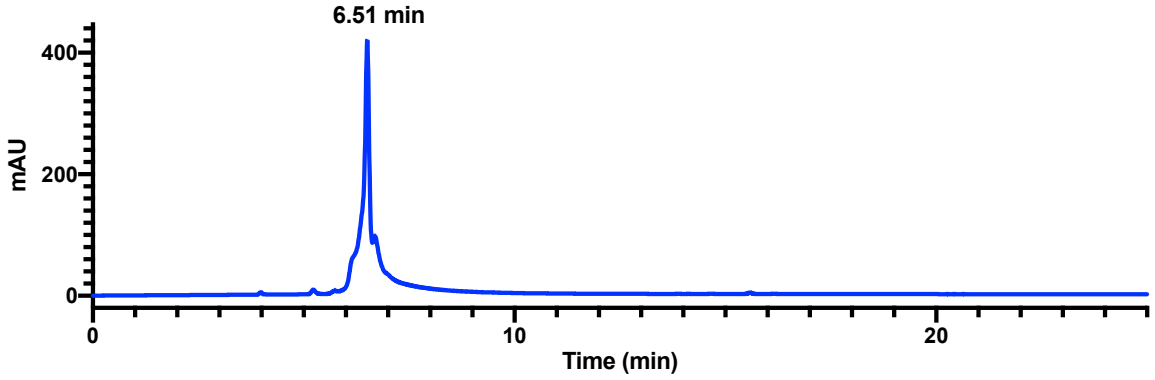


Figure S70: Mass spectrum of scrambled splicing thyclotide 20

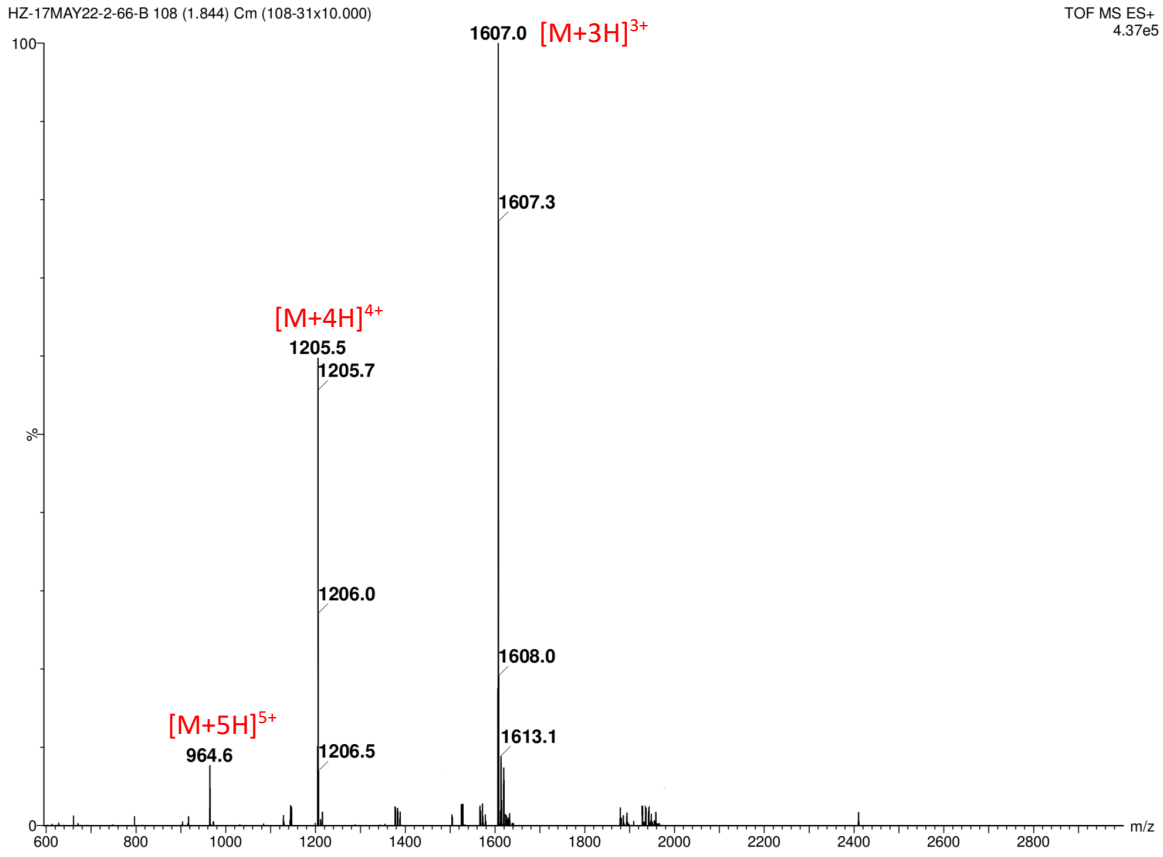


Figure S71: HPLC chromatogram of MDM2 splicing thyclotide 21

NH_2-$T_{thf}G_{thf}C_{thf}A_{thf}C_{thf}A_{thf}T_{thf}T_{thf}T_{thf}G_{thf}C_{thf}C_{thf}T_{thf}A_{thf}C_{thf}$-AEEA-$CONH_2$

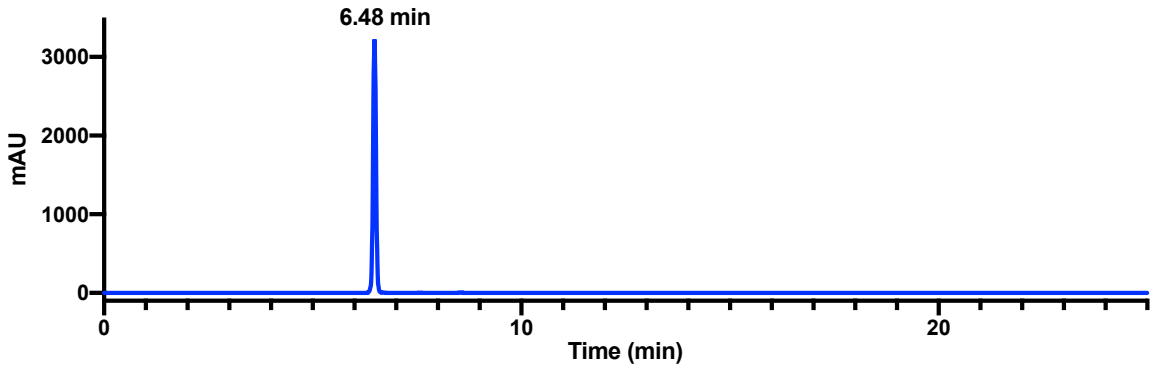
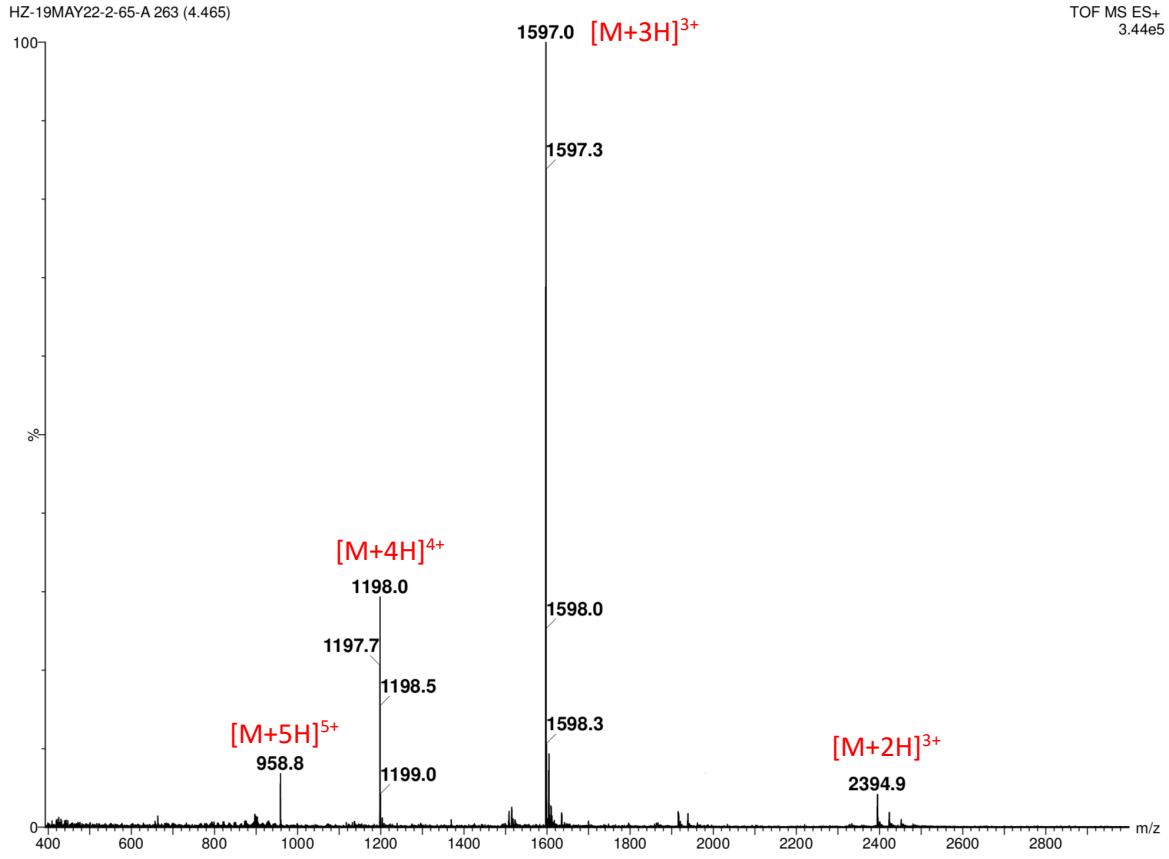


Figure S72: Mass spectrum of MDM2 splicing thyclotide 21



Supplementary method for splicing experiments

Effect of thyclotide 21 on the splicing of intron 2 of MDM2 pre-mRNA:

SKHEP1 and HepG2 cells were either non treated, treated with 5 µM of scrambled thyclotide **20**, with 5 µM of splicing *aeg*PNA **19,** or with 5 µM of splicing thyclotide **21**. 24 hours after treatment, cells were collected, and total RNA was extracted with Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). All RNA samples were treated with RNaseOUT recombinant ribonuclease inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) and were stored at -80°C following extraction. For β-actin and MDM2 intron 2 PCRs, the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and the Platinum SuperFi II Green PCR Master Mix were used (Invitrogen, Carlsbad, CA, USA). The primers (5' to 3') for β-actin were CCCTGGAGAAGAGCTACGAG (forward) and ATGCCAGGGTACATGGTGGT (reverse). The primers for MDM2 pre-mRNA were CGATTGGAGGGTAGACCTGT (forward) and CACGATGAAAACTGGAAATCA (reverse). PCR products were analyzed on a 2% agarose gel with a low molecular weight DNA ladder (New England Biolabs, Ipswich, MA, USA) and imaged using the ChemiDoc XRS+ Imaging System (BioRad, Hercules, CA, USA).

Figure S73: Thyclotide's sequence is critical for cell uptake efficiency. (**A**) FACS of SKHEP1 and HepG2 cells treated with 5 µM of either MDM2 splicing *aeg*PNA **17** (blue) or MDM2 splicing thyclotide **18** (red). (**B**) Agarose gel of PCR products of β-actin or Intron 2 splicing variant. SKHEP1 or HepG2 cells were either non treated (1), treated with 5 µM of scrambled thyclotide **20** (2), with 5 µM of splicing *aeg*PNA **19** (3) or with 5 µM of splicing thyclotide **21** (4). (**C**) FACS of SKHEP1 and HepG2 cells treated with 5 µM of either MDM2 splicing thyclotide **18** (blue) or 14-mer antimir-21 thyclotide **5** (red).