Synthesis of Oligoribonucleic Acid Conjugates Using a Cyclooctyne Phosphoramidite

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General methods and materials

Chemicals were purchased from Acros Organics, Sigma Aldrich and Nova Biochem and used as received. Dichloromethane was distilled over CaH_2 and stored on 4 Å molecular sieves. DIPEA was distilled and stored on KOH pellets. Compounds used in reactions requiring anhydrous conditions were co-evaporated with 1,4-dioxane, pyridine or toluene three times. All reactions were performed at ambient temperature under an argon atmosphere unless stated otherwise. Peptide synthesis was performed on a Applied Biosystems 433A peptide synthesizer. Oligonucleotides were synthesized on an ÄKTA Oligopilot Plus oligonucleotide synthesizer (GE Healthcare Life Sciences).

Reactions were monitored by TLC on Kieselgel 60 F254 (Merck). Compounds were visualized by using UV light (254 nm) or applying a solution of $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ 25 g/L, $(NH_4)_4Ce(SO_4)_4\cdot 2\ H_2O$ 10 g/L, 10% H_2SO_4 in H_2O followed by charring (+/- 150 ^oC). LC/MS analysis was performed on a Jasco HPLC system (UV detection simultaneously at 214 and 254 nm) coupled to a PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer). Alternatively a Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan) was used. An analytical Gemini C_{18} column (Phenomex, 50 x 4.60 mm, 3 micron) or ReproSil-Pur C_{18}-Aq (Dr. Maisch, 150 x 4.6 mm, 5 micron) was used in combination with eluents A: H_2O; B: MeCN and C 0.1 M aq. NH_4OAc or D: 1% aq. TFA as the solvent system. Analytical anion-exchange was performed on a GE ÄKTAexplorer 10 using a Dionex DNA-PAC PA-200 4x250 mm column with eluents A: 50 mM NaOAc and 50 mM $NaClO_4$ and B: 500 mM NaOAc and 500 mM $NaClO_4$ using a linear gradient (0 - 20%). Anion exchange purification was performed on a GE ÄKTAexplorer 10 using a GE Q-sepharose HR 26 x 10 column with eluents A: 50 mM NaOAc and 50 mM $NaClO_4$ and B: 500 mM NaOAc and 500 mM $NaClO_4$ followed by a desalting procedure using a sephadex G25 column with 150 mM NH_4OAc as the solvent system. RP HPLC was performed on a Gilson GX-281 HPLC system. A preparative Gemini C_{18} column (Phenomex, 150 x 21.2 mm, 5 micron) or Platinum EPS C_{18} (Grace, 150 x 10 mm, 5 micron) was used in combination with eluents A: 0.1 aq TFA or 10 mM aq. NH_4OAc and B: MeCN as the solvent system. ^1H, ^{13}C and ^{31}P NMR were recorded on a Bruker AV-400 instrument. Chemical shifts (δ) of ^1H and

^{13}C spectra are relative to tetramethylsilane. ^{31}P chemical shifts are relative to phosphoric acid. $CDCl_3$ was neutralized by filtration over neutral Al_2O_3 (Merck). HRMS spectra were recorded by direct injection (2 μL of a μM solution in H_2O or MeCN and 0.1% formic acid) on a Thermo Finnigan LTQ Orbitrap equipped with a electro spray ion source in positive mode. IR spectra were recorded on a Shimadzu FT-IR 8300 and are reported in cm^{-1}. The yields of the oligonucleotides and the conjugates were determined specrophotometrically using Optical Density measurements on a Varian Cary 50 Bio UV-VIS Spectrophotometer at 260 nm.

Carbonic acid 7,8-didehydro-1,2:5,6-dibenzocyclooctene-3-yl ester (6'-((*tert*-butyldiphenylsilyl)oxy) hexyl)-1'-amide (3)

To a solution of alcohol **1** (882 mg, 4 mmol) in dichloromethane (120 mL) were added pyridine (1.6 mL, 20 mmol, 5 eq) and p-nitrophenyl chloroformiate (1.6 g, 8 mmol, 2 eq). The reaction mixture was stirred overnight obtaining a pink slurry. The reaction mixture was quenched with brine (25 mL), transferred into a separatory funnel, the organic layer was washed with brine (25 mL), dried ($MgSO_4$) and concentrated under reduced pressure. To a solution of crude p-nitrophenyl carbonate in DMF (30 mL) was added dropwise a solution of 6-(*tert*-butyldiphenylsilyloxy)-hexan-1-amine (2.84 g, 8 mmol, 2 eq) and triethylamine (4.4 mL, 16 mmol, 4 eq) in DMF (17 mL). The reaction mixture was stirred for 20 hrs. The reaction mixture was concentrated under reduced pressure and the residue purified by silica gel column chromatography (petroleum ether / ethyl acetate 85/15, v/v) to afford protected **3** (2.37 g, 3.9 mmol, 97%).

^{1}H NMR (400 MHz, CDCl3) δ = 7.71 – 7.63 (m, 4H), 7.52 – 7.45 (m, 1H), 7.44 – 7.21 (m, 15H), 5.49 (s, 1H), 4.93 (s, 1H), 3.71 – 3.58 (m, 2H), 3.24 – 3.09 (m, 2H), 2.97 – 2.81 (m, 1H), 1.68 – 1.24 (m, 8H), 1.05 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 155.3, 152.2, 151.0, 135.5, 134.0, 129.9, 129.5, 128.0, 127.8, 127.6, 127.0, 126.2, 125.9, 123.8, 123.7, 121.3, 112.9, 109.9, 76.6, 63.7, 46.2, 41.1, 32.4, 30.0, 26.9, 26.5, 25.5, 19.2.
IR cm^{-1}: 3341.9; 2931.6; 2857.2; 1699.7; 1427.9; 1238.2; 1105.4; 755.0. HRMS: calculated for $[C_{39}H_{43}NO_3Si + Na]^+$: 624.29044; $[C_{39}H_{43}NO_3Si + NH_4]^+$: 619.33505 found: 624.29005 $[M + Na]^+$; 619.33523 $[M + NH_4]^+$

Carbonic acid 7,8-didehydro-1,2:5,6-dibenzocyclooctene-3-yl ester, ((6'-hydroxy)hexyl)-1'-amide (4)

To a solution of **3** (0.6 g, 1 mmol) in THF (9 mL) was added TBAF (1.6 mL, 1 M solution in THF, 1.6 eq). After stirring for 5 hours the reaction mixture was quenched upon addition of H_2O (10 mL), diluted with dichloromethane (50 mL) and washed with H_2O (2 x 10 mL). The aqueous layers were extracted with dichloromethane (10 mL), the combined organic layers were washed with brine (10 mL) dried (Na_2SO_4) and

concentrated under reduced pressure. Silica gel column chromatography (petroleum ether / ethyl acetate, 70 : 30 → 25 : 75) afforded the title compound **4** (0.29 g, 0.79 mmol, 79%).

^{1}H NMR (400 MHz, CDCl3) δ = 7.54 (d, *J* = 8.0, 1H) 7.38 – 7.25 (m, 8H), 5.41 (bs, 1H), 3.52 (m, 2H), 3.18 (d, *J* = 15.2 Hz, 1H), 3.09 (m, 2H), 2.82 – 2.77 (m, 4H), 1.36 – 1.35 (m, 4H). ^{13}C NMR (101 MHz, MeOD) δ 157.9, 153.6, 152.4, 130.9, 123.0, 129.2, 129.1, 128.2, 128.1, 127.1, 126.8, 124.9, 124.9, 122.4, 113.8, 111.0, 77.7, 62.8, 47.2, 41.9, 41.7, 33.5, 30.8, 27.6, 26.5. IR cm^{-1}: 3321.9; 3062.2; 2932.9; 2858.4; 2362.2; 1694.6; 1253.3; 1026.8. HRMS: calculated for $[C_{23}H_{25}NO_3 + Na]^+$: 386.17268; found: 386.17283 $[M + Na]^+$

Carbonic acid 7,8-didehydro-1,2:5,6-dibenzocyclooctene-3-yl ester, 6'-(2-cyanoethoxy-*N,N*-diisopropylamino)phosphine 1'-amide (5)

To a solution of **4** (0.29 g, 0.79 mmol) in dichloromethane were added DIPEA (265 µL, 1.6 mmol, 2 eq) and 2-cyanoethoxy-*N,N*-diisopropylamino-chlorophosphine (196 µL, 0.88 mmol, 1.1 eq). The reaction mixture was stirred for 6 hours, diluted with ethyl acetate (50 mL) washed with sat. aq. $NaHCO_3$ (3 x 10 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Silica gel column chromatography (toluene / ethyl acetate / triethylamine 69 : 30 : 1) afforded the title compound **5** (0.44 g, 0.78 mmol, 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (d, *J* = 7.6 Hz, 1H), 7.39 – 7.22 (m, 7H), 5.48 (s, 1H), 5.04 (s, 1H), 3.90 – 3.72 (m, 2H), 3.69 – 3.51 (m, 4H), 3.25 – 3.07 (m, 2H), 2.89 (dd, *J* = 15.0, 3.5 Hz, 1H), 2.67 – 2.36 (m, 2H), 1.76 – 1.47 (m, 4H), 1.47 – 1.26 (m, 4H), 1.26 – 1.06 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.4, 152.3, 151.0, 123.0, 129.0, 128.1, 127.9, 127.0, 126.9, 126.2, 125.9, 123.8, 123.8, 121.3, 117.7, 112.9, 110.0, 77.4, 77.1, 76.8, 76.7, 63.6, 63.5, 58.4, 58.2, 46.3, 43.1, 42.9, 41.1, 31.1, 31.1, 30.0, 26.5, 25.7, 24.7, 24.6, 24.6, 20.4, 20.4. ^{31}P NMR (162 MHz, $CDCl_3$) δ 148.8. IR cm^{-1}: 3339.7; 2965.1;

2933.3; 1706.1; 1521.0; 1238.6; 1183.7; 1126.8; 1025.8; 974.9. HRMS: calculated for $[C_{39}H_{43}NO_3Si + H]^+$: 564.29857; $[C_{39}H_{43}NO_3Si + Na]^+$: 586.28051 found: 564.29857 $[M + H]^+$; 586.27982 $[M + Na]^+$

N^{α}-Azidopropionyl penetratin carboxamide (11)

Rink Amide Tentagel (RAPP Polymer, 0.24 mmol/g, 208 mg, 50 μmol) was subjected to solid phase Fmoc peptide synthesis using standard Fmoc protected amino acid building blocks (Nova Biochem, 0.25 mmol, 5 eq), HCTU as the activating agent and Fmoc cleavage as the final step. The resin was transferred into a vial equipped with a filter and containing azidopropionic acid succimidyl ester (64 mg, 0.3 mmol, 6 eq) in NMP (2 mL) and shaken for 6 hours. The resin was washed with NMP and DCM (3 x 5 mL) and shaken in a solution of TFA/H_2O/TIS (95 : 2.5 : 2.5, v/v/v, 3 mL) for 4 hours. The solution was separated from the resin by filtration and transferred into cold Et_2O (4 mL) followed by centrifugation (4400 rpm, 10 minutes). Decantation of the supernatant afforded the crude peptide which was purified by means of RP HPLC (10% → 50% MeCN in 0.1% aq TFA) yielding the title peptide.

LC/MS retention time (10% → 90% MeCN in 0.1% aq TFA, 13.5 min run) 7.16 min; Mass (ESI): m/z 1171.93 $[M + 2H]^{2+}$; 1399.13 $[M + 8\ TFA + 2\ H]^{2+}$. HRMS: calculated for $[C_{107}H_{172}N_{38}O_{20}S + 2H]^{2+}$: 1172.17517; found: 1172.17636 $[M + 2H]^{2+}$.

Cyclooctyne-T_5 (6)

Synthesis was performed on a 1 μmol scale using preloaded CPG. Coupling was performed using 10 eq of commercially available amidites and 15 eq of alkyne amidite **5** at a 0.1 M concentration. 5-mercaptobenzyltetrazole (BMT, 0.3 M) was used as the activating agent in 3 minute coupling cycles. Oxidation and capping were performed by means of standard procedures using I_2/pyridine/H_2O and Ac_2O respectively. Deprotection and cleavage was performed with concentrated aqueous NH_4OH for 1 hour at rt. Concentration and subsequent HPLC purification yielded the target oligo-2'-deoxynucleotide (OD, 0.28 μmol, 28%).

LC/MS: (MeCN :10 mM aq NH_4OAc, 10 → 90 v/v) 4.35 min; ESI-MS: m/z 1884.8 $[M + H]^+$; 943.2 $[M + 2H]^{2+}$.

4,4-Difluoro-1,3-dimethyl-2-(2-(benzylaminocarbonylethyl))-7-(4-(3-azido-propoxy)-phenyl)-4-bora-3a,4a-diaza-s-indacene (7)

Azido-BODIPY-acid (42 mg, 0.09 mmol) was dissolved in DCM. 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (45 mg, 0.18 mmol, 2 eq.) and benzylamine (15 μL, 0.14 mmol, 1.5 eq.) were added and the mixture was stirred for 16 h. Next, the mixture was washed with aq. HCl (pH 3), water and brine, dried ($MgSO_4$) and concentrated in vacuo. Silica column chromatography (toluene: EtOAc 10:1 → 4:1) yielded the title compound as a red solid (48 mg, 0.086 mmol) in 95% yield.

^{1}H NMR ($CDCl_3$, 400 MHz): δ ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, *J* = 8.2 Hz, 2H), 7.29 (t, *J* = 8.9 Hz, 3H), 7.22 (d, *J* = 7.4 Hz, 2H), 7.08 (s, 1H), 6.99 (d, *J* = 8.0 Hz, 3H), 6.57 (d, *J* = 3.9 Hz, 1H), 5.78 (s, 1H), 4.41 (d, *J* = 5.6 Hz, 2H), 4.13 (t, *J* = 5.8 Hz, 2H), 3.56 (t, *J* = 6.6 Hz, 2H), 2.79 (t, *J* = 7.4 Hz, 2H), 2.54 (s, 3H), 2.32 (t, *J* = 7.4 Hz, 2H), 2.19 (s, 3H), 2.10 (p, *J* = 6.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.51, 159.48, 159.28, 155.36, 139.76, 137.98, 135.06, 133.90, 130.77, 130.42, 128.71, 127.93, 127.76, 127.53, 125.74, 122.91, 118.32, 114.22, 77.36, 77.04, 76.72, 64.49, 48.26, 43.72, 36.35, 28.78, 20.01, 13.23, 9.72. IR (thin film): 2930, 2097 (N_3), 1645, 1605, 1527, 1460, 1398, 1294, 1232, 1183, 1144, 1057, 995, 784, 668, 536 cm^{-1}. HRMS: calculated for $[C_{30}H_{31}BFN_6O_2]^+$ = 537.25801; found 537.25817 $[M - F]^+$

T_5-Bodipy conjugate (8)

To a stirred solution of oligonucleotide **6** (0.28 μmol) in H_2O/dioxane (2 mL, 4 : 1) was added bodipy-azide **7** (0.28 μmol) in dichloromethane (41 μL). The mixture was allowed to stir overnight followed by lyophilization. HPLC purification yielded the title compound as a pink powder (0.06 μmol)

LC-MS: (MeCN : 10 mM aq NH_4OAc 10 → 90 v/v) retention time: 5.19 min. ESI-MS: 1211.2 $[M - F + 2H]^{2+}$ HRMS: calculated for $[C_{103}H_{121}BF_2N_{17}O_{40}P_5 + 2H]^{2+}$: 1221.34425; found: 1221.34563 $[M + 2H]^{2+}$.

Cyclooctyne-UCAGACUUUUAAUCUG (9)

Synthesis was performed on a 10 μmol scale using preloaded CPG. Coupling was performed using 5 eq of commercially available amidites and 6 eq of alkyne amidite **5** at a 0.1 M concentration. 5-mercaptobenzyltetrazole (BMT, 0.3 M) was used as the activating agent in 15 minute coupling cycles. Oxidation and capping were performed by means of standard procedures using I_2/pyridine/H_2O and Ac_2O respectively. Deprotection and cleavage was performed with an aqueous solution of concentrated $NH_4OH/MeNH_2$ (1 : 1 v/v) overnight at rt. Concentration. The reaction mixture was concentrated, taken up in DMSO (1 mL), a solution of TEA·HF (5 mL) was added and deprotection was allowed to progress overnight. Precipitation using cold (0 ^oC) n-butanol and subsequent centrifugation yielded a pellet which was purified using HPLC purification yielding the target oligo-2'-deoxynucleotide (0.5 μmol).

LC-MS: (MeCN : 10 mM aq NH_4OAc 00 → 50 v/v) retention time: 5.62 min. ESI-MS: 1810.9 $[M + 3H]^{3+}$; 1358.3 $[M + 4H]^{4+}$.

RNA-hyaluronan conjugate (12)

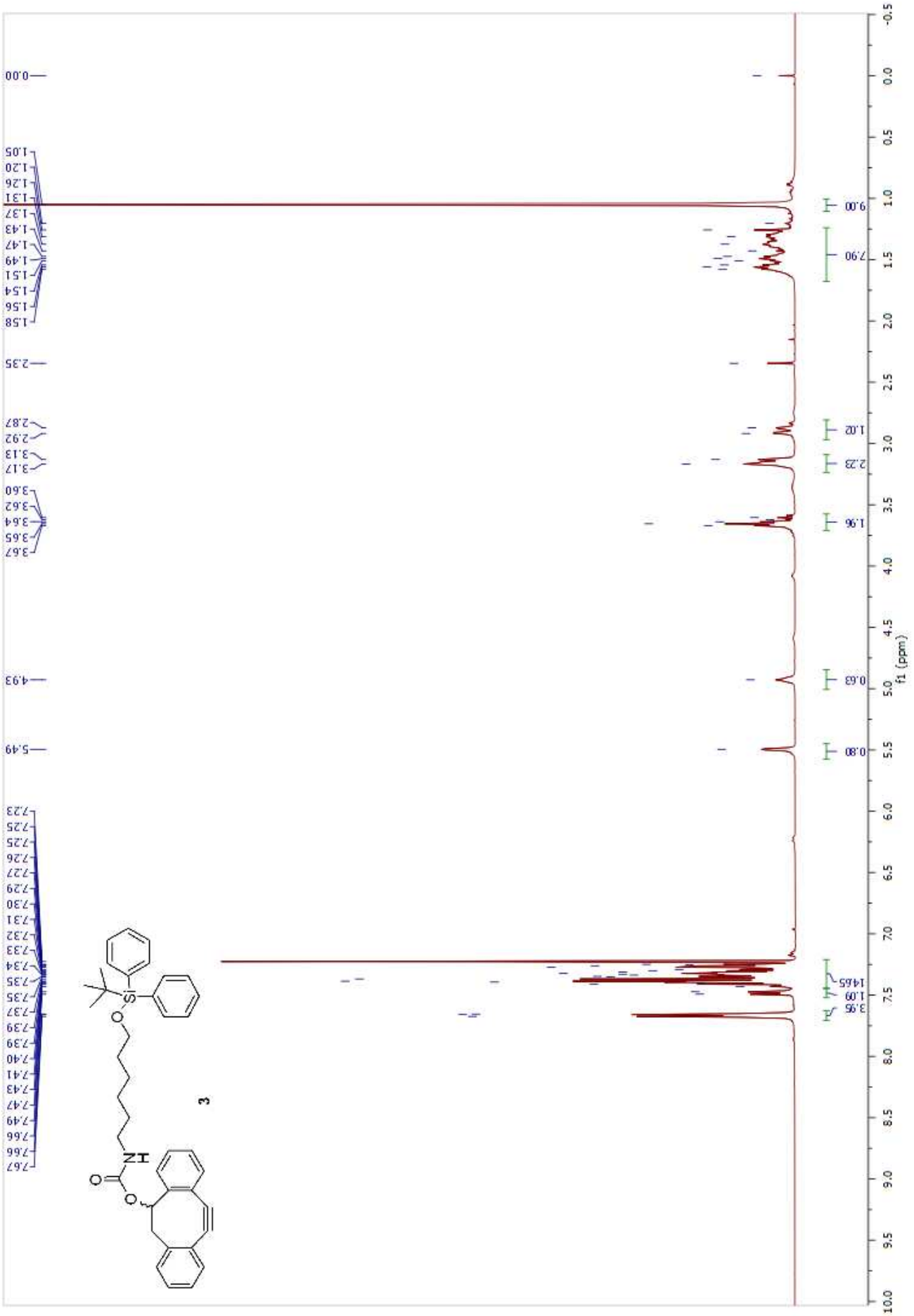
Oligonucleotide **9** (0.1 μmol) was dissolved in H_2O (20 μL) and hyaluronan azide **10** (0.1 μmol) in H_2O (10 μL) was added. The mixture was shaken for 5 hrs followed by lyophilization. HPLC purification yielded the title conjugate (0.035 μmol).

LC-MS: (MeCN : 10 mM aq NH_4OAc 00 → 20 v/v) retention time: 7.94. ESI-MS: 1573.4 $[M + 4H]^{4+}$; 1258.9 $[M + 5H]^{5+}$. HRMS: calculated for $[C_{204}H_{259}N_{59}O_{141}P_{16} + 3H]^{3+}$: 2097.03287; $[C_{204}H_{259}N_{59}O_{141}P_{16} + 2H + NH_4]^{3+}$: 2102.70867 found: 2097.03335 $[M + 3H]^{3+}$; 2102.70991 $[M + 2H + NH_4]^{3+}$.

RNA-penetratin conjugate (13)

Oligonucleotide **9** (0.1 μmol) was dissolved in PBS buffer (0.2 M, pH 7.3, 10 μL) and penetratin azide **11** (0.1 μmol) in H_2O (28 μL) was added. The mixture was shaken for 5 hrs. Dilution followed by HPLC purification yielded the title conjugate (0.013 μmol).

LC-MS: (MeCN : 10 mM aq NH_4OAc 00 → 90 v/v) retention time: 6.26 min. ESI-MS: 1944.6 $[M + 4H]^{4+}$; 1555.4 $[M + 5H]^{5+}$; 1296.8 $[M + 6H]^{6+}$. HRMS: calculated for $[C_{280}H_{381}N_{91}O_{139}P_{16}S + 3H]^{3+}$: 2591.71209; $[C_{280}H_{381}N_{91}O_{139}P_{16}S + 4H]^{4+}$: 1944.03589 found: 2591.71101 $[M + 3H]^{3+}$; 1944.03937 $[M + 4H]^{4+}$.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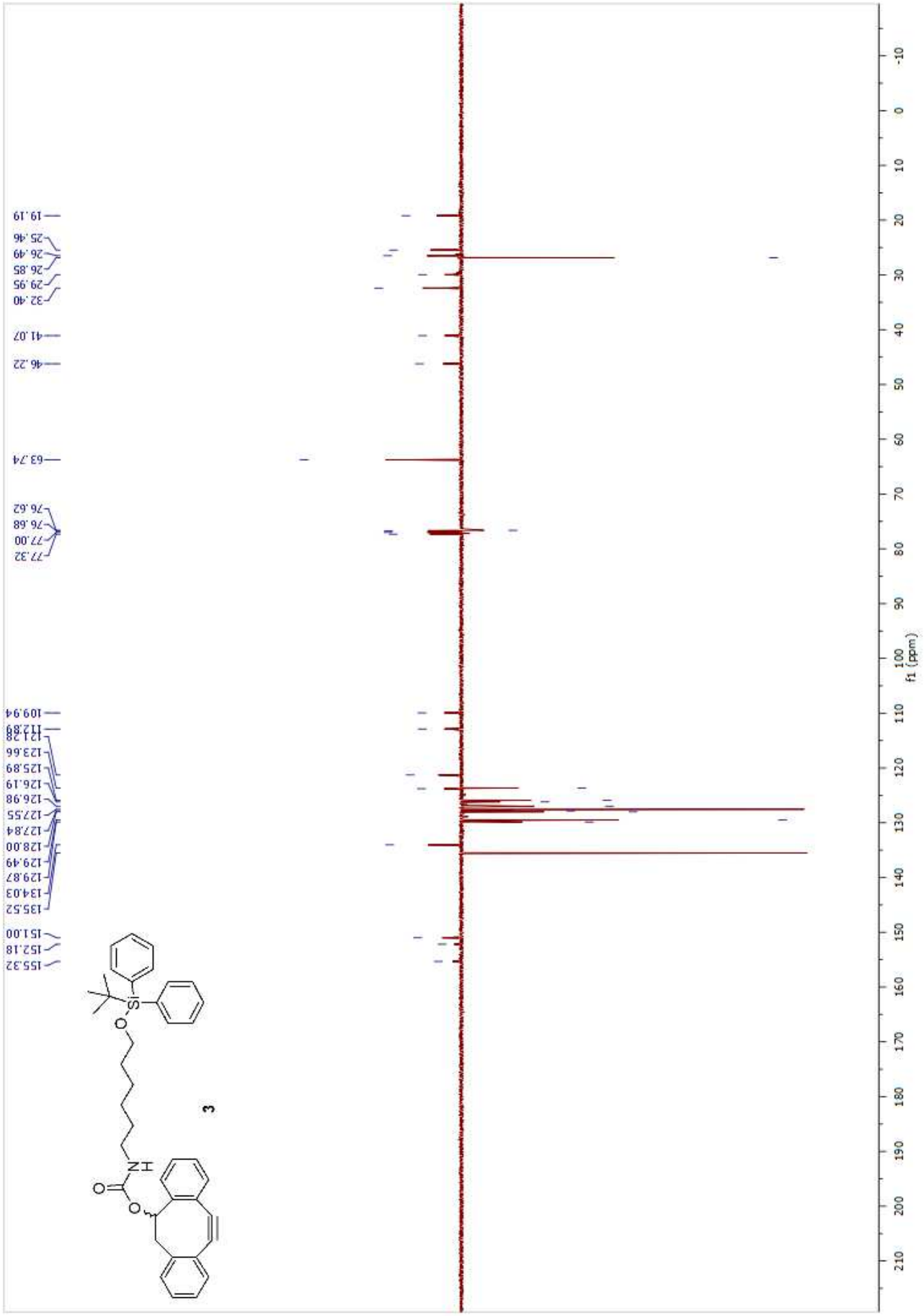
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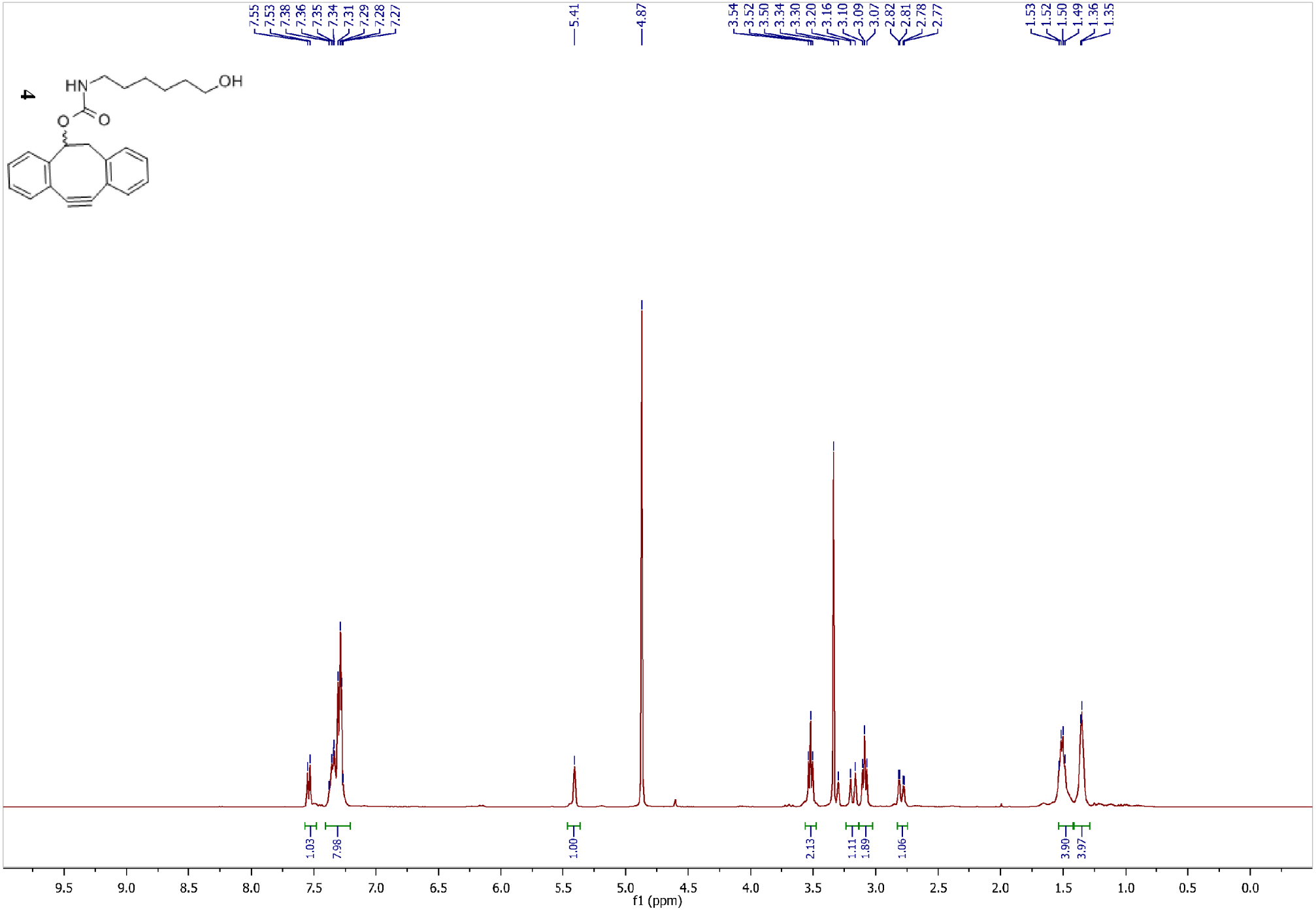
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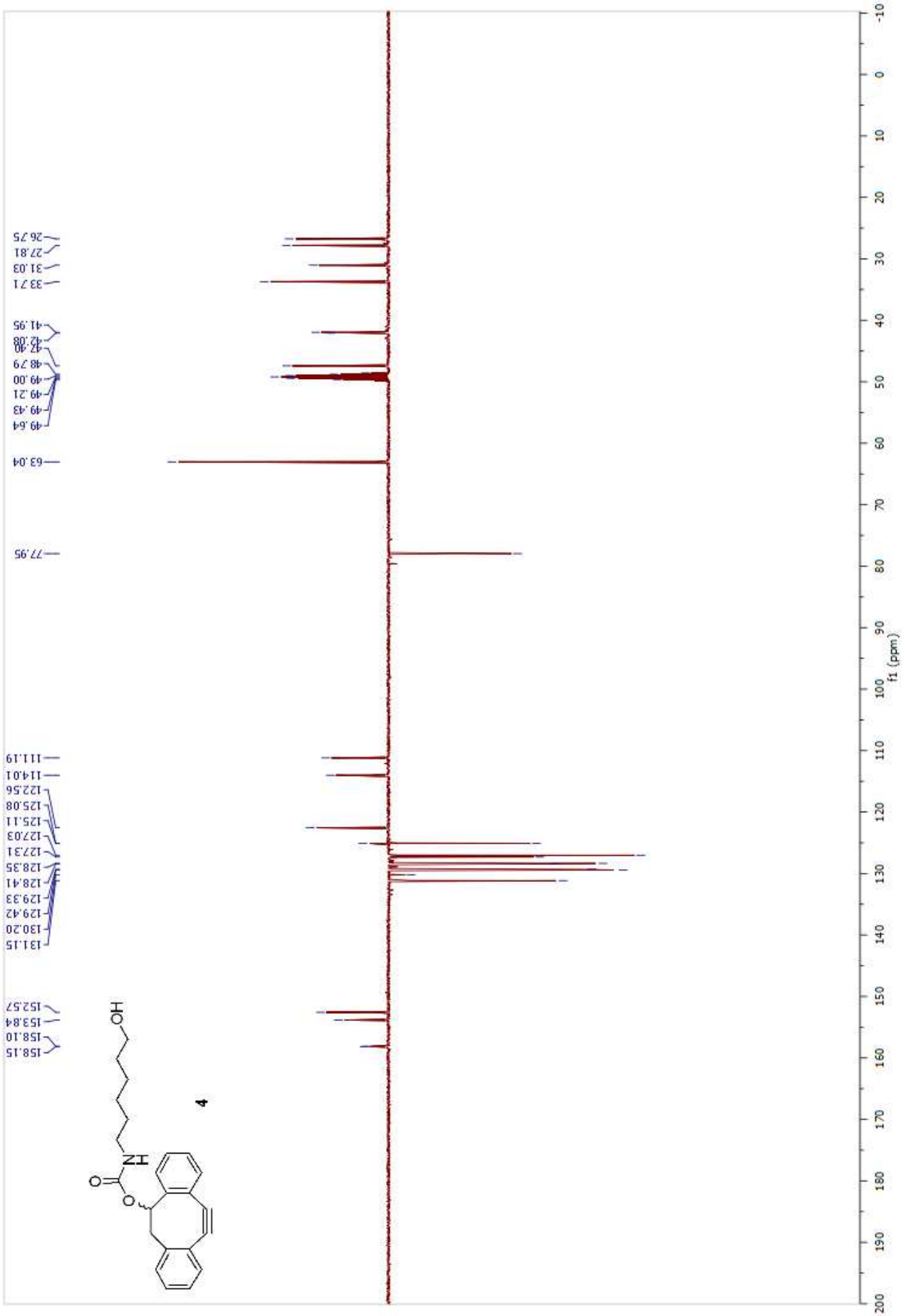
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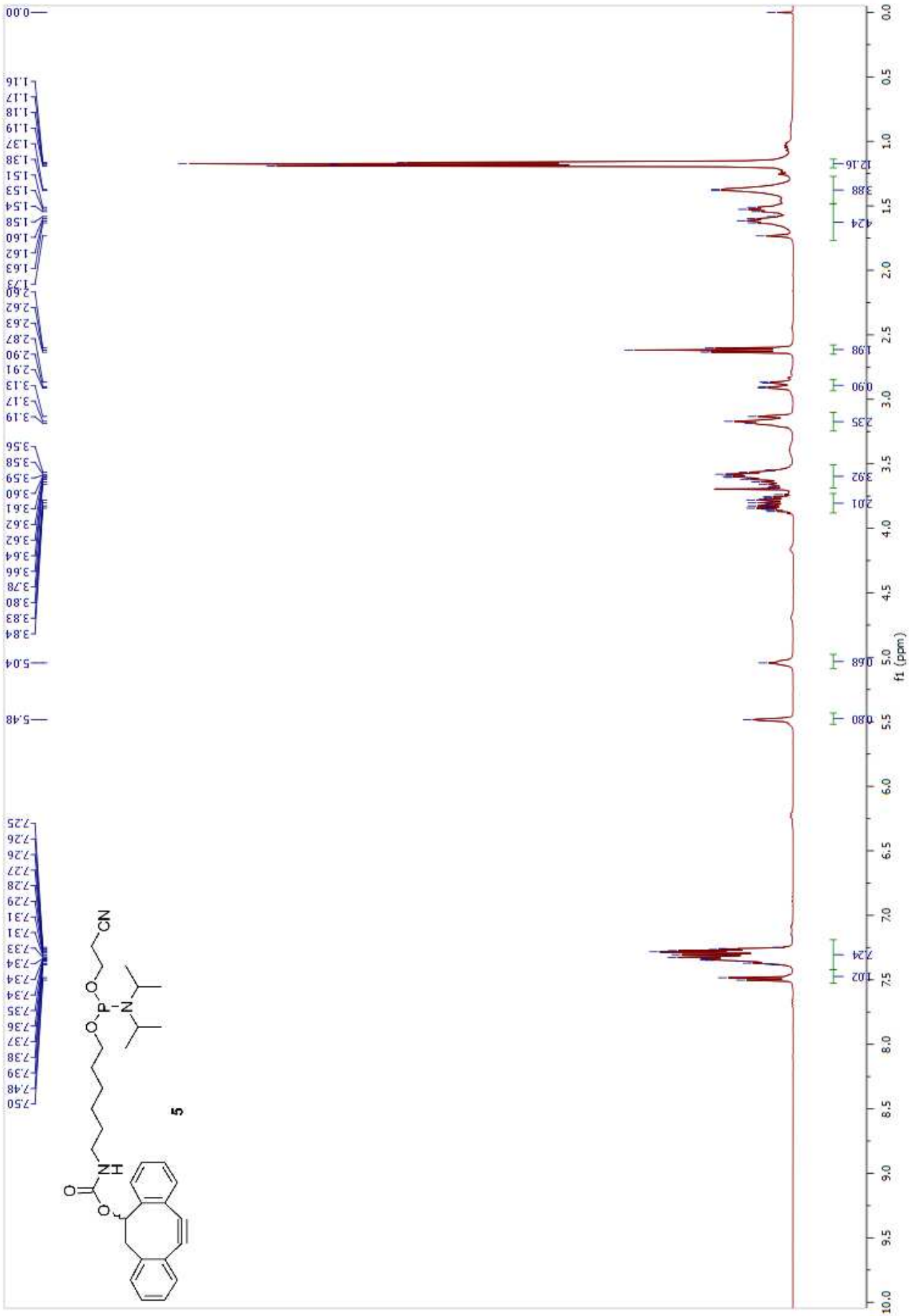
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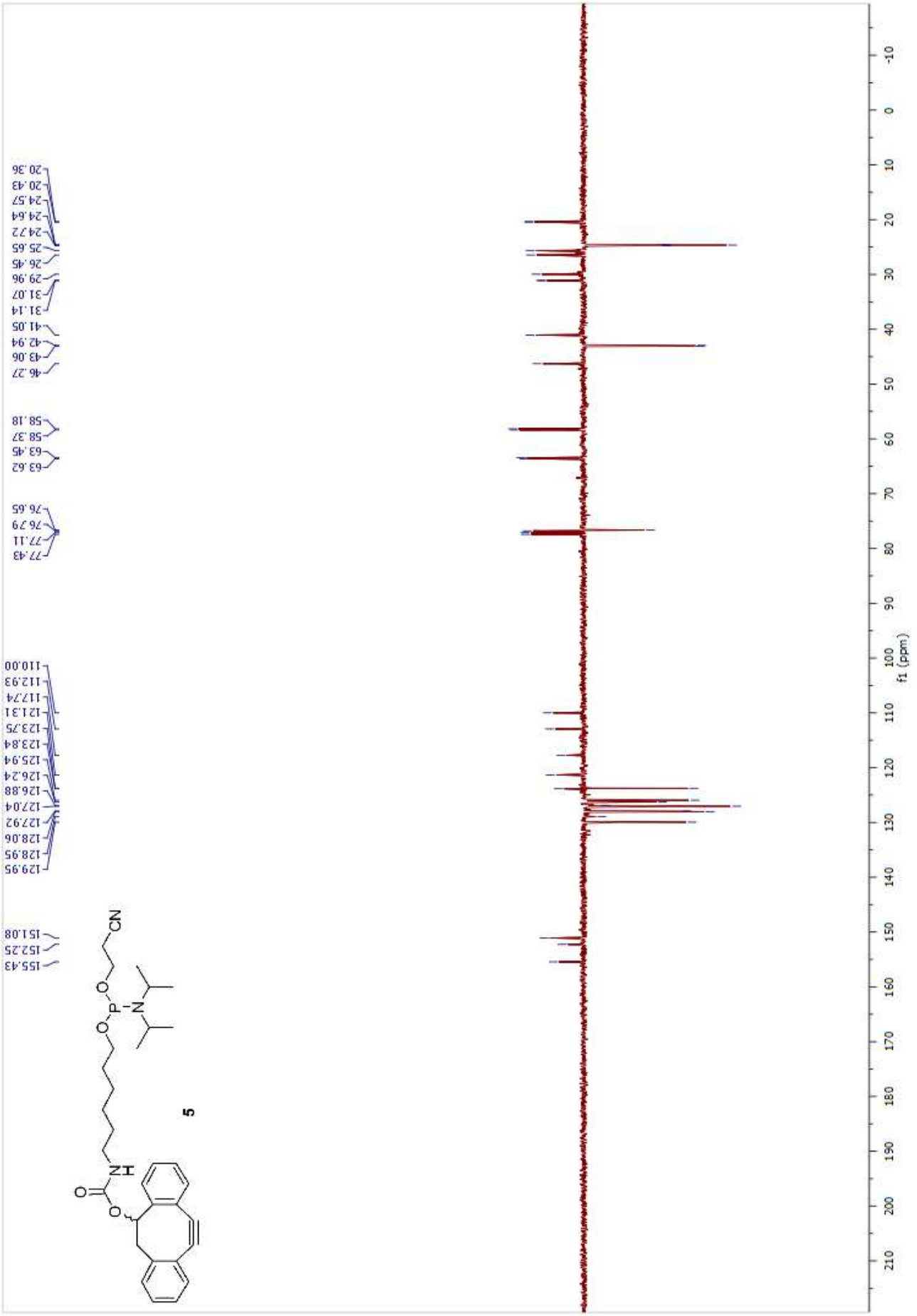
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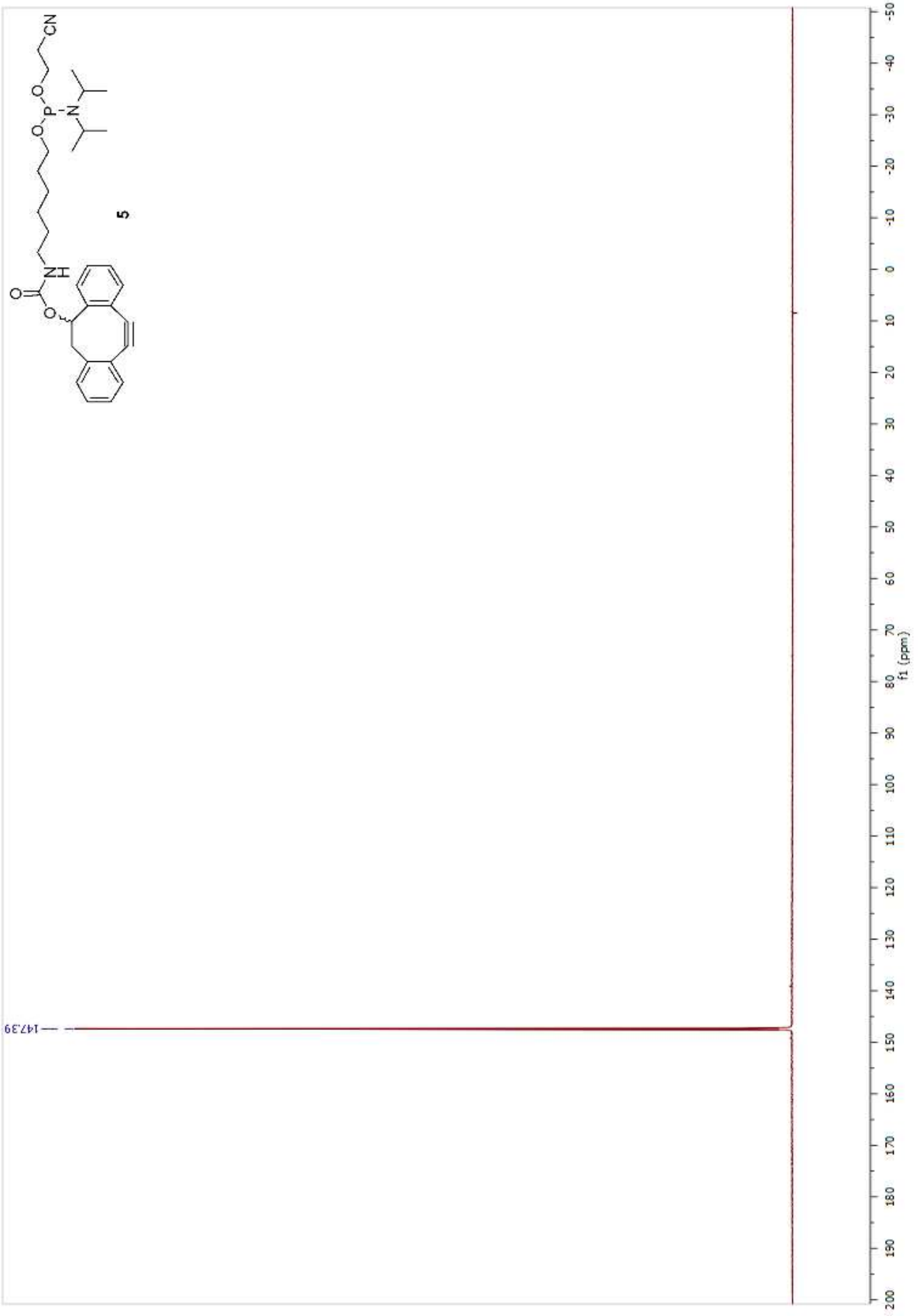
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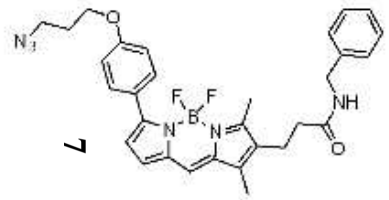

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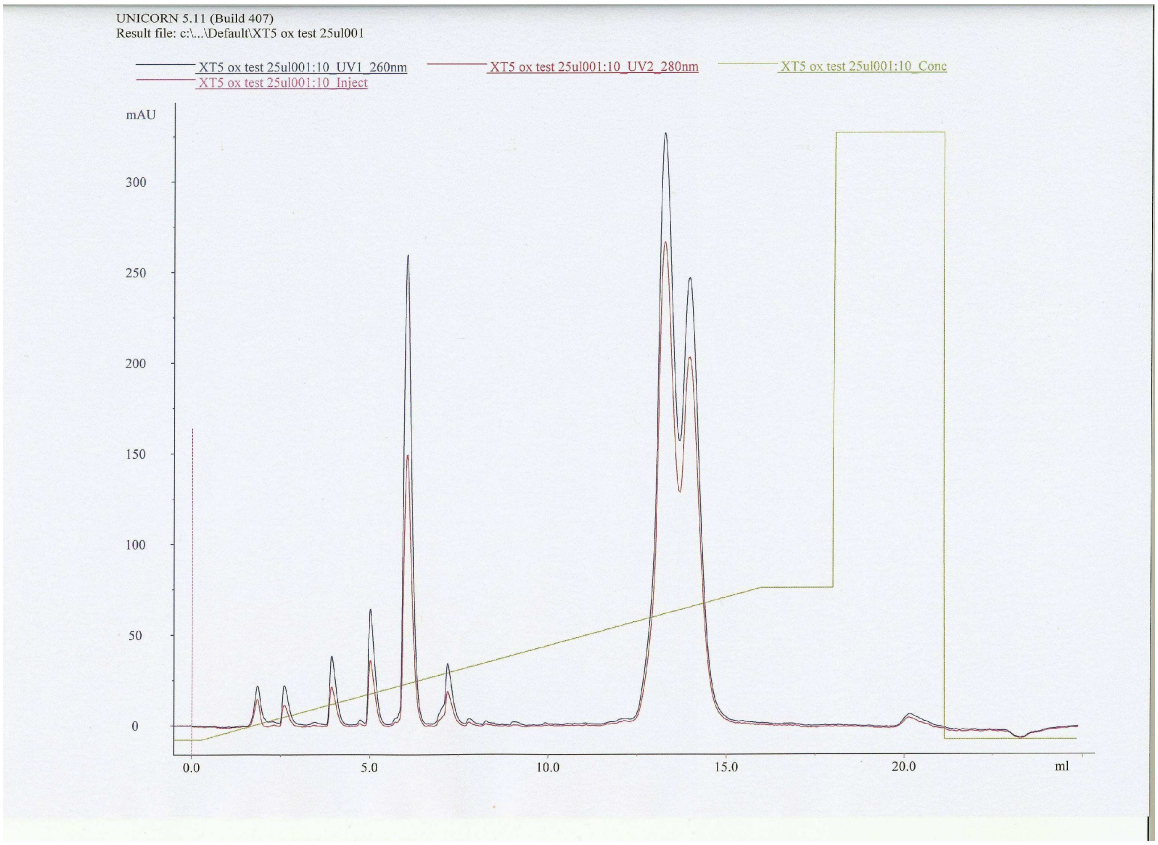
Analysis of Cyclooctyne-T_5 (6) after cleavage from the solid support

A) anion-exchange chromatography



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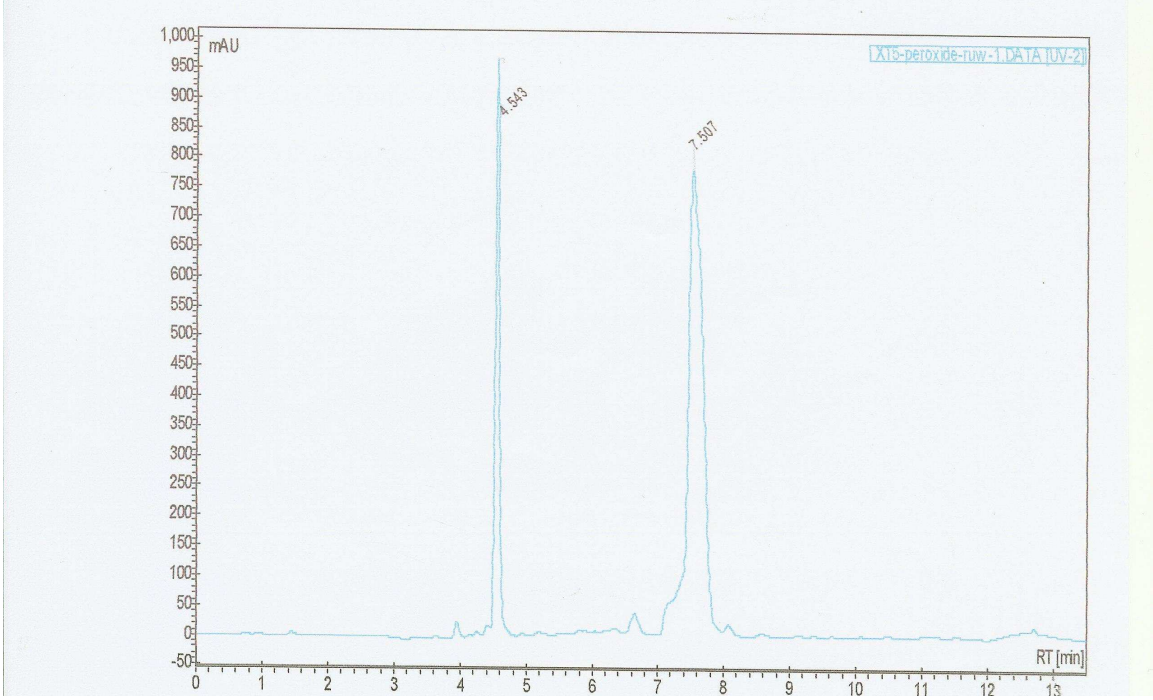
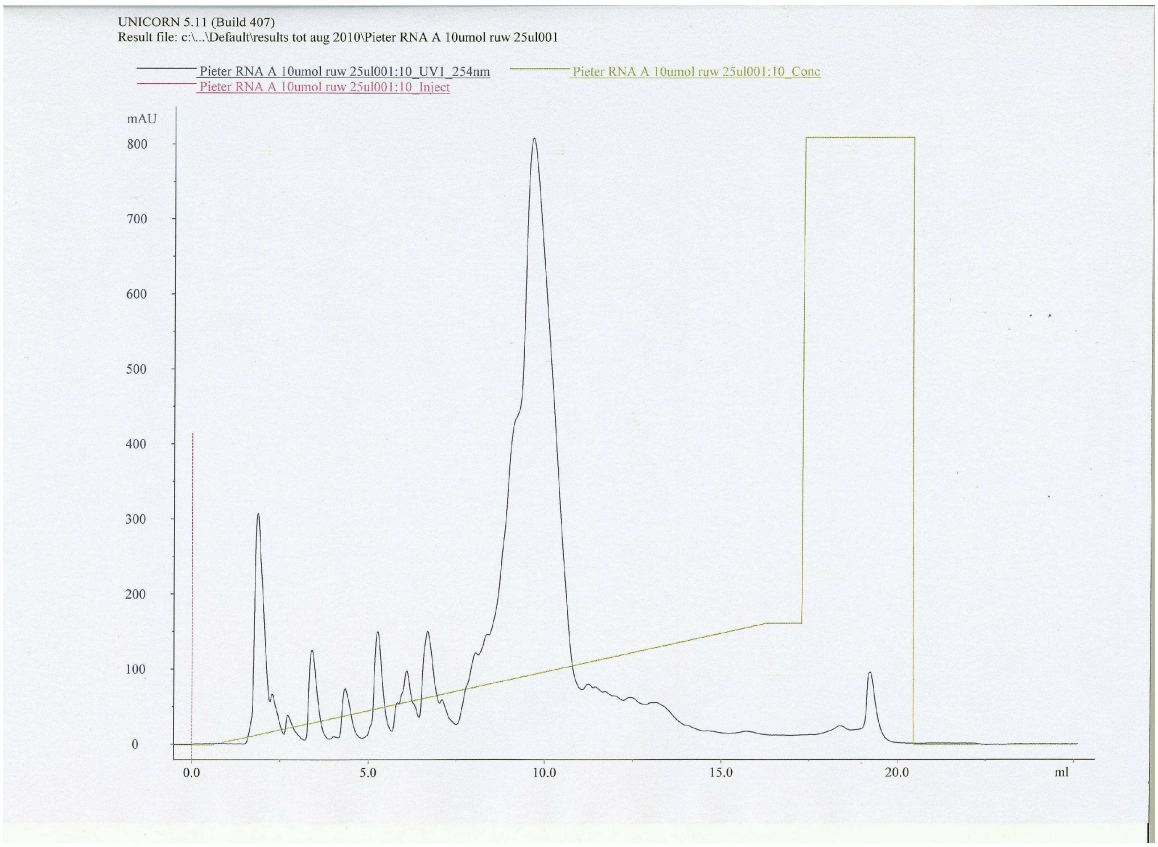
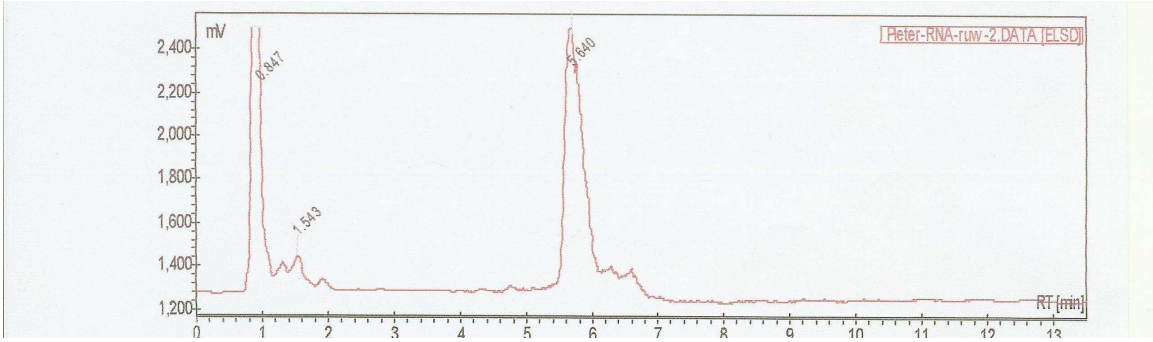


Analysis of crude Cyclooctyne-UCAGACUUUUAAUCUG (9) after cleavage from the solid support

A) anion-exchange chromatography

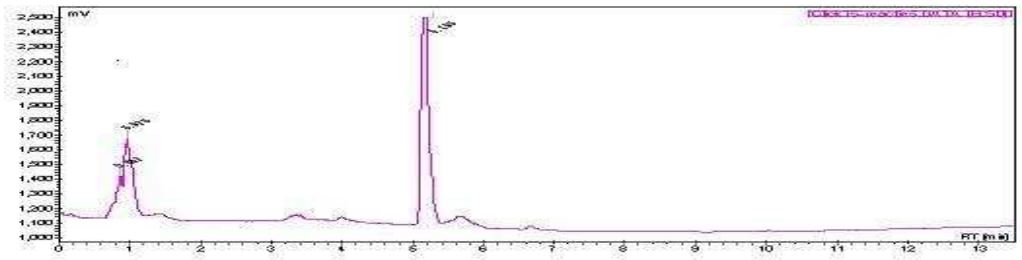


B) RP-HPLC

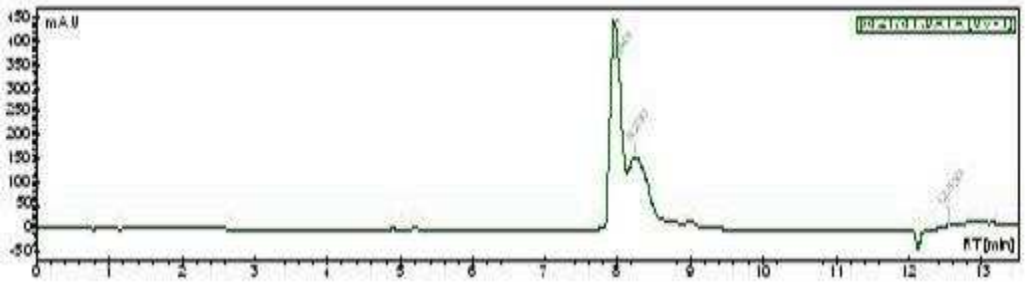


Crude LC traces

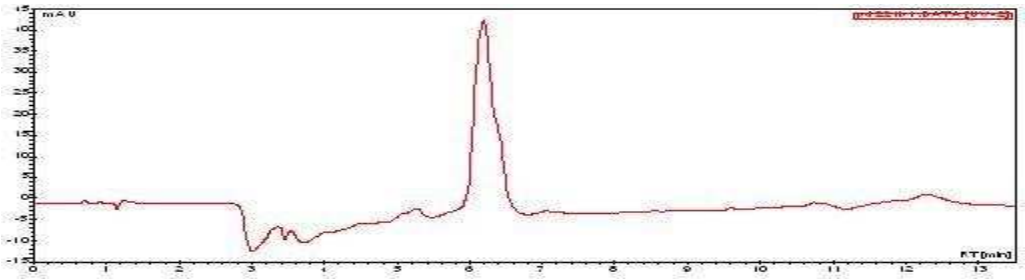
T_5 – Bodipy conjugate (10)



Hyaluronan – RNA conjugate (15)

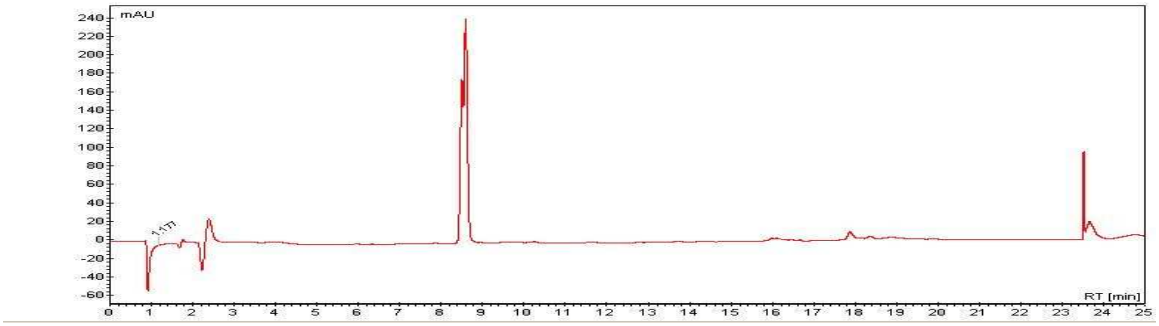


Azidoacyl penetratin – RNA conjugate (16)

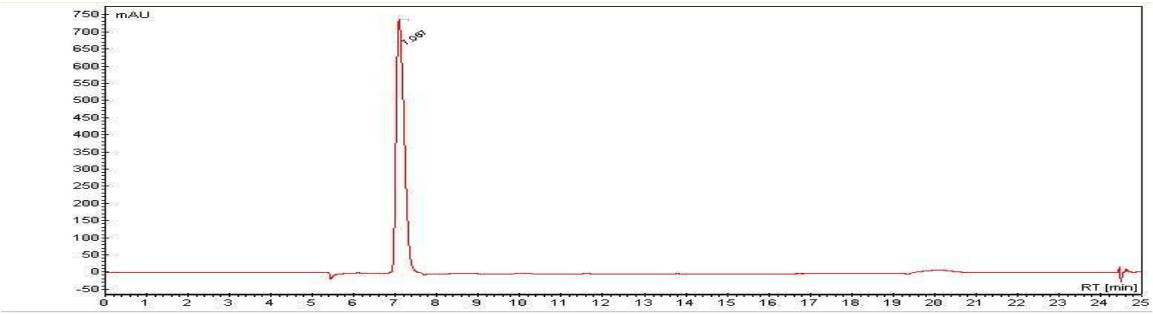


LC Traces of Purified Conjugates

T_5 – Bodipy conjugate (10)



Hyaluronan – RNA conjugate (15)



Azidoacyl penetratin – RNA conjugate (16)