Supporting Information

Improved synthesis of the unnatural base NaM, and evaluation of its orthogonality in in vitro transcription and translation

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General Information

Unless otherwise specified, commercial reagents and solvents were used as received without further purification. Reactions that required heating were heated via oil bath. All glassware and stir bar were dried via oven at 120 °C overnight or Bunsen burner and then purged with argon before use. All products and solids were dried under vacuum overnight. TLC was taken using silica gel 60 W F_{254s} with aluminum back. Dowex® 50WX8-200 was purchased from fisher-scientific and used for the cation exchange columns ^{1}H NMR (400 MHz) and ^{13}C NMR (400 MHz) were recorded on a Bruker AV 400 (400 MHz) spectrometer at Biotech 1 Richmond VA. Chemical shifts for protons are reported in ppm and are referenced to the NMR solvent peak. Chemical shifts for carbons are reported in ppm and are referenced to the carbon resonances of the NMR solvent peak. Multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublet), ddd (doublet of doublet of doublet), m (multiplet). Coupling constants J are reported in Hz. High resolution mass spectrometry (HRMS) was obtained Agilent 6520 Q-TOF LC/MS with ESI source. MALDI-TOF was carried through Voyager DE-Pro, samples were prepared with *4-chloro-α-cyanocinnamic acid.* 2-iodo-3-methoxynaphthalene was synthesized in 1 step from 3-methoxynaphthalene[1] Compound 1-Methyl-3-benzenesulfonylimidazolium triflate was synthesized via the literature protocol referenced.[2]

General Synthetic Protocols

General Protocol A: Protection of 2 with silyl chlorides

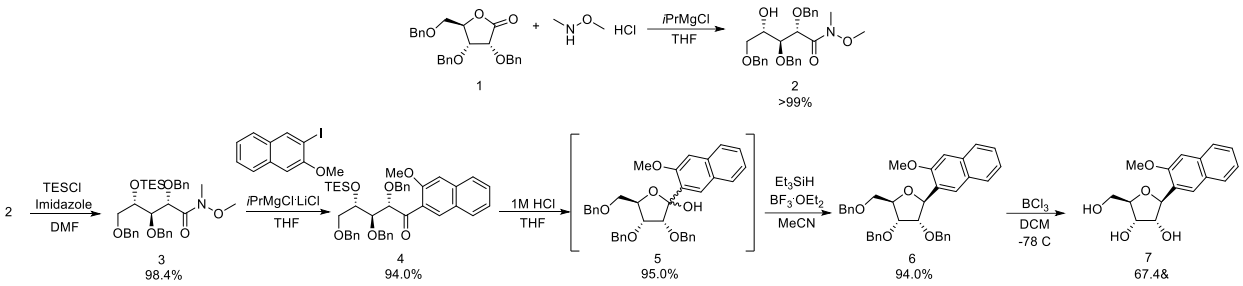
To a dry and Ar purged round bottom flask, **2** (1 eq) and R_3Si-Cl (1.2-2eq) was dissolved in anhydrous DMF (0.1 M). The solution was stirred and cooled to 0 C. Then imidazole (1.8 eq) was then added to the stirring solution. The solution was stirred for an additional 2h – 16h and allowed to warm up to RT. Then the reaction was then monitored via TLC. The solution was then diluted with DCM, washed with water and concentrated. Then Dissolved in Diethyl Ether and washed with water and brine and then dried over NaSO4 and then concentrated. The reaction was purified via Column Chromatography. The purified fractions were then combined and concentrated via rotary vacuum to a clear oil to give the titled compound **3a-3b**.

General Protocol B: Weinreb Amide Ketone synthesis of 3

To a dried 50 mL round bottom flask, 2-iodo-3-methoxynapthalene was added and dissolved in THF. to the stirring solution, iPrMgClLiCl$_2$ in 1.3 M anhydrous THF was then added to the stirring solution. The magnesium halogen exchange was monitored via TLC in a 95:5 (Hexanes:Ether) solvent system. The solution was activated instantly and then **3a-3c** was added. The reaction was then stirred for an additional 1 hr and monitored via TLC. The solution was then concentrated and then purified via column chromatography using eluents (95:5) Hexanes:Ether followed by (9:1) hexanes:EtOAc. The pure fractions were then combined and then concentrated via rotary evaporation.

The material was then characterized via ^{1}H NMR (400 MHz d-DMSO) to obtain the final product as a clear oil to give the titled compound **4a-4b**.

Experimental Steps for the Synthesis of NaM Nucleoside



(2S,3S,4S)-2,3,5-tris(benzyloxy)-4-hydroxy-N-methoxy-N-methylpentanamide. (2)

To a dry Ar purged 100 mL round bottom flask equipped with a stir bar, tri-o-benzylribolactone **(1)** (1215 mg, 2.903 mmol) and N,O-dimethylhydroxylamine hydrochloride (425 mg, 4.355 mmol) were added along with THF (12 mL). Then 2 M *i*PrMgCl in THF (360 µmol, 8.710 mmol) was added in portions and stirred for an additional 5 mins. The reaction was monitored via TLC in Hexanes:EtOAc (7:3 Rf = 0.2). Once the reaction was finished, it was then quenched with saturated NH_3Cl (30 mL) and extracted with EtOAc. The combined organic layer was then washed with water and brine and dried over $NaSO_4$. The organic layer was then concentrated down to give the final product **2** (1.39 g, >99.9% yield) as a yellow oil. ^{1}H NMR (400 MHz, d_6-DMSO: δ 7.22-7.36 (16 H, m), 5.04-5.05 (1H, d, J = 4.98 Hz), 4.66-4.70 (1H, m), 4.63-4.65 (1H, d, J = 11.5 Hz), 4.47 (2H, s), 4.41-4.46 (2H, m), 4.35-4.38 (1H, d, J=11.6 Hz), 4.05-4.09 (1H, m), 3.79-3.82 (1H, m), 3.63-3.67 (1H, dd, J = 9.9 Hz), 3.52 (3H, s), 3.48-3.50 (1H, dd, J = 9.9 Hz), 3.11 (3H, s). ^{13}C NMR (400 MHz, d6-DMSO): Data matched literature reference.[3]

(2S,3R,4S)-2,3,5-tris(benzyloxy)-N-methoxy-N-methyl-4-((triethylsilyl)oxy)pentanamide. (3a)

General protocol A was applied using the following modifications TESCl (2 eq) as the electrophile. TLC monitoring (Hexanes:EtOAc 8:2 Rf = 0.3). Column chromatography eluents Hexanes:EtOAc (9:1 – 8:2). To give the titled compound (4.19 g, 98.4% yield). ^{1}H NMR (400 MHz, d_6-DMSO): δ 7.21-7.35(15H, m), 4.62-4.68 (1H, m) 4.62-4.65 (1H, d, *J = 11.4 Hz*), 4.35-4.48 (2H, m), 4.45 (2H, s), 4.38-4.41 (2H, d, *J =11.5 Hz*), 4.22-4.25 (1H, m), 3.79-3.80 (1H, d, J = 8.5 Hz), 3.67-3.71 (1H, dd, *J = 6.80 Hz*), 3.53 (3H, s), 3.44-3.49 (1H, dd, *J = 5.4, 4.6 Hz*), 3.10 (3H, s), 0.87-0.91 (9H, t, J = 7.9 Hz), 0.53-0.59 (6H, q, J = 7.8 Hz). ^{13}C NMR: 138.82, 138.71, 138.09, 128.61, 128.55, 128.05, 127.89, 127.86, 127.83, 127.80, 127.76, 82.35, 73.76, 72.79, 72.37, 72.07, 71.54, 7.22, 7.16, 6.15, 4.92. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{34}H_{47}O_6Si$ = 616.3070; observed = 616.3040. This data matched that in the literature report.[3]

Synthesis of (2S,3R,4S)-2,3,5-tris(benzyloxy)-N-methoxy-N-methyl-4-((tert-butyldimethylsilyl)oxy)pentanamide. (**3b)**

General Protocol A was applied using the following modifications TBDMSCl (2 eq) as the electrophile. TLC monitoring using Hexanes:Acetone (95:5 – 9:1 Rf = 0.3) Column chromatography eluents Hexanes:Acetone

(95:5 – 9:1) to give the titled compound (1.97 g, 68.9% yield). ^{1}H NMR (400 mHz, d-DMSO): 7.21-7.35 (15H, m), 4.62-4.65 (1H, d, *J = 11.3 Hz*), 4.45-4.48 (1H, d, *J = 11.8* Hz), 4.35-4.39 (2H, dd, *J = 4.1*, *7.4 Hz*) 4.21-4.24 (1H, m), 3.77-3.79 (1H, d, *J = 8.9* Hz), 3.65-3.68 (1H, dd, *J = 4.4, 5.6 Hz*), 3.50 (3H, s), 3.45-3.50 (1H, dd, *J = 2.9, 7.0 Hz*) 3.29 (3H, s), 3.26 (1H, m), 3.46 (3H, s), 3.10 (3H, s), 0.86 (9H, s), .84 (6H, s), .04 (3H, s), .02 (3H, s). -.04 (3H, s). ^{13}C NMR: 138.34, 138.20, 137.38, 137.56, 128.33, 128.30, 128.25, 128.16, 128.14, 128.08, 127.85, 127.82, 127.69, 127.59, 127.44, 127.38, 127.33, 127.30, 127.28, 81.66, 81.17, 75.27, 73.08, 72.29, 72.02, 71.77, 71.09, 25.68, 17.79, -4.65, -4.97. **HRMS** (LCMS): m/z $[M+H]^+$ calculated for $C_{34}H_{47}O_6Si$ = 594.3245; observed = 594.3259.

(2S,3R,4S)-2,3,5-tris(benzyloxy)-1-(3-methoxynaphthalen-2-yl)-4-((triethylsilyl)oxy)pentan-1-one. (4a)

General protocol B was applied with the following modifications. **3a** (3.40 g, 7.17 mmol) was used as the weinreb amide compound. The purified fraction was then evaporated to give the titled compound 98.4% yield as a yellow oil. ^{1}H NMR (400 MHz, d_6-DMSO): δ 7.88 (1H, s), 7.85-7.81 (1H, d, *J = 8.1 Hz*), 7.76-7.78 (1H, d, *J = 8.1 Hz*), 7.53-7.57 (1H, t, *J = 8.0 Hz*), 7.42 (1H, s), 7.40-7.41 (1H, t, *J = 8.0 Hz*), 7.13-7.35 (13H, m), 7.03-7.05 (2H, d, *J = 7.3 Hz*), 5.22-5.24 (1H, d, J =5.3 Hz), 4.55-4.73 (2H, m), 4.43-4.49 (2H, q, J = 11.4 Hz), 4.38 (2H, s), 4.06-4.09 (1H, m), 3.84-3.87 (1H, t, J = 4.9 Hz), 3.83 (3H, s), 3.61-3.65 (1H, dd, *J = 3.3, 6.9 Hz*), 3.41-3.45 (1H, dd, *J = 6.0, 4.1 Hz*) 0.79-0.83 (9H, t, *J = 7.9 Hz*), 0.45-0.52 (6H, q =J = 2.7 Hz) ^{13}C NMR (400MHz, d_6-DMSO): 202.29, 154.72, 138.70, 138.57, 138.31, 135.87, 130.94, 130.40, 128.97, 128.59, 128.54, 128.49, 128.40, 128.00, 127.96, 127.93, 127.87, 127.76, 127.72, 127.69, 126.99, 124.85, 106.90, 82.37, 82.10, 72.86, 72.76, 72.31, 72.28, 72.14, 55.94, 55.36, 7.09, 4.84. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{43}H_{50}O_6Si$ = 713.3274; observed = 713.3247.

Synthesis of (2S,3R,4S)-2,3,5-tris(benzyloxy)-4-((tert-butyldimethylsilyl)oxy)-1-(3-methoxynaphthalen-2-yl)pentan-1-one. (**4b)**

General Protocol B was applied with the following modifications. **3b** (900 mg, 1.51 mmol) was used as the weinreb amide compound to deliver the titled compound 94.4% yield. ^{1}H NMR (400 mHz, d-DMSO): 7.86 (1H, s), 7.83-7.86 (1H, d, *J =8.1 Hz*) 7.73-7.76 (1H, d *J =8.1 Hz*), 7.52-7.56 (1H, t, *J = 7.2 Hz*), 7.11-7.32 (16H, m), 7.00-7.02 (2H, d, *J = 6.6 Hz*), 5.22-5.24 (1H, d, *J = 6.4 Hz*), 4.64-4.67 (1H, d, *J = 11.7 Hz*), 4.52-4.57 (1H, d, *J = 11.7 Hz*), 4.54-4.55 (1H, d, *J = 3.8 Hz*), 4.39-4.41 (3H, m), 4.10-4.12 (1H, m), 3.81-3.85 (1H, dd, *J = 3.1, 3.6 Hz*), 3.81 (3H, s), 3.64-3.67 (1H, dd, *J = 3.7, 10.0 Hz*). 3.43-3.48 (1H, dd, *J = 6.7 Hz, 10.0 Hz*), 0.79 (9H, s), -0.02 (3H, s), -0.11 (3H, s). ^{13}C NMR: 202.49, 154.36, 138.25, 137.96, 137.76, 135.43, 130.50, 129.88, 128.54, 128.15, 128.10, 128.05, 127.88, 127.52, 127.47, 127.38, 127.28, 127.27, 127.25, 126.46, 124.32, 106.42, 81.42, 72.47, 72.23, 72.09, 71.74, 55.45, 25.64, 17.75, -4.64, -5.19. **HRMS** (LCMS): m/z $[M+H]^+$ calculated for $C_{43}H_{50}O_6Si$ = 691.3449; observed = 691.3419.

(3R,4R,5R)-3,4-bis(benzyloxy)-5-((benzyloxy)methyl)-2-(3-methoxynaphthalen-2-yl)tetrahydrofuran-2-ol. **(5)**

To a 50 mL round bottom flask equipped with a stir bar and purged with Ar, **4a** (1060 mg, 1.5 mmol) was added and dissolved in THF (10 mL). To the stirring solution, 1M HCl (6 mL) was then added and stirred for 5 h. The reaction was monitored via TLC using eluents 4:1 Hexanes:EtOAc and once the starting material was consumed, the reaction was then quenched with saturated $NaHCO_3$ (20 mL) and extracted with 3x EtOAc (30 mL) and then the organic layer was washed with DI Water (50 mL) and brine (50 mL) and dried over $NaSO_4$. The organic layer was then concentrated and then purified via Columned Chromatography Using Eluents 4:1 Hexanes:EtOAc Rf = 0.24 and then 7:3 Hexanes:EtOAc. The purified fraction was then concentrated to give **the titled compound** 95.0% yield as a yellow oil. ^{1}H NMR: (600 MHz d_6-DMSO) revealed an isomeric mixture ratio of the linear ketone (1.00) and an unknown isomeric mixture of α and β containing a mixture of 0.76:0.40 (see Fig. S69). ^{13}C NMR: (400 Mhz d_6-DMSO): 201.69, 155.29, 154.14, 138.57, 138.46, 138.38, 138.32, 138.25, 138.11, 138.08, 135.08, 134.15, 133.98, 131.64, 130.90, 130.83, 129.91, 128.46, 128.16, 128.05, 127.98, 127.87, 127.76, 127.67, 127.57, 127.49, 127.39, 127.33, 127.27, 127.14, 127.11, 127.03, 126.97, 126.47, 126.35, 126.21, 125.99, 124.18, 123.49, 106.40,

106.02, 105.49, 104.84, 102.81, 82.84, 81.20, 81.11, 80.80, 78.93, 78.93, 78.46, 78.09, 76.87, 72.66, 72.44, 72.28, 72.16, 72.12, 71.88, 71.81, 71.26, 69.45, 68.77, 55.48, 55.20, 54.83, **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{37}H_{26}O_6$ =599.2410; observed = 599.2465.

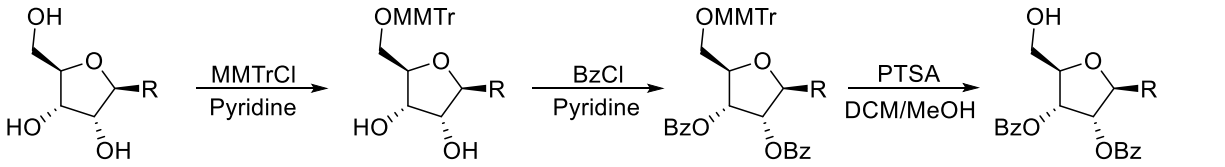
NaM-Tribenzyl. (6)

To a dry 50 mL Round bottom flask purged with Ar, **5** (840 mg, 1.457 mmol) was added and co-evaporated 2x with anhydrous MeCN. Then MeCN (10 mL) was added to the round bottom flask and then the solution was cooled to -40 °C. To the stirring cooled solution, Triethylsilane (442 µmol, 2.768 mmol) followed by BF_3OEt_2 (194 µmol, 1.529 mmol) were added and stirred for 30 mins. The reaction was monitored via TLC using eluents 4:1 Hexanes:EtOAc until the starting material was consumed. The reaction was then quenched with saturated $NaHCO_3$ and extracted with EtOAc 3x. The organic layer was then washed with DI Water, brine and dried over $NaSO_4$. The organic layer was then concentrated and then purified via flash chromatography using eluents 8:2 Hexanes:EtOAc. The purified fraction was then concentrated to give the title compound in 94.0% yield as a yellow oil. α:β (1:7.3) ^{1}H NMR (400 MHz d_6-DMSO): δ 8.03 (1H, s), 7.77-7.79 (1H, d *J = 8.2 Hz*), 7.21-7.43 (21H, m), 5.42 (1H, s), 4.60-4.86 (2H, m), 4.67-4.70 (2H, d, J = 11.8 Hz), 4.39-4.54 (2H, m), 4.22-4.25 (1H, m), 4.03-4.10 (2H, m), 3.94 (3H, s), 3.90-3.92 (1H, d, *J = 9.8 Hz)*, 3.76-3.80 (1H, dd, *J = 3.81, 7.3 Hz*), ^{13}C NMR: 154.38, 138.61, 138.42, 138.18, 133.52, 130.25, 128.27, 128.14, 128.11, 127.89, 127.57, 127.55, 127.47, 127.43, 127.38, 126.19, 126.04, 125.88, 123.47, 105.15, 80.94., 79.18, 78.99, 76.25, 72.46, 71.07, 70.91, 69.13, 55.39. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{37}H_{26}O_5$ =583.2463; observed = 583.2532

NaM (7)

To a 100 mL round bottom flask equipped with a stir bar, **6** (1.29 g, 2.30 mmol) was dissolved in anhydrous DCM (27 mL) and cooled to -78 °C. BCl_3 1M in DCM (10.60 mL, 10.6 mmol) was added dropwise and stirred for 3 h and monitored via TLC using eluents 5% DCM:MeOH. The reaction was then quenched with MeOH (30 mL). The solution was then concentrated down to an orange oil and was purified via flash chromatography using eluents DCM, DCM:MeOH 3%-5%. The purified fractions were then collected and concentrated down to give the title compound in 67.4% yield as a white solid. The product was then characterized via ^{1}H NMR (400 MHz, d-MeOH): δ 7.98 (1H, s), 7.71-7.73 (1H, d), 7.69-7.71 (1H, d), 7.32-7.36 (1H, t), 7.23-7.27 (1H, t), 7.19 (1H, s) 5.19-5.20 (1H, d, J = 3.1 Hz), 4.01-4.03 (1H, dd, J = 3.8 Hz), 3.93-3.97 (2H, m), 3.92 (3H, s), 3.89-3.90 (1H, d, J = 2.4 Hz), 3.76-3.81 (1H, dd, *J =4.2 11.7 Hz*). ^{13}C NMR: 156.69, 135.59, 131.52, 130.06, 128.73, 127.44, 127.41, 127.18, 124.71, 106.19, 83.99, 83.35, 77.76, 72.00, 63.34, 55.85. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{16}H_{18}O_5$ =313.1054; observed = 313.1048.

Protocol for the Protection of 5SICS & NaM



R = NaM, 5SICS

8
a: NaM (93.8%)
b: 5SICS (93.0%)

9
a: NaM (96.1%)
b: 5SICS (99.0%)

10
a: NaM (98.8%)
b: 5SICS (83.7%)

5'-O-methoxy trityl NaM. (8a)

To a 25 mL round bottom flask equipped with a stir bar, **NaM** (**7)** (110 mg, 379 µmol) was added followed by 4-Methoxytriphenylmethyl chloride (152 mg, 493 µmol). The flask was then lyophilized overnight and then purged with Ar. Then pyridine (1 mL) was added to the flask and the solution was allowed to stir overnight. The reaction was monitored via TLC using 1:1 hexanes:EtOAc Rf = 0.25. Then MeOH was added to the solution and allowed to stir for 5 mins and then the solution was concentrated down to an oil. Then the crude material was dissolved in DCM (10 mL) and extracted with saturated $CuSO_4$ (10 mL). The aqueous layer was then extracted with DCM (10 mL) 2x and the combined organic layer was washed with saturated $NaHCO_3$ DI water and brine and drive over $NaSO_4$. The organic layer was then dried via rotary evaporation and then the crude material was purified via column chromatography using eluents Hexanes:EtOAc (7:3) followed by 1:1. The purified fractions were combined and concentrated down to give the title compound in 93.8% yield as a white solid. ^{1}H NMR (MeOD): δ 8.12 (1H, s), 7.70 (1H, d, *J = 8.2 Hz*), 7.55-7.58 (5H. m), 7.43 (2H, d, *J = 8.7 Hz*), 7.32 (1H, t, *J = 7.1 Hz*), 7.10-7.26 (14H, m), 6.78-6.81 (2H, m), 5.35, (1H, s), 4.17-4.20 (2H, m), 4.11-4.12 (1H, m), 3.91 (3H, s), 3.52-3.57 (1H, m), 3.43-3.46 (1H, dd, *J = 4.0, 10.5* Hz). ^{13}C NMR ($CDCl_3$): δ 171.98, 165.24, 165.13, 158.57, 155.07, 144.38, 144.26, 135.56, 134.31, 133.78, 133.11, 133.09, 130.55, 130.50, 130.23, 129.87, 129.80, 129.74, 129.46, 129.42, 129.00, 128.73, 128.66, 128.58, 128.55, 128.51, 128.45, 128.37, 128.25, 127.95, 126.93, 126.64, 126.50, 126.34, 123.78, 113.23, 105.36, 86.77, 80.00, 79.63, 76.33, 71.69, 62.94, 55.44, 55.15, 29.74 **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{36}H_{34}O_6$ = 585.2245; observed = 585.2219

5'-O-methoxy trityl 5SICS. (8b)

A dry 50 mL round bottom flask equipped with a stir bar, compound **5SICS (20)** (540 mg, 1.85 mmol) and MMTrCl (687 mg, 2.22 mmol) were added and lypholized overnight. The contents were then purged with Ar, and freshly distilled pyridine (8 mL) was added and allowed to stir at room temperature overnight. The reaction was then monitored via TLC. Once completed, the solution was diluted with DCM and then extracted with saturated $CuSO_4$. The aqueous layer was then extracted 2x with DCM (30 mL) and then the combined organic layers were then washed with DI Water, brine and dried over $NaSO_4$. The organic layer was then concentrated down to a crude oil and then purified via column chromatography using eluents Hexanes:EtOAc (7:3; 1:1) Rf = 0.25. The pure fractions were then combined and concentrated down to give the title compound in 93.0% yield as a yellow solid. The compound was characterized via NMR (400 Mhz MeOD) ^{1}H NMR : δ 8.87 (1H, d, *J =8.6 Hz*), 8.52 (1H, d *J =2.7 Hz*), 8.43 (1H, d *J =7.5 Hz*), 7.50 (4H, d *J =2.8 Hz*), 7.41 (1H, t *J =2.9 Hz*), 7.25-7.38 (10H m), 6.99 (1H, s), 6.88 (2H, d *J =8.8 Hz*), 6.46 (1H, d *J =7.5 Hz*), 4.50 (1H, dd *J =8.9 Hz*), 4.25-4.29 (2H, m), 3.77 (3H, s), 3.55-3.64 (2H, ddd), 2.43 (3H, s). ^{13}C NMR: 183.54, 160.39, 150.04, 145.76, 145.44, 144.95, 138.37, 136.27, 133.95, 133.18, 132.44, 131.83, 130.91, 130.07, 129.79, 129.76, 128.98, 128.22, 128.20, 127.21, 125.54, 114.27, 112.07, 97.21, 88.40, 83.84, 76.98, 69.73, 62.50, 55.76, 21.60. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{35}H_{33}NO_5S$ = 602.1979; observed = 602.1966

2',3'-O-dibenzoyl-5'-O-methoxy trityl NaM (9a)

To a dry 25 mL round bottom flask purged with Ar, **8a** (180 mg, 311 µmol) was added followed by DMAP (3.0 mg, 25 µmol), anhydrous MeCN (0.16 M) and TEA (433 µmol, 3.10 mmol). Then freshly distilled benzoyl chloride (216 µmol, 1.86 mmol) was added and allowed to stir overnight. The reaction was monitored via TLC using eluents Hexanes:EtOAc (9:1) Rf = 0.20. Once completed the reaction was quenched with saturated $NaHCO_3$ (10 mL) and extracted with diethyl ether (10 mL) 3x. The combined organic layer was then washed with DI water (10 mL) , brine (10 mL) and dried over $NaSO_4$. The organic layer was then concentrated down and purified via flash chromatography using eluents 95:5 Hexanes:EtOAc and 9:1 Hexanes:EtOAc. The combined purified fractions were then combined and concentrated down to give the title compound in 96.1% yield as a white solid. ^{1}H NMR (400 Mhz, d_6-DMSO): δ 8.18 (1H, s), 7.93 (3H, d *J = 7.8 Hz*), 7.86 (1H, d *J = 8.0 Hz*). 7.78 (2H, d *J = 7.5 Hz*). 7.61-7.69 (2H, m), 7.40-7.51 (12H, m), 7.21-7.34 (10H, m), 6.80 (2H, d *J = 8.8 Hz*), 5.73-5.77 (2H, m), 5.58 (1H, d), 4.59-4.60 (1H, m), 3.82 (3H, s), 3.65 (3H, s), 3.56 (1H, d *J = 12.6 Hz*), 3.38 (1H, d *J = 9.6 Hz*). ^{13}C NMR(400 Mhz $CDCl_3$): 171.98, 165.24, 165.13, 158.57, 155.07, 144.38, 144.26, 135.56, 134.31, 133.78,

133.11, 133.09, 130.55, 130.50, 130.23, 129.87, 129.80, 129.74, 129.46, 129.42, 129.00, 128.73, 128.66, 128.58, 128.55, 128.51, 128.45, 128.37, 128.25, 127.95, 126.93, 126.64, 126.50, 126.34, 123.78, 113.23, 105.36, 86.77, 80.00, 79.63, 76.33, 71.69, 62.94, 55.44, 55.15, 29.74. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{50}H_{42}O_8$ = 793.2780; observed = 793.2714.

2',3'-O-dibenzoyl-5'-O-methoxy trityl 5SICS (9b)

To a dry 100 mL round bottom flask equipped with a stir bar, compound **8b** (900 mg, 1.55 mmol) and DMAP (15.0 mg, 124 µmol) were added and lypholized overnight. The flask was then purged with Ar and then anhydrous MeCN (0.10 M) and then freshly distilled TEA (2.16 mL, 15.5 mmoL) were added followed by freshly distilled BzCl (1.01 mL 9.31 mmol). The solution was stirred at room temperature for 3 h. The reaction was monitored via TLC and once completed, the reaction was quenched with saturated $NaHCO_3$ and extracted with Diethyl Ether 3x (50 mL). The organic layers were then combined and washed with DI Water, brine and dried over $NaSO_4$. The organic layer was then concentrated down via rotary evaporation to a crude oil. The contents were further purified via columned chromatography using eluents Hexanes:EtOAc (95:5; 9:1) Rf =0.20 to give the title compound **in** 99.0% yield as a whitish-yellow solid. The product was characterized via NMR (400 Mhz, d_6-DMSO) ^{1}H NMR : δ 8.76 (1H, d *J =8.6 Hz*), 8.29 (1H, d *J =7.5 Hz*), 7.91-7.96 (3H, m), 7.82 (2H, d *J =4.0 Hz*), 7.61-7.69 (4H, m), 7.42-7.52 (10H, m), 7.23-7.35 (8H, m), 7.96 (1H, d, *J =7.5 Hz*), 6.88 (2H, d *J =8.8 Hz*), 5.94 (2H, d *J =4.3 Hz*), 4.76-7.78 (1H, m), 3.69 (3H, s), 3.53-3.60 (2H, m), 2.47 (3H, s). ^{13}C NMR: 183.82, 171.56, 165.23, 164.99, 158.83, 143.89, 143.81, 143.73, 134.73, 133.74, 133.46, 133.36, 132.50, 132.27, 132.23, 130.62, 130.21, 130.05, 129.82, 129.43, 129.29, 128.97, 128.61, 128.49, 128.46, 128.41, 128.37, 128.14, 127.29, 127.25, 126.21, 113.41, 112.15, 92.24, 87.63, 81.36, 75.70, 69.60, 61.33, 55.24, 21.71. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{49}H_{41}NO_7S$ = 810.2504; observed = 810.2425

2',3'-O-dibenzoyl NaM. (10a)

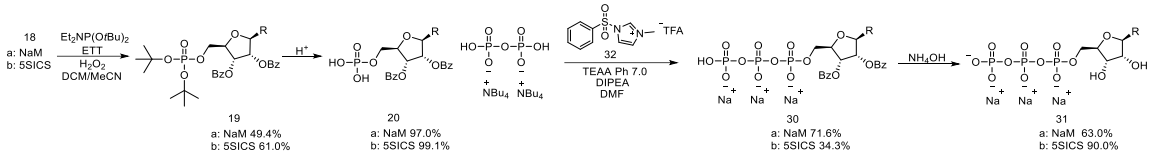
To a 25 mL round bottom flask, **9a** (180 mg, 233 µmol) was added and dissolved in 2% TsOH in DCM/MeOH 4:1 (1.7 mL). The solution was stirred and monitored via TLC using eluents Hexanes:EtOAc (7:3) Rf = 0.33 until the reaction was complete. The reaction was quenched with Saturated $NaHCO_3$ (10 mL) and extracted with DCM (10 mL) 3x. The combined organic layers wre then washed with DI water (10 mL) and Brine (10 mL) and dried over $NaSO_4$. The organic layer was then concentrated down and purified on flash column chromatography SiO_2 using eluents 9:1 Hexanes:EtOAc and 7:3 Hexanes:EtOAc. The purified fractions were then combined and evaporated down to obtain the title compound in 98.8% yield as a white solid. ^{1}H NMR (400 Mhz d-MeOH): δ 8.18 (1H, s), 7.95 (2H, d *J = 7.2 Hz*), 7.90 (2H, d *J = 7.2 Hz*), 7.84 (1H, d *J = 8.1 Hz*), 7.78 (1H, d *J = 8.1 Hz*), 7.55-7.61 (2H, m), 7.33-7.45 (6H, m), 7.27 (1H, s), 5.72-5.75 (1H, dd *J = 5.2 Hz*), 5.62-5.67 (2H, dd *J = 6.5 Hz*), 4.47-4.50 (1H, ddd *J = 7.0 Hz*), 4.05-4.09 (1H, dd *J = 12.4 Hz*), 3.93-3.97 (1H, dd *J = 12.4 Hz*), 3.84 (3H, s). ^{13}C NMR: 166.94, 166.64, 156.48, 135.88, 134.54, 130.79, 130.64, 130.60, 130.07, 130.01, 129.76, 129.59, 129.53, 129.46, 129.24, 128.92, 127.78, 127.56, 127.52, 124.90, 106.44, 83.05, 80.66, 77.80, 73.01, 62.47, 55.94. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{30}H_{26}O_7$ =521.1569; observed =521.1563.

2',3'-dibenzoyl 5SICS. (10b)

To a 25 mL round bottom flask, **9b** (790 mg, 1.02 mmol) was added and dissolved in 2% TsOH in DCM/MeOH 4:1 (7.5 mL). The solution was stirred and monitored via TLC using eluents Hexanes:EtOAc (7:3) Rf = 0.33 until the reaction was complete. The precipitate was purified via vacuum filtration using DCM (20 mL) as the wash.To further increase yield, the filtrate was evaporated down to a red crude oil and purified via SiO_2 flash chromatography using eluents 9:1 Hexanes:EtOAc and 7:3 Hexanes:EtOAc. The purified fractions were then combined and evaporated down to obtain the title compound in 91.2% yield and a light-yellow solid. ^{1}H NMR (400 Mhz, d_6-DMSO): δ 8.74 (1H, d J = 8.6 Hz), 8.45 (1H, d J = 7.5 Hz), 7.85-7.90 (5H, m), 7.60-7.68 (3H, m), 7.43-7.50 (5H, m), 7.23 (1H, d J = 7.5 Hz), 5.81-5.84 (1H, dd J = 5.1 Hz), 5.74-5.77 (1H, dd J = 5.2 Hz), 5.69 (1H, t J = 4.7 Hz), 4.67 (1H, ddd J= 5.0 Hz), 3.83-3.99 (2H, dd J = 12.5 Hz), 2.47 (3H, s). ^{13}C NMR: 183.82, 171.56, 165.23, 164.99, 158.83, 143.89, 143.81, 143.73,

134.73, 133.74, 133.46, 133.36, 132.50, 132.27, 132.23, 130.62, 130.21, 130.05, 129.82, 129.43, 129.29, 128.97, 128.61, 128.49, 128.46, 128.41, 128.37, 128.17, 127.29, 127.25, 126.21, 113.41, 112.15, 92.24, 87.63, 81.36, 75.70, 69.60, 61.33, 55.24, 21.71. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{29}H_{25}NO_6S$ = 538.1293; observed = 538.1285.

Protocol for the Synthesis of 5SICSTP & NaMTP



(2R,3R,4S,5S)-2-(((di-tert-butoxyphosphoryl)oxy)methyl)-5-(3-methoxynaphthalen-2-yl)tetrahydrofuran-3,4-diyl dibenzoate. (11a)

To a dry 25 mL round bottom flask equipped with a stir bar, **10a** (110 mg, 215 µmol) and 5-(ethylthio)-1H-tetrazole (49.5 mg, 386 µmol) was added followed by anhydrous DCM:MeCN 1:1 (0.11 M). Then, ditertbutyl diethylphosphoaramidite (69.5 mg, 279 µmol) was added and stirred overnight. The reaction was monitored via TLC using eluents Hexanes:EtOAc (7:3). Once completed, the solution was cooled down to -40 C and 30% H_2O_2 (197 µmol, 1.93 mmol) was added to the stirring solution. The solution was then allowed to warm up to room temperature and was allowed to stir for 1 hr. Once the reaction was completed (monitored via TLC using eleunts Hexanes:EtOAc (1:1)), the solution was then diluted with DCM (10 mL) and extracted with saturated $NaHCO_3$ (10 mL). The aqueous layer was extracted with DCM (10 mL) 3x and then the combined organic layers were then washed with DI Water (10 mL) and brine (10 mL) and dried over $NaSO_4$. The organic layer was then concentrated down to a thick oil and was purified via flash chromatography using eluents Hexanes:EtOAc (7:3->1:1) Rf = 0.50 (1:1). The purified fractions were then combined and concentrated down to give the title compound in 61.0 % yield as a clear oil. ^{1}H NMR (400 Mhz $CDCl_3$): δ 8.12 (1H, s), 7.99 (2H, d J =), 7.89 (2H, d J =), 7.85 (1H, d J =), 7.73 (1H, d J =), 7.30-7.58 (9H, m), 7.11 (1H, s), 5.83-5.86 (1H, dd J =), 5.64-5.67 (2H, dt J=), 4.62-4.66 (1H, m), 4.35-4.46 (2H, ddd J =), 3.85 (3H, s), 1.52 (9H, s), 1.49 (9H, s). ^{13}C NMR: 165.31, 165.07, 155.11, 134.37, 133.28, 133.21, 129.81, 129.78, 129.77, 129.35, 129.09, 128.76, 128.67, 128.56, 128.53, 128.42, 128.36, 128.23, 128.21, 126.82, 126.59, 126.40, 126.15, 123.92, 105.36, 82.79, 82.76, 79.85, 79.62, 76.13, 71.70, 65.83, 55.45, 30.45, 30.40, 30.01, 29.97, 29.94. ^{31}P NMR: -9.62 (1P, t, $J_{P,H}$ *= 5.8 Hz*). ^{31}P CPD NMR -9.62 (1P, s). **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{38}H_{43}O_{10}P$ =713.2484; observed =713.2438.

(2R,3R,4R,5R)-2-(((di-tert-butoxyphosphoryl)oxy)methyl)-5-(6-methyl-1-thioxoisoquinolin-2(1H)-yl)tetrahydrofuran-3,4-diyl dibenzoate. (11b)

To a dry 25 mL round bottom flask equipped with a stir bar, **10b** (450 mg, 873 µmol) and 4,5-Dicyanoimidazole (186 mg, 1.57 mmol) was added followed by anhydrous DMF (0.19 M). Then, ditertbutyl diethylphosphoaramidite (240 mg, 960 µmol) was added and stirred overnight. The reaction was monitored via TLC using eluents Hexanes:EtOAc (7:3). Once completed, the solution was cooled down to -40 C and 30% H_2O_2 (802 µmol, 7.86 mmol) was added to the stirring solution. The solution was then allowed to warm up to room temperature and was allowed to stir for 1 hr. Once the reaction was completed and monitored via TLC using eleunts Hexanes:EtOAc (1:1), the solution was then diluted with DCM (20 mL) and extracted with saturated NaHCO3 (20 mL). The aqueous layer was extracted with DCM (20 mL) 3x and then the combined organic layer was then washed with DI Water (20 mL) and brine (20 mL) and dried over NaSO4. The organic layer was then concentrated down to a thick oil and was purified via flash chromatography using eluents Hexanes:EtOAc (7:3-1:1) Rf = 0.50. The purified fractions were then combined and concentrated down to give the title compound in 49.4 % yield as a yellow oil. ^{1}H NMR (400 Mhz, CDCl3): δ 8.95 (1H, d *J = 8.4 Hz*), 8.10 (1H, d *J = 7.5 Hz*), 8.01-8.05 (3H, m), 7.89 (2H, d *J = 8.2 Hz*), 7.50-7.56 (2H, m), 7.31-7.41 (6H, m), 6.94 (1H, d *J = 7.3 Hz*), 5.77-5.84 (2H, m), 4.66-4.69 (1H, dt *J = 5.7 Hz*), 4.37-4.48 (2H, ddd J = 11.7 Hz), 2.48 (3H, s), 1.56 (9H, s), 1.54 (9h, s). ^{13}C NMR: 184.59, 165.26, 164.96, 143.86, 133.58, 133.40, 132.62, 132.50, 132.19, 130.26, 130.11, 129.85, 129.13, 128.89, 128.49, 128.47, 128.11, 126.25, 112.35, 91.42, 83.46, 83.39, 83.24, 83.17, 81.37, 81.28, 75.58, 70.15,

64.94, 64.88, 30.02, 30.00, 21.73. ^{31}P NMR: -9.96 (1P, t, J_H = *3.7 Hz*), ^{31}P CPD NMR: -9.96 (1P, s). **HRMS** (LCMS): m/z [M+Na]$^+$ calculated for $C_{37}H_{42}NO_9S$ = 731.2296; observed = 731.2287.

5'-O-phosphate-2',3'-dibenzoyl NaM. (12a)

To a 25 mL round bottom flask equipped with a stir bar, **11a** (90.0 mg, 130 µmol) was added and then dissolved in DCM (0.13 M) and then TFA (50.0 µmol, 652 µmol) was added and was allowed to stir overnight. The solution was then concentrated down to a clear oil and then precipitated out with DI water (3 mL) and sonication. The contents were then filtered off and the precipitate was collected washed with DI water (5 mL) and dried under vacuum to give the title compound in (73.1 mg, 97.0%) yield as a white solid. ^{1}H NMR (400 Mhz, MeOD): δ 8.24 (1H, s), 7.82-7.91 (7H, m), 7.61-7.67 (2H, m), 7.37-7.50 (7H, m), 7.36 (1H, s), 5.65-5.68 (1H, dd *J = 4.7 Hz*), 5.56-5.58 (2H, dd *J = 6.7 Hz*), 4.62-4.66 (1H, m), 4.32-4.37 (1H, m), 4.17-4.22 (1H, m), 3.80 (3H, s). ^{13}C NMR: 165.52, 165.25, 155.09, 134.49, 133.14, 129.40, 129.25, 129.20, 128.68, 128.62, 128.37, 128.20, 128.14, 128.06, 127.85, 127.52, 126.39, 126.17, 126.13, 123.51, 105.05, 81.66, 79.27, 76.40, 71.62, 61.08, 54.55. ^{31}P NMR: 0.77 (1P, m). ^{31}P CPD NMR: 0.77 (1P, br). **HRMS** (LCMS): m/z [M-H]$^+$ calculated for $C_{30}H_{27}O_{10}P$ =577.1269; observed =577.1266.

5'-O-phosphate-2',3'-dibenzoyl 5SICS. (12b)

20b To a 25 mL round bottom flask equipped with a stir bar, **11b** (295 mg, 417 µmol) was added and then dissolved in Dioxane (0.42 M) and then HCl in 4M Dioxane (281 µmol, 1.13 mmol) was added and was allowed to stir overnight. The solution was then concentrated down to a yellow oil and then precipitated out with DI water (5 mL) and sonication. The contents were then filtered off and the precipitate was collected, washed with DI Water (5 mL) and dried under vacuum to give the title compound in 99.1% yield as a yellow solid. ^{1}H NMR (400 Mhz, d_6-DMSO): 8.73 (1H, d *J = 8.5 Hz*), 8.23 (1H, d *J = 7.4 Hz*) 7.84-7.90 (5H, m), 7.62-7.68 (3H, m), 7.43-7.49 (5H, m) 7.18 (1H, d, *J = 7.4 Hz*), 5.82 (1H, t *J = 4.7 Hz*), 5.71 (1H, t *J = 5.3 Hz*), 4.84 (1H, s), 4.33 (2H, m), 2.46 (3H, s).^{13}C NMR: 183.09, 164.61, 164.24, 143.94, 133.92, 132.32, 131.62, 130.97, 130.30, 129.38, 129.27, 128.83, 128.80, 128.69, 128.57, 128.38, 126.51, 112.16, 90.75, 74.86, 70.18, 21.18. ^{31}P NMR: -1.07 (1P, t, $J_{P,H}$ = *8.7 Hz*). ^{31}P CPD NMR: -1.05 (1P, s). **HRMS** (LCMS): m/z [M-H]$^+$ calculated for $C_{29}H_{26}NO_9PS$ = 594.0993; observed = 594.0997.

5'-O-triphosphate-2'3'-dibenzoyl NaM (13a)

To a dry 4 mL vial purged with Ar, **12a** (90.0 mg, 156 µmol) and 1-Methyl-3-benzenesulfonylimidazolium triflate (69.5 mg, 187 µmol) were added and dissolved in DMF (1.2 mL). To the stirred solution anhydrous DIPEA (81.3 µmol, 467 µmol) was added and stirred for 1 min. This solution was added to a premade solution of TBA_2 Pyrophosphate (206 mg, 311 µmol) in DMF (1.2 mL) at 0 °C. The solution was allowed to stir for 3 h at rt and monitored via ^{31P} NMR by periodic removal of 100 µmol and adding 450 µmol of D_2O. Once completed the reaction was cooled to 0 °C and then quenched with 50 mM TEAA buffer (2 mL) Ph 7.0. The solution was then extracted with Chloroform (10 mL) 3x and then the aqueous layer was purified via reverse phase flash column chromatography C_{18} Using eluents 50 mM TEAA:MeCN 5%-40%. The purified fractions were combined and lyophilized down to a white solid. The product was passed through a Dowex 50W X8 cation exchange chromatography in Na^+ form. The eluted filtrate was then lypholized to give the title compound in (93.4 mg, 74.6% yield) as a white solid. ^{1}H NMR (400 Mhz, D_2O): δ 7.78 (1H, s), 7.63 (2H, d *J =7.4 Hz*), 7.54 (1H, d *J = 8.0 Hz*), 7.33 (1H, d *J =7.3 Hz*), 7.27 (2H, d *J =7.6 Hz*), 7.14-7.22 (2H, m), 7.07 (1H, t J =6.8 Hz), 7.00 (2H, t *J =7.2 Hz),* 6.73 (1H, s), 6.64 (1H, t *J = 6.5 Hz*), 6.47 (1H t *J = 7.0 Hz*), 5.88-5.91 (1H, dd *J = 6.1 Hz*), 5.79-5.82 (1H, dd *J = 4.2 Hz*), 5.36 (1H, d *J = 6.9 Hz*), 4.39-4.48 (3H, m), 3.37 (3H, s). ^{31}P NMR: -5.15 (1P, d J_P = *18.5 Hz*), -10.39 (1P, m), -20.31 (1P, t J_P = *18.5 Hz*). ^{31}P CPD NMR: -5.15 (1P, d J_P = *18.9 Hz*), -10.39 (1P, d J_P = *18.1 Hz*), -20.31 (1P, t J_P = *17.7 Hz*). Product is not soluble enough to obtain a carbon NMR. No detectable HRMS available.

5'-O-triphosphate-2',3'-dibenzoyl 5SICS. (13b)

To a dry 4 mL vial purged with Ar, **12b** (150 mg, 252 µmol) and 1-Methyl-3-benzenesulfonylimidazolium triflate (113 mg, 302 µmol) was added and dissolved in DMF (3.0 mL). To the stirring solution anhydrous

DIPEA (81.3 µmol, 467 µmol) was added and stirred for 1 min. This solution was added to a premade solution of TBA_2 Pyrophosphate (206 mg, 302 µmol) in DMF (3.0 mL) at 0 C°. The solution was allowed to stir for 3 h at room temperature and monitored via ^{31P} NMR by taking a 100 µmol aliquot out and 450 µmol of D_2O. Once completed the reaction was cooled to 0 C° and then quenched with 50 mM TEAA buffer (2 mL) Ph 7.0. The solution was then extracted with Chloroform (15 mL) 3x and then the aqueous layer was purified via reverse phase flash column chromatography C_{18} Using eluents 50 mM TEAA:MeCN 5%-40%. The purified fractions were combined and lypholized down to a yellow solid. The eluted filtrate was then lypholized to give the title compound in 34.3% yield as a yellow solid. The sodium salt was only partly soluble in water so only the ^{1}H and ^{31P} was concentrated enough to obtain a spectrum ^{1}H NMR (400 Mhz, D_2O): δ 8.45 (1H, d *J = 8.1 Hz*), 8.15 (1H, d *J = 6.5 Hz*), 8.07 (1H, d *J = 3.6 Hz*), 7.67 (2H, d *J = 7.5 Hz*), 7.60 (2H, d *J = 7.5 Hz*), 7.26-7.33 (2H, m), 7.07-7.17 (4H, m), 6.95 (2H, t *J = 6.7 Hz*), 6.85 (1H, d *J = 8.0 Hz*), 5.84 (2H, d *J = 19.1 Hz*), 4.74 (1H, m) 4.43 (2H, m), 2.69 (3H, s). ^{31}P NMR: -10.88 (1P, m), -11.82 (1P, m), -23.17 (1P, m). ^{31}P CPD NMR: -10.87 (1P, d J_P *= 18.3 Hz*), -11.83 (1P, d, J_P *= 14.1 Hz*), -23.15 (1P, t J_P *= 21.2 Hz*). Product is not soluble enough to obtain a carbon NMR. No detectable HRMS available.

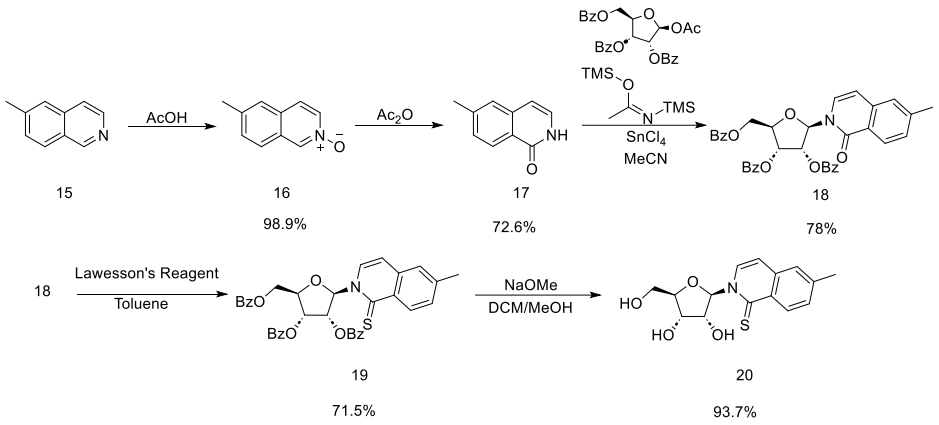
NaM-Triphosphate. (14a)

To a 20 mL vial equipped with a stir bar, **13a** was added (72.0 mg, 88 µmol) and then dissolved in DI Water (3 mL) then NH_4OH 30wt% (3 mL, 22.6 mmol) was added. The solution was allowed to stir over night and then lyophilized to a white powder. The contents were then purified via C18 Reverse Phase column chromatography using eluents 50 mM TEAA:MeCN 5%-30%. The purified fractions were combined and lyophilized down and further purified via EtOAc liquid extraction. The water layer was then subjected to a Dowex 50W X8 cation exchange column packed with 1M NaCl and washed with water. The filtrate was then lyophilized down to give the title compound in 63.0% yield as a white powder. ^{1}H NMR (400 Mhz, D_2O): δ 8.10 (1H, s), 7.99 (1H, d *J = 8.1 Hz*), 7.86 (1H, d *J = 8.1 Hz*), 7.55 (1H, t *J = 7.6 Hz*), 7.47 (1H, t *J = 7.6 Hz*), 7.38 (1H, s), 5.34 (1H, d *J = 2.6 Hz*), 4.32-4.43 (5H, m), 4.00 (3H, s). ^{13}C NMR: 181.51, 155.23, 134.02, 128.60, 128.20, 128.09, 126.92, 126.60, 126.37, 124.26, 105.84, 81.12, 75.44, 70.22, 65.22, 55.55. ^{31}P NMR: -5.52 (1P, d J_P = 19.6 Hz), -10.55 (1P, m), -21.17 (1P, *t* J_P *= 19.0 Hz*). ^{31}P CPD NMR: -5.52 (1P, d J_P *= 19.5 Hz*), -10.55 (1P, d J_P *= 18.5 Hz*), -21.17 (1P, t J_P *= 19.4 Hz*). No detactable HRMS available.

5SICS-Triphosphate(14b)

To a 20 mL vial equipped with a stir bar, **13b** was added (65.0 mg, 79 µmol) and then dissolved in DI Water (5 mL) then NH_4OH 30wt% (5 mL, 37.7 mmol) was added. The solution was allowed to stir over night and then lyophilized to a yellow powder. The contents were then purified via C_{18} Reverse Phase column chromatography using eluents 50 mM TEAA:MeCN 5%-40%. The purified fractions were combined and lyophilized down and further purified via EtOAc liquid extraction. The water layer was then subjected to a Dowex 50W X8 cation exchange column packed with 1M NaCl and washed with water. The filtrate was then lyophilized down to give the title compound in 90.0% yield as a yellow powder. ^{1}H NMR (400 MHz, D_2O): δ 8.62 (1H, d J = 8.5 Hz), 8.19 (1H, d J = 7.5 Hz), 7.42 (1H, s), 7.41 (1H, d J = 9.3 Hz), 7.21 (1H, d J = 7.5 Hz), 7.09-7.17 (1H, dd J = 18.5 Hz), 4.42-4.55 (5H, m), 2.46 (3H, s). ^{13}C NMR: 181.52, 145.04, 133.05, 130.57, 130.54, 130.49, 128.28, 126.37, 114.25, 95.06, 82.26, 75.53, 67.87, 63.93, 20.89. ^{31}P NMR: -5.33 (1P, d J_P *= 19.5 Hz*), -10.76 (1P, m), -20.86 (1P, t J_P *= 19.3 Hz*). ^{31}P CPD NMR: -5.33 (1P, d J_P *= 18.8 Hz*), -10.75 (1P, d J_P *= 19.6 Hz*), -20.87 (1P, t J_P *= 18.6 Hz*). No detectable HRMS available.

Experimental Steps for the Synthesis of 5SICS Nucleoside



6-methylisoquinoline N-oxide. (16)

To a 25 mL round bottom flask with a stir bar, **15** (100 mg, 698 µmol) was added followed by AcOH (3.00 mL, 52.4 mmol) and 30% H_2O_2 (185 µmol, 1.82 mmol). The solution was then heated to 90 ^oC and was allowed to stir for 3 h. The reaction was monitored via TLC then the solution was concentrated down and diluted with DCM and extracted with saturated $NaHCO_3$. The aqueous layer was extracted 3x with DCM and the combined organic layer was washed with DI water and brine and dried over $NaSO_4$. The organic layer was then concentrated down and the crude material was purified over column chromatography using eluents Hexanes:EtOAc:MeOH (3:6:1) Rf = 0.2. The purified fractions were then concentrated down to give the title compound in 98.9% yield as a white solid. ^1H NMR (400 MHz, $CDCl_3$): δ 8.71 (1H, s), 8.09 (1H, d *J =7.1 Hz*), 7.62 (1H, d *J =8.5 Hz*) 7.56, (1H, d *J = 7.4 Hz*), 7.55 (1H, s), 7.54 (1H d *J = 8.4 Hz*), 2.51 (3H, s). ^{13}C NMR: 139.88, 136.77, 136.23, 131.89, 129.39, 127.85, 125.89, 125.01, 123.74, 21.93. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{10}H_9NO$ = 182.0582; observed = 182.0574

6-methylisoquinolone. (17)

To a dry 100 mL round bottom flask equipped with a stir bar, **16** (1350 mg, 8.48 mmol) was added and purged with Ar. Then, freshly distilled Ac_2O (20 mL, 0.210 mol) was added and the solution was refluxed for 4 h. The reaction was monitored with TLC and was concentrated down via rotary evaporation. Then the crude material was dissolved in THF (5 mL) and 1M NaOH (10 mL) and the reaction was stirred and refluxed overnight. The reaction was followed by TLC using EtOAc (Rf = 0.5). Then, THF was evaporated via rotary evaporation and the crude material was dissolved in DCM and extracted with 1M NaOH. The aqueous layer was extracted 2x (20 mL) with DCM and then the combined organic layer was then washed with DI Water and brine and dried over $NaSO_4$. The organic layer was then concentrated down to a thick oil and was further purified via column chromatography using eluents Hexanes:EtOAc (7:3 –>0:1). The purified fractions were combined and concentrated down to give the title compound in 72.6% yield as a yellow solid. The product was characterized via NMR: (400 Mhz, CDCl3): δ ^1H NMR : 10.21 (1H br), 8.28 (1H, d J = *8.1 Hz*), 7.33 (1H, s), 7.31 (1H, d), 7.08 (1H, d *J = 7.1 Hz*) 6.47 (1H, d *J = 7.2 Hz),* 2.47 (3H, s). ^{13}C NMR: 163.89, 143.48, 138.37, 128.67, 127.52, 126.17, 126.12, 124.20, 106.66, 21.99. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{10}H_9NO$ = 182.0582; observed = 182.0574

(2R,3R,4R,5R)-2-((benzoyloxy)methyl)-5-(6-methyl-1-oxoisoquinolin-2(1H)-yl)tetrahydrofuran-3,4-diyl dibenzoate. (18)

To a dry 100 mL round bottom flask equipped with a stir bar and purged with Ar, **17** (0.970 g, 6.09 mmol) followed by anhydrous MeCN (0.125 M) then N,Obis(trimethylsilyl)acetamide (1.94 mL, 7.92 mmol) was added and allowed to stir for 10 mins. Then 1-O-acetyl-2,3,5-tri-O-benzoylbeta-D-ribofuranose (3.22 g,

6.39 mmol) The solution was then cooled to 0 °C and then $SnCl_4$ (3.58 mL, 30.5 mmol) was added and the solution was allowed to stir overnight at room temperature. The reaction was monitored via TLC using eluents Hexanes:EtOAc, then concentrated down via rotary evaporation. The crude mixture was then diluted with EtOAc and then saturated $NaHCO_3$ was added and shaken until a white precipitated stopped forming. The two organic and aqueous layer were then filtered and the filtrate was separated. The aqueous layer was then extracted with EtOAc 3x (20 mL) and then the combined organic layer was washed with DI water and brine and dried over $NaSO_4$. The organic layer was then concentrated down to a thick oil and was further purified with column chromatography using eluents Hexanes:EtOAc (3:1) Rf = 0.43. The pure fractions were then combined and concentrated down to give the title compound in 78.8% yield as a white foam. The product was characterized via NMR (400 MHz $CDCl_3$) ^{1}H NMR : δ 8.25 (1H, d *J =8.2 Hz*), 8.13 (1H, d *J =7.8 Hz*), 7.95 (4H, t, *J=8.7 Hz*), 7.59 (1H, t, *J=7.2 Hz*), 7.45-7.54 (4H, m), 7.35 (4H, t J =*7.7 Hz*), 7.26 (1H, d *J = 7.9 Hz*), 7.23 (1H, s), 6.77 (1H, d *J = 5.0 Hz*), 6.32 (1H, d *J = 7.5 Hz*), 5.97 (1H, t *J = 5.6 Hz*), 5.86 (1H, t *J = 5.5 Hz*), 4.85-4.87 (1H, dd, *J = 12.3 Hz*), 4.73-4.75 (1H, ddd), 4.67-4.70 (1H, dd, *J= 12.3 Hz*), 2.50 (3H, s) ^{13}C NMR:166.31, 165.47, 165.39, 162.12, 143.62, 136.96, 133.67, 133.62, 133.52, 130.06, 129.97, 129.92, 129.67, 128.99, 128.97, 128.75, 128.68, 128.57, 128.55, 128.24, 126.61, 125.96, 123.93, 107.06, 88.12, 80.13, 74.65, 71.36, 64.02, 60.49. 21.89, 21.14, 14.32. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{36}H_{29}NO_8$ = 626.1791; observed = 626.1761

(2R,3R,4R,5R)-2-((benzoyloxymethyl)-5-(6-methyl-1-thioxoisoquinolin-2(1H)-yl)tetrahydrofuran-3,4-diyl dibenzoate. 5SICS-tribenzoyl (19)

To a dry 100 mL round bottom flask equipped with a stir bar and purged with Ar, compound **18** (1.50 g, 2.54 mmol) was added followed by Lawesson's reagent (2.06 g, 5.09 mmol) and dissolved in anhydrous dioxane (0.13 M). The solution was then heated to reflux for 16 h and monitored via TLC. The solution was then cooled to room temperature and then concentrated via rotary evaporation. The crude material was then purified via column chromatography using eluents Hexanes:DCM (7:3; 1:1; 0:1). To give the title compound in 71.5% yield as a yellow solid. The product was characterized via NMR (400 MHz $CDCl_3$) ^{1}H NMR : δ 8.91 (1H, d *J =8.5 Hz*), 8.13 (2H, d *J =7.2 Hz*), 8.05 (2H, d *J =7.2 Hz*), 7.90 (1H, d *J =7.5 Hz*), 7.85 (2H, d *J= 7.2 Hz*), 7.81 (1H, d *J =2.9 Hz*), 7.63 (1H, t *J =7.2 Hz*), 7.57 (1H, t *J =7.2 Hz*), 7.49 (3H, t *J =7.9 Hz*), 7.43 (2H, t *J =7.7 Hz*), 7.28-7.36 (4H, m), 6.64 (1H, d *J =7.6 Hz*), 4.94-4.97 (1H, dd *J =5.3 Hz*), 5.82-5.86 (1H, dd *J =7.2 Hz*), 4.93-4.97 (1H, dd *J =12.5 Hz*), 4.84-4.86 (1H, ddd), 4.67-4.71 (1H, dd *J =12.3 Hz*), 2.46 (3H, s). ^{13}C NMR: 184.11, 166.07, 165.22, 164.94, 143.87, 133.59, 133.45, 132.28, 132.21, 130.38, 130.07, 129.94, 129.86, 129.78, 129.45, 129.12, 128.72, 128.65, 128.45, 128.43, 126.99, 126.17, 112.23, 92.11, 79.85, 75.49, 69.70, 62.81, 21.65. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{36}H_{29}NO_7S$ = 642.1565; observed = 642.1488

5SICS. **(20)**

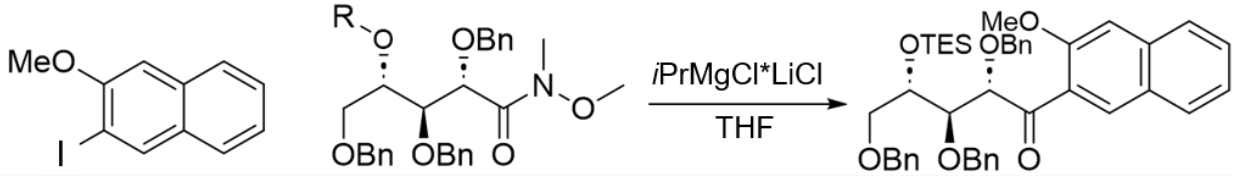
15. To a 50 mL round bottom flask equipped with a stir bar, **19** (532 mg, 858 µmol) in anhydrous DCM (10 mL) was cooled to 0 °C and 30% NaOMe (141 mg, 2.61 mmol) was added. The mixture was stirred for 2 hr at 0 °C and then 30 min at room temperature. The reaction mixture was extracted with EtOAc (50 mL × 3) and saturated $NaHCO_3$ aq. (50 mL). The combined organic layers were washed with DI Water and brine and dried over anhydrous Na_2SO_4. The organic layer was then concentrated via rotary evaporation to give the title compound in 93.7% yield as a yellow solid. The product was characterized via NMR (400 MHz $CDCl_3$) ^{1}H NMR : δ 8.91 (1H, d *J =8.7 Hz*), 8.39 (1H, d *J =7.7 Hz*), 7.46 (1H, s), 7.41 (1H, d *J =8.8 Hz*), 7.07 (1H, s), 7.01 (1H, d *J =6.8 Hz*), 4.22 (1H m), 4.17 (1H, m), 4.05 (1H, m), 3.88 (1H, m), 2.48 (3H, s). ^{13}C NMR: 183.87, 144.99, 134.19, 132.58, 130.87, 130.01, 127.26, 112.71, 96.90, 85.40, 77.28, 69.53, 60.98, 21.58. **HRMS** (LCMS): m/z $[M+Na]^+$ calculated for $C_{15}H_{17}NO_4S$ 330.0778; observed = 330.0764.

Tables S1-S2:Optimization of the Weinreb Amide Ketone Synthesis

Table S1. Optimization of the synthesis of protected weinreb amides.

Entry	SiRCl	Equivalence	Solvent	Yield %	Product
1	TBDPSCl	1.20	CH_2Cl_2	NR	-
2	TBDMSCl	1.20	CH_2Cl_2	49.3	3b
3	TBDMSCl	2.60	CH_2Cl_2	71.0	3b
4	TBDMSCl	5.00	CH_2Cl_2	53.2	3b
5	TBDMSCl	6.00	CH_2Cl_2	68.9	3b
6	TESCl	2.00	DMF	98.4	3a

Table S2. Optimization of Weinreb amide ketone synthesis with various protecting groups.



Entry	Reactant	Equivalence of 2-iodo-3-methoxynapthalene	Yield %	Product
1	3b	1.05	53.5	4b
2	3b	1.05	60.7	4b
3	3b	1.50	75.2	4b
4	3b	2.00	94.4	4b
5	3a	2.00	98.4	4a

Tables S3-S4 oligonucleotide and RNA sequences

Table S3. List of Oligonucleotide Sequences.

Oligo # / Sequence	UBP Oligo Template 5'-3'
Oligo # 01	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**CAG**ATGGTGATGGTGATGATGCATTCTT
CAG	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 02	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**AYA**ATGGTGATGGTGATGATGCATTCTT
AYA	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 03	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**AYC**ATGGTGATGGTGATGATGCATTCTT
AYC	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 04	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**AYG**ATGGTGATGGTGATGATGCATTCTT
AYG	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 05	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**AYT**ATGGTGATGGTGATGATGCATTCTT
AYT	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 06	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**CYA**ATGGTGATGGTGATGATGCATTCTT
CYA	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 07	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**CYC**ATGGTGATGGTGATGATGCATTCTT
CYC	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 08	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**CYG**ATGGTGATGGTGATGATGCATTCTT
CYG	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 09	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**CYT**ATGGTGATGGTGATGATGCATTCTT
CYT	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 10	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**GYA**ATGGTGATGGTGATGATGCATTCTT
GYA	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 11	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**GYC**ATGGTGATGGTGATGATGCATTCTT
GYC	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 12	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**GYG**ATGGTGATGGTGATGATGCATTCTT
GYG	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 13	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**GYT**ATGGTGATGGTGATGATGCATTCTT
GYT	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 14	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**TYA**ATGGTGATGGTGATGATGCATTCTT
TYA	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 15	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**TYC**ATGGTGATGGTGATGATGCATTCTT
TYC	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 16	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**TYG**ATGGTGATGGTGATGATGCATTCTT
TYG	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 17	CTACTACACCCAGCTATCCACCGGCTGCGGGCTCAT**TYT**ATGGTGATGGTGATGATGCATTCTT
TYT	TCCTCCTTCGGTAAAGTTAACCCTATAGTGAGTCGTATTACGCC
Oligo # 18	T(mG)TGCGAGGGGGGGGACTTGAACCCCCACGTCCGTAAGGACACTAACAC**GXC**AAGCTAGCG
GXC	CGTCTACCAATTCCGCCACCTTCGCTATAGTGAGTCGTATTACGCC
Oligo # 19	T(mG)TGCGAGGGGGGGGACTTGAACCCCCACGTCCGTAAGGACACTAACAC**AXC**AAGCTAGCG
AXC	CGTCTACCAATTCCGCCACCTTCGCTATAGTGAGTCGTATTACGCC
Oligo # 20	T(mG)TGCGAGGGGGGGGACTTGAACCCCCACGTCCGTAAGGACACTAACAC**GXT**AAGCTAGCG
GXT	CGTCTACCAATTCCGCCACCTTCGCTATAGTGAGTCGTATTACGCC

Table S4. List of RNA sequences prepared by transcription from table S3.

RNA Sequence	RNA Template 5'-3'
mRNA # 01 CUG	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**CUG**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 02 AXA	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**AXA**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 03 AXC	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**AXC**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 04 AXG	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**AXG**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 05 AXU	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**AXU**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 06 CXA	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**CXA**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 07 CXC	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**CXC**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 08 CXG	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**CXG**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 09 CXU	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**CXU**AGGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 10 GXA	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**GXA**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 11 GXC	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**GXC**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 12 GXG	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**GXG**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 13 GXU	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**GXU**AGGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 14 UXA	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**UXA**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 15 UXC	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**UXC**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 16 UXG	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**UXG**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
mRNA # 17 UXU	GGGUUAACUUUACCGAAGGAGGAAAGAAUGCAUCAUCACCAUCACCAU**UXU**AUGAGCCCGCAGCCGGUGGAUAGCUGGGUGUAGUAG
tRNA # 01 GYC	GCGAAGGUGGCGGAAUUGGUAGACGCGCUAGCUU**GYC**GUGUUAGUGUCCUUACGGACGUGGGGGUUCAAGUCCCCCCCCUCGCACA
tRNA # 02 GYU	GCGAAGGUGGCGGAAUUGGUAGACGCGCUAGCUU**GYU**GUGUUAGUGUCCUUACGGACGUGGGGGUUCAAGUCCCCCCCCUCGCACA
tRNA # 03 AYC	GCGAAGGUGGCGGAAUUGGUAGACGCGCUAGCUU**AYC**GUGUUAGUGUCCUUACGGACGUGGGGGUUCAAGUCCCCCCCCUCGCACA

Tables S5 and S6: MALDI-MS calculated and observed peaks

Table S5. Mass Spec Table summary of the digestion products of NaM mRNAS NXN

UBP Codon	AXA	AXG	GXA	GXG
[Calc+H] Fragments	1641.25 – AAAAU 1657.25 – AGAAU 1664.27 - AXAAU	1657.25 – AAGAU 1673.24 – AGGAU 1680.27 - AXGAU	1657.25 – GAAAU 1673.24 – GGAAU 1680.27 - GXAAU	1673.24 – GAGAU 1689.24 – GGGAU 1696.26 - GXGAU
-NaMTP Fragments Detected	1641.61 - AAAU 1657.40 - AGAAU	1657.43 - AAGAU 1673.43 - AGGAU	1657.47 - GAAAU 1673.47 - GAGAU	1673.16 - GAGAU 1689.14 - GGGAU
+NaMTP Fragments Detected	1664.32 - AXAAU	1680.23 - AXGAU	1680.43 - GXGAU	1696.14 - GXGAU

Table S6. Mass spec data table of the calculated and observed misincorporations or UBP incorporation at various concentrations of 5SICS-tRNA$_{GYC}$ using NaM-mRNA$_{GXC}$.

Positive Control	MH_6LMSPNPVDSWV EM [M+H]: 2240.00	MH_6LMSPNPVDSWV Obsd [M+H]: 2240.26
0 uM 5SICS-tRNA$_{GYC}$	MH_6VMSPNPVDSWV EM [M+H]: 2225.99 MH_6DMSPNPVDSWV EM [M+H]: 2241.84	MH_6VMSPNPVDSWV Obsd [M+H]: 2225.95 MH_6DMSPNPVDSWV Obsd [M+H]: 2241.95
25 uM 5SICS-tRNA$_{GYC}$	MH_6VMSPNPVDSWV EM [M+H]: 2225.99 MH_6DMSPNPVDSWV EM [M+H]: 2241.84	MH_6VMSPNPVDSWV Obsd [M+H]: 2225.95 MH_6DMSPNPVDSWV Obsd [M+H]: 2241.84
50 uM 5SICS-tRNA$_{GYC}$	MH_6VMSPNPVDSWV EM [M+H]: 2225.99 MH_6LMSPNPVDSWV EM [M+H]: 2240.00 MH_6DMSPNPVDSWV EM [M+H]: 2241.84	MH_6VMSPNPVDSWV Obsd [M+H]: 2225.76 MH_6LMSPNPVDSWV Obsd [M+H]: 2240.69 MH_6DMSPNPVDSWV Obsd [M+H]: 2241.36
80 uM 5SICS-tRNA$_{GYC}$	MH_6LMSPNPVDSWV EM [M+H]: 2240.00 MH_6DMSPNPVDSWV EM [M+H]: 2241.84	MH_6LMSPNPVDSWV Obsd [M+H]: 2239.55 MH_6DMSPNPVDSWV Obsd [M+H]: 2241.95
100 uM 5SICS-tRNA$_{GYC}$	MH_6LMSPNPVDSWV EM [M+H]: 2240.00	MH_6LMSPNPVDSWV Obsd [M+H]: 2240.26

In Vitro Transcriptions of UBP Supporting Data Fig S1-S3

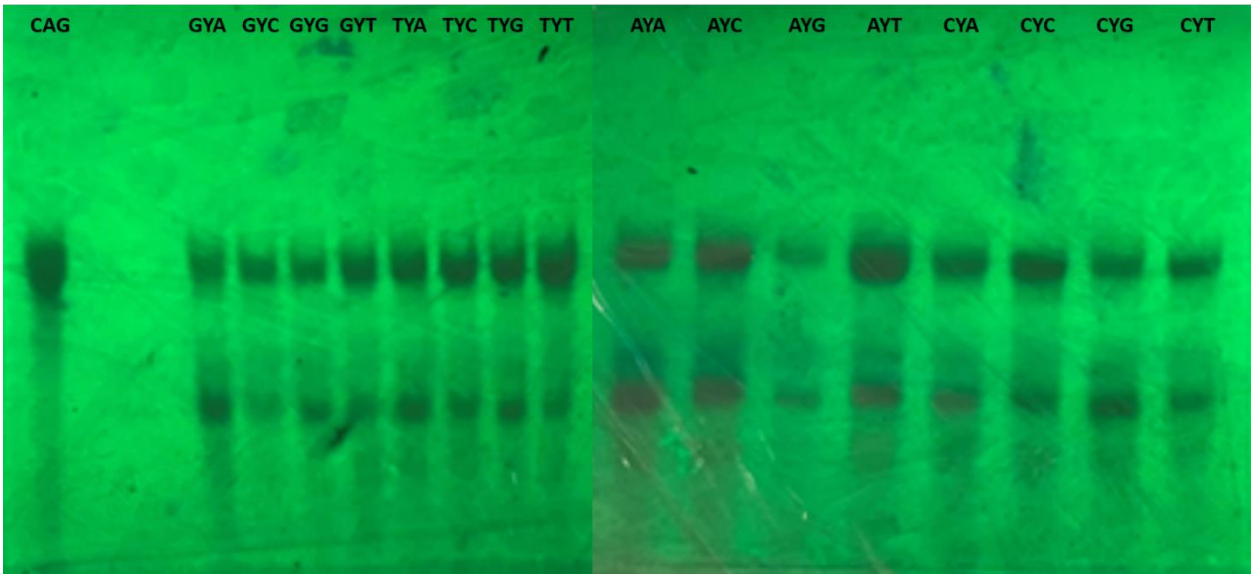


Figure S1. In vitro transcription of 5SICS Oligos 1-17 NYN without the presence of NaM-TP. Oligo # 01 CAG was added as a positive control. The gels were imaged by UV-shadowing.

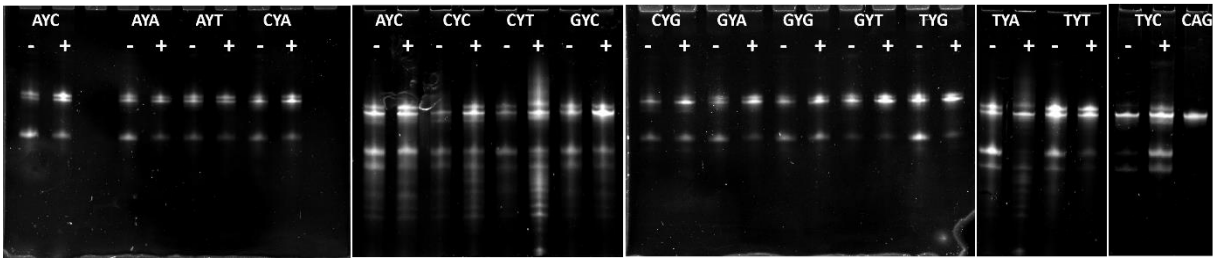


Figure S2. In vitro transcriptions of 5SICS Oligos 1-17 NYN with (+) and without (-) NaMTP. Lanes are labeled by the oligonucleotide template codon name (Table S2). Gels were Sybr green stained and imaged.

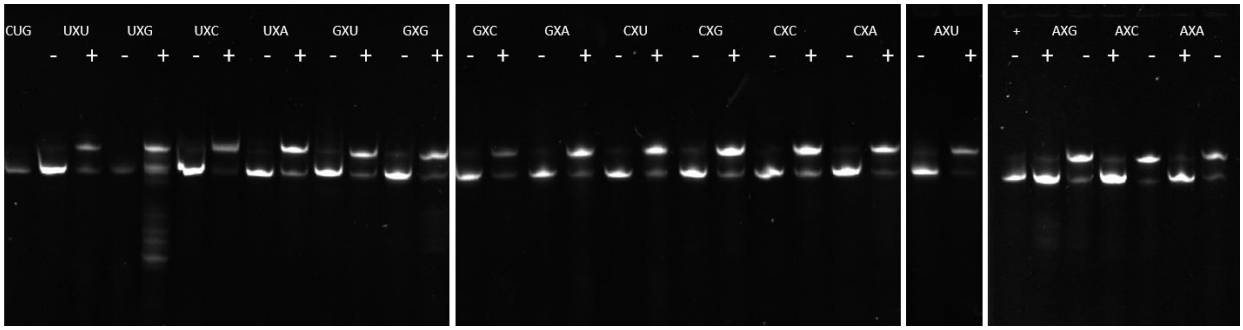


Figure S3. Reverse transcriptions of NaM mRNAs 1-17 NXN (Table S3) that were previously transcribed with and without NaMTP. Lanes labeled "-" are the NaM mRNA NXN templates transcribed without NaMTP and "+" are the NaM mRNA NXN templates transcribed with 2.5 mM NaMTP. Gels were Sybr green stained and imaged.

UBP Reverse Transcription Sequencing Data Fig S4

Figure S4. UBP DNA Sequencing bar graph of mRNA 11 GXC after reverse transcription using SSII and PCR amplification. Each bar graph represents which letter was read at the UBP site from the reverse transcription of mRNA 11 GXC. Fraction read is the percentage of reads with each of these 5 outcomes.

RNAse A Digestion NaM mRNA Fragments Fig. S5-S12

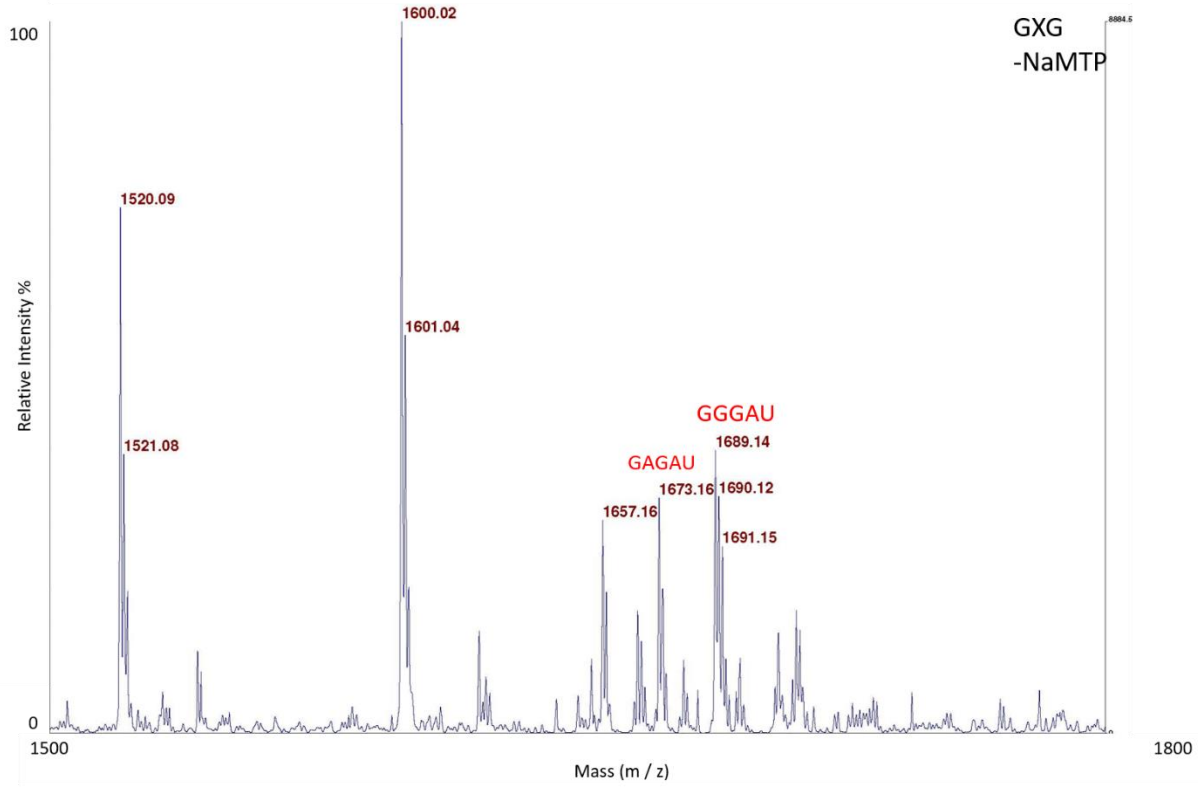


Figure S5. MALDI-MS spectrum of 5SICS Oligo-7 CYC forward transcribed without NaMTP after RNAse digestion. GAGAU calculated: 1673.24, observed: 1673.16. GGGAU calculated: 1689.24, observed: 1689.14.

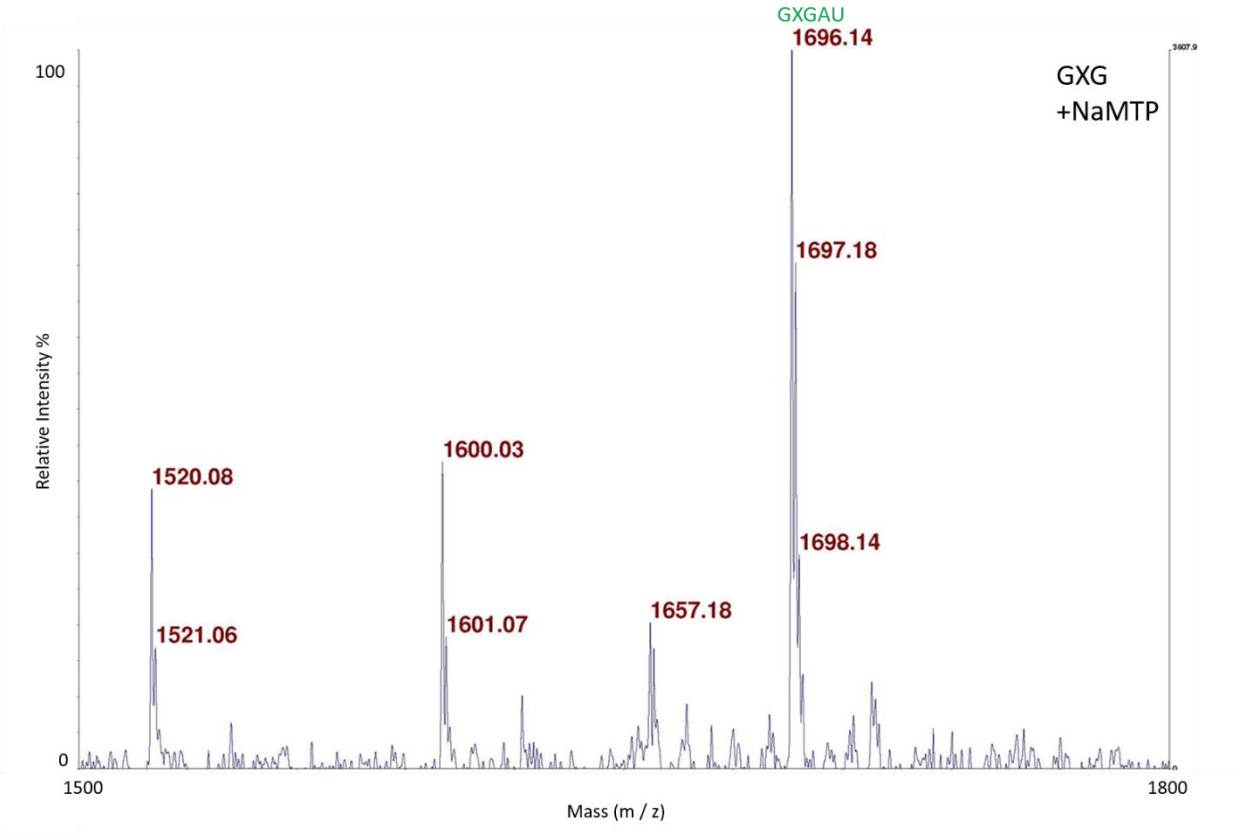


Figure S6. MALDI-MS spectrum of 5SICS Oligo-7 CYC forward transcribed with 2.5 mM NaMTP after RNAse digestion. GXGAU calculated: 1696.26, observed: 1696.14.

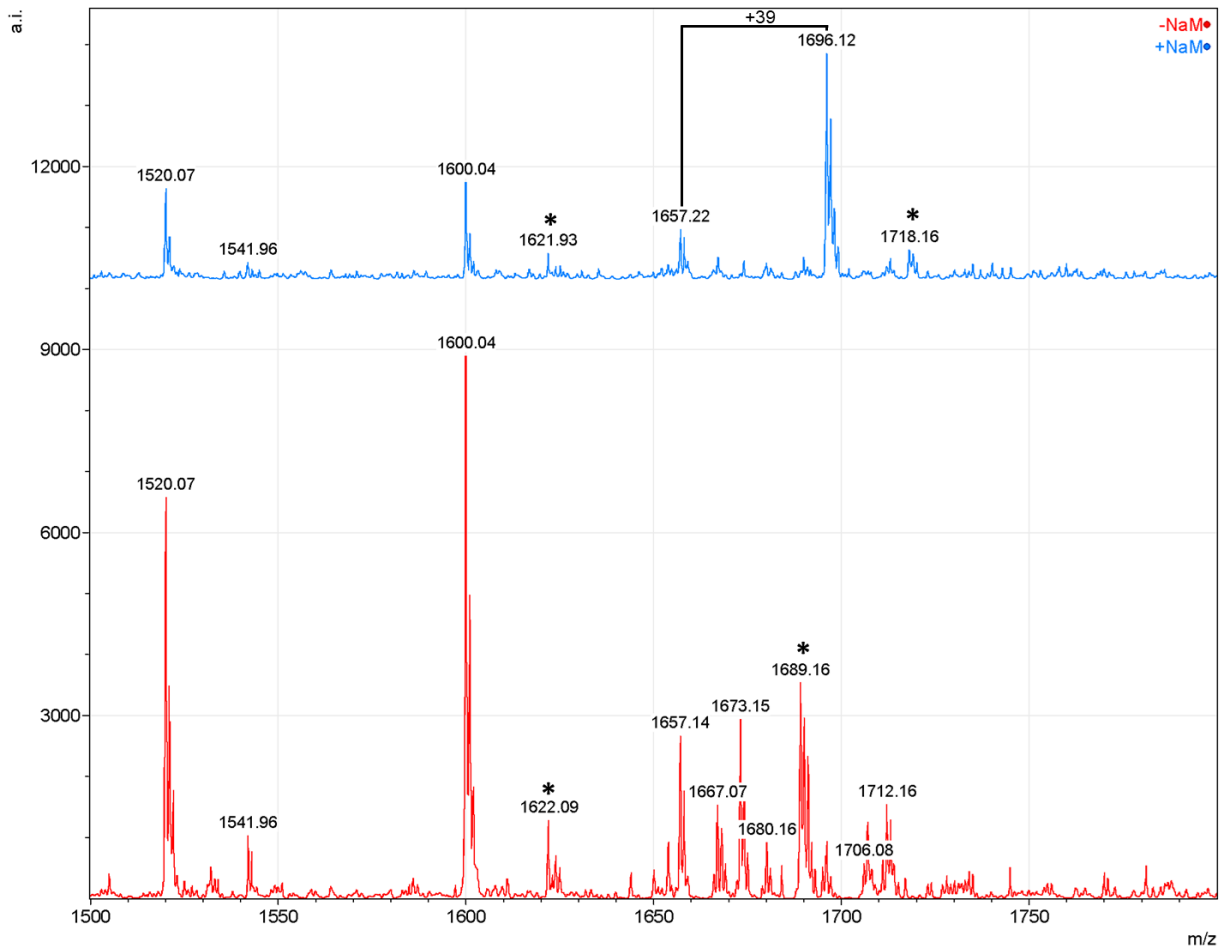


Figure S7. Overlayed MALDI-MS spectrum of Figure S5-6. Detected +22 Da Na^+ adducts are labeled in black asteriks. The expected NaM Fragment labeled with +39 (blue spectrum) from 1657.22.

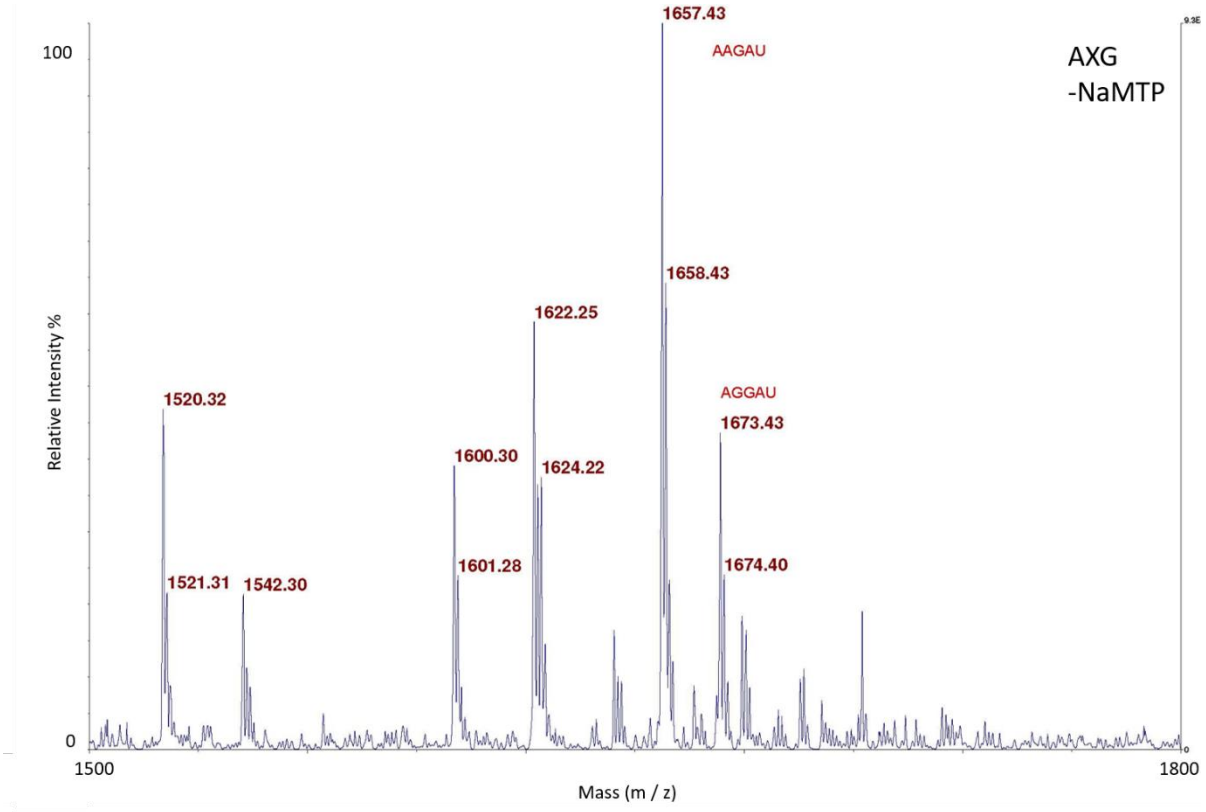


Figure S8. MALDI-MS spectrum of 5SICS Oligo-9 CYT forward transcribed without NaMTP after RNAse digestion. AAGAU calculated: 1657.25, observed: 1657.43. AGGAU calculated: 1673.24, observed: 1673.43.

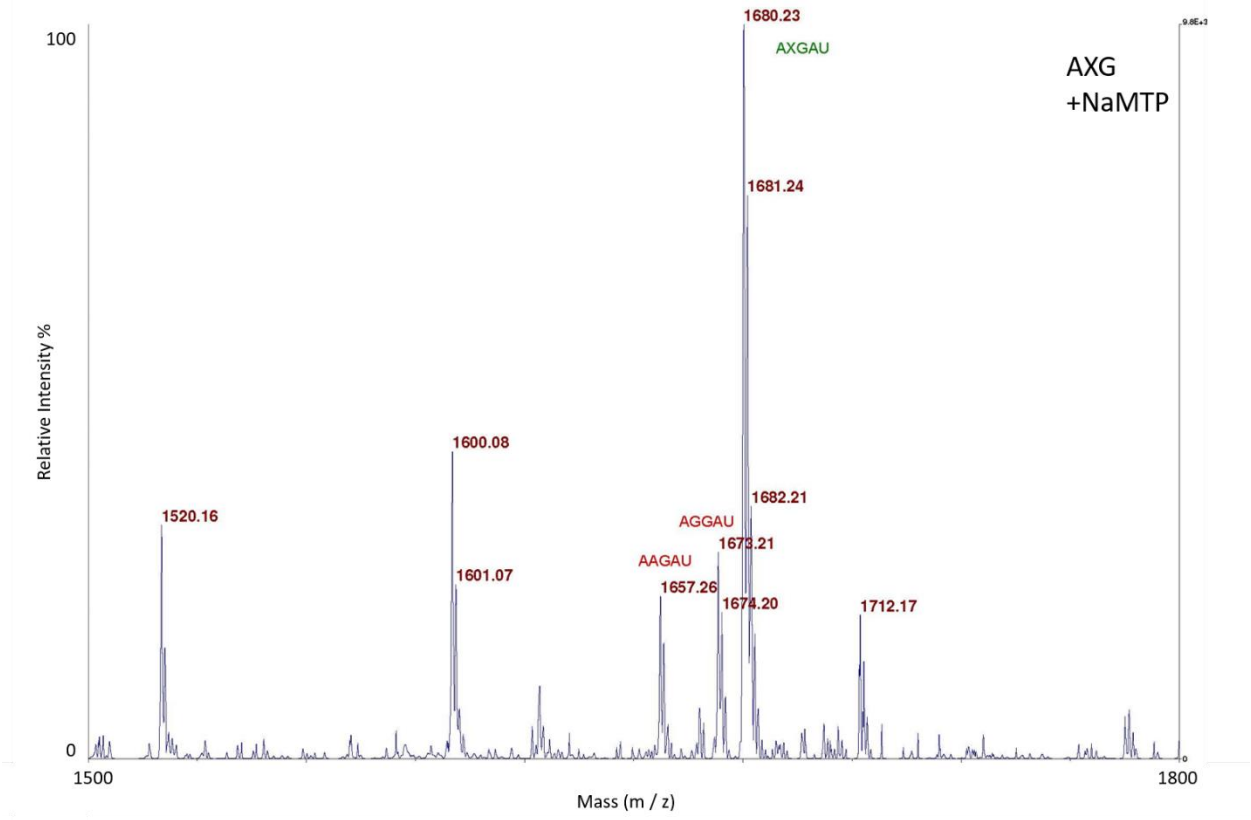


Figure S9. MALDI-MS spectrum of 5SICS Oligo-9 CYT forward transcribed with 2.5 mM NaMTP after RNAse digestion. AAGAU calculated: 1657.25, observed: 1657.26 (Background). AGGAU calculated: 1673.24, observed: 1673.43. AXGAU calculated: 1680.27, observed: 1680.23.

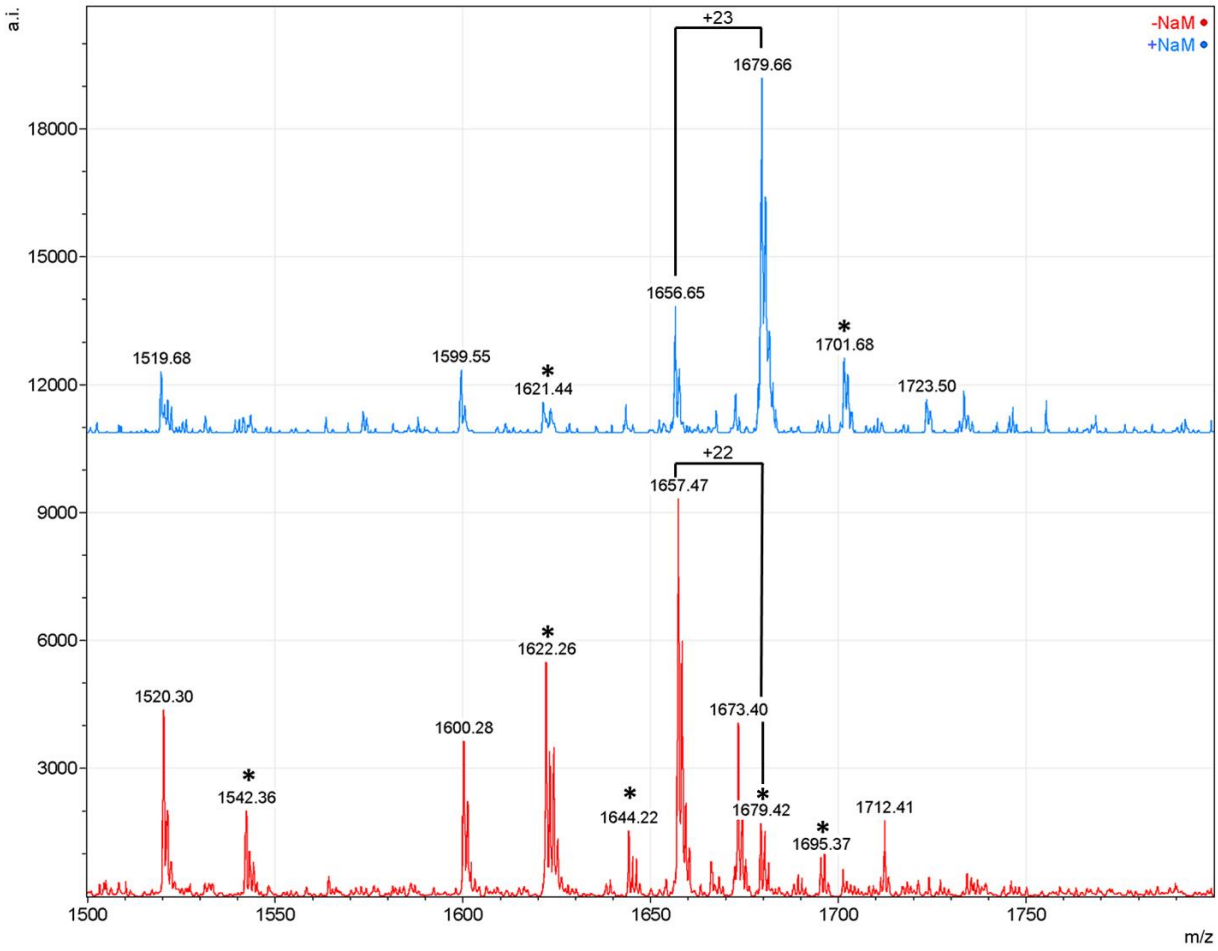


Figure S10. Overlayed MALDI-MS spectrum of Figure S8-9. Detected +22 Da Na^+ adducts are labeled in black asterisk. The expected NaM Fragment labeled with +23 (blue spectrum) and +22 Na^+ adduct is labeled from 1657.47 to 1679.42 peak.

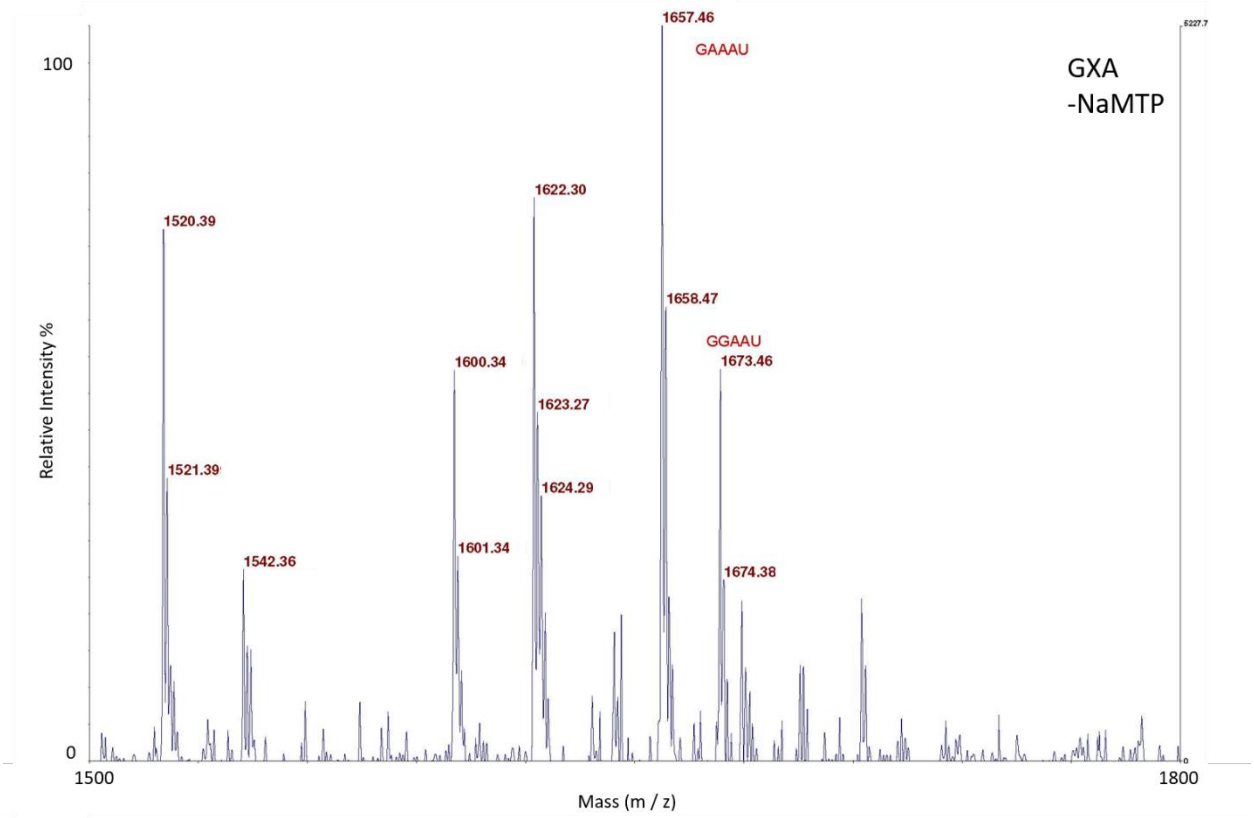


Figure S11. MALDI-MS spectrum of 5SICS Oligo-15 TYC forward transcribed without NaMTP after RNAse digestion. GAAAU calculated: 1657.25, observed: 1657.47. GGAAU calculated: 1673.24, observed: 1673.47.

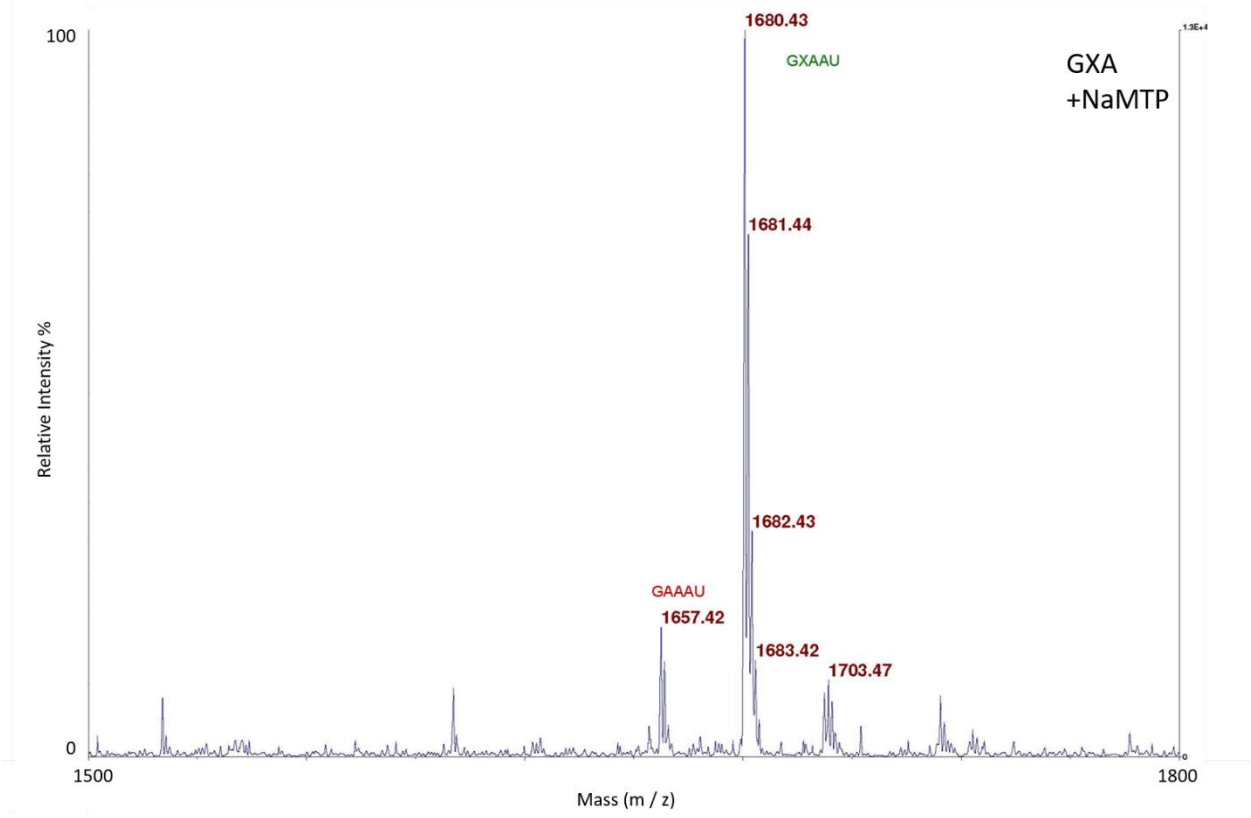


Figure S12. MALDI-MS spectrum of 5SICS Oligo-15 TYC forward transcribed with 2.5 mM NaMTP after RNAse digestion. GAAAU calculated: 1657.25, observed: 1657.42 (Background). GXAAU calculated: 1680.27, observed: 1680.43.

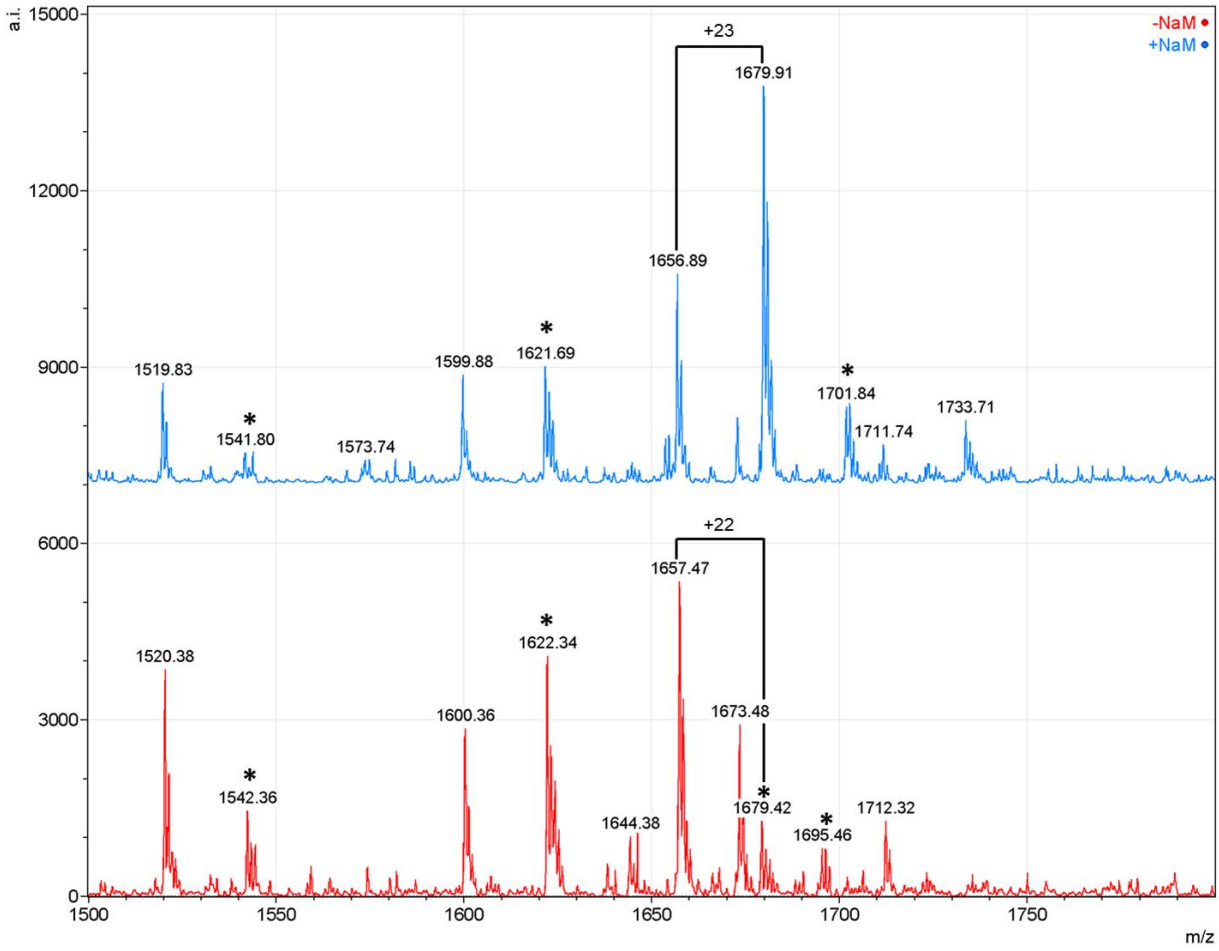


Figure S13. Overlayed MALDI-MS spectrum of Figure S11-12. Detected +22 Da Na^+ adducts are labeled in black asterisk. The expected NaM Fragment labeled with +23 (blue spectrum) and +22 Na^+ adduct is labeled from 1657.47 to 1679.42 peak.

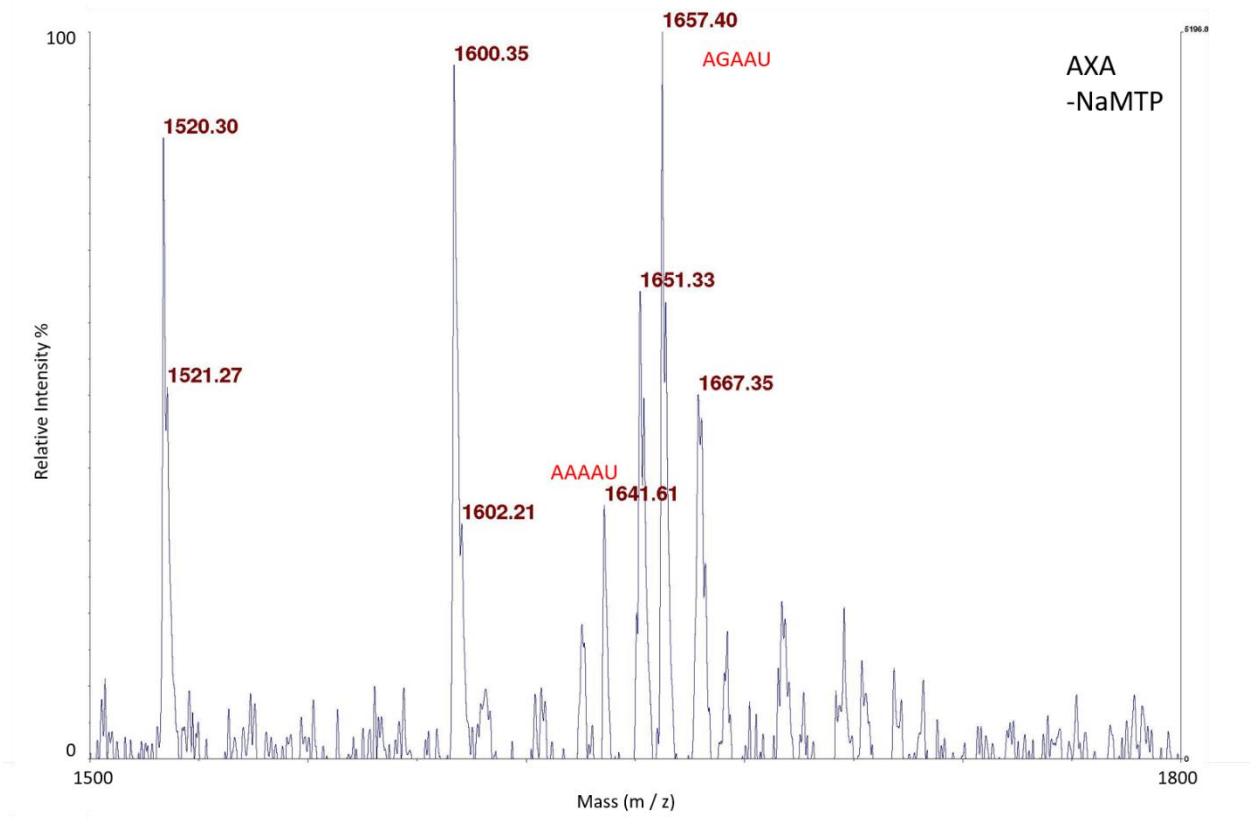


Figure S14. MALDI-MS spectrum of 5SICS Oligo-17 TYT forward transcribed without NaMTP after RNAse digestion. AAAAU calculated: 1641.25, observed: 1641.61; AGAAU calculated: 1657.25, observed: 1657.40.

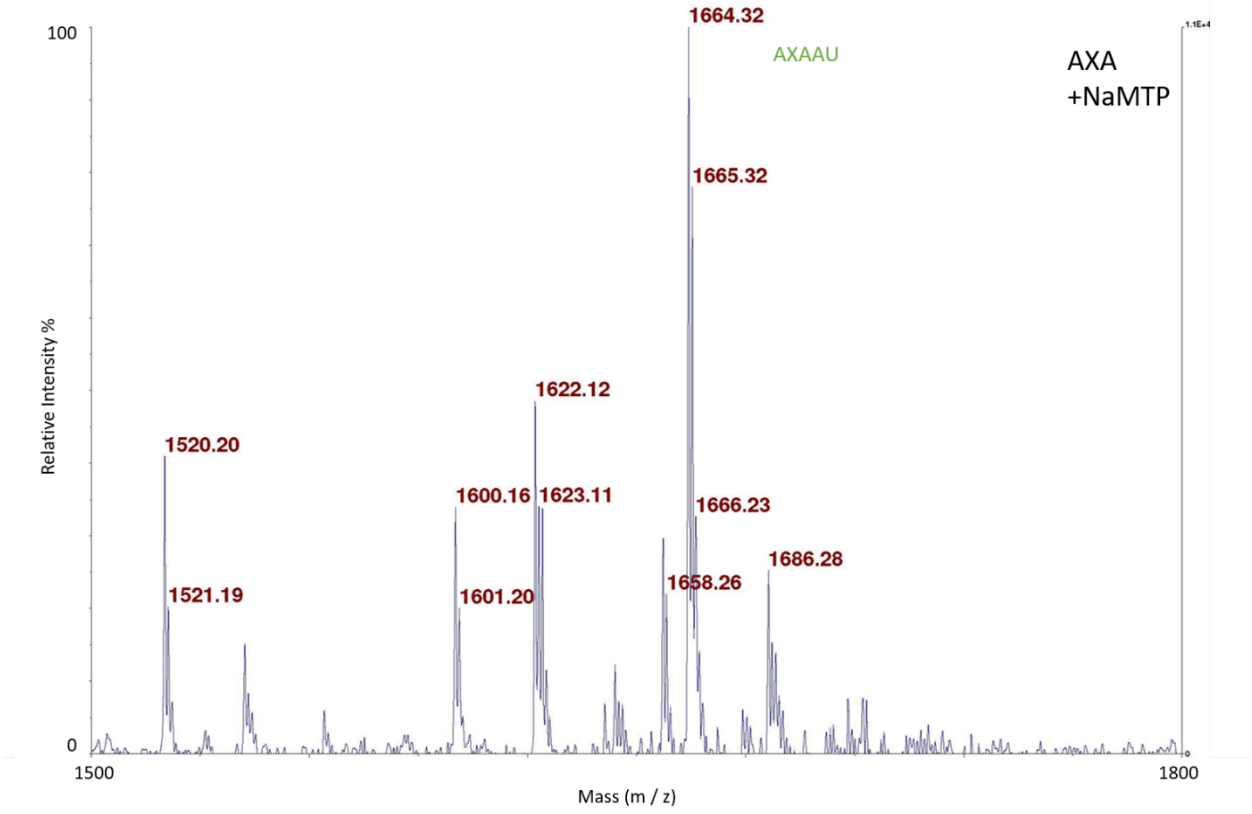


Figure S15. MALDI-MS spectrum of 5SICS Oligo-17 TYT forward transcribed with 2.5 mM NaMTP after RNAse digestion. AGAAU calculated: 1657.25, observed:(Background). AXAAU calculated: 1664.27, observed: 1664.32.

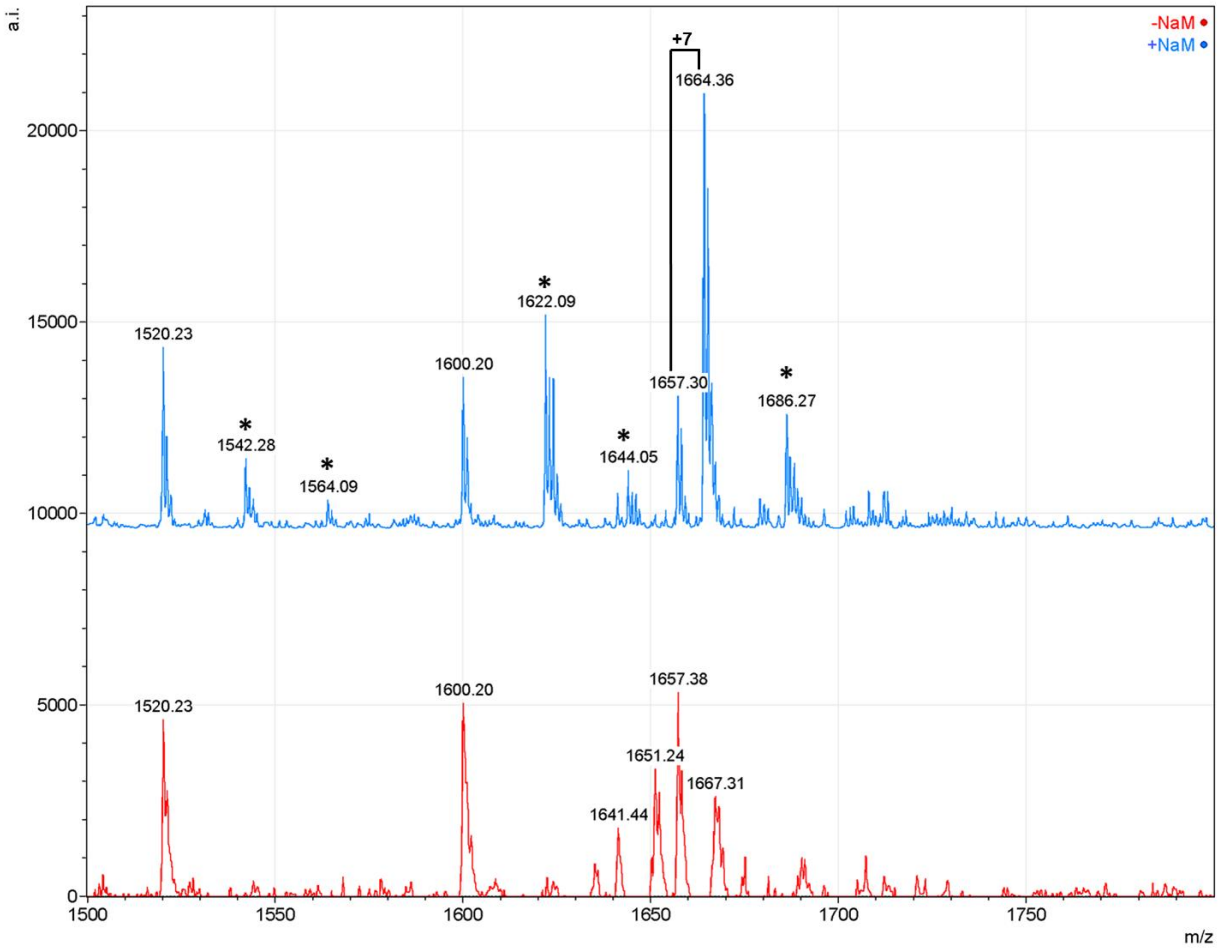


Figure S16. Overlayed MALDI-MS spectrum of Figure S14-15. Detected +22 Da Na^+ adducts are labeled in black asterisk. The expected NaM Fragment labeled with +7 (blue spectrum) from 1657.30.

Translation Monitoring of NaM Containing mRNAs using Affinity Clamp S17-S20

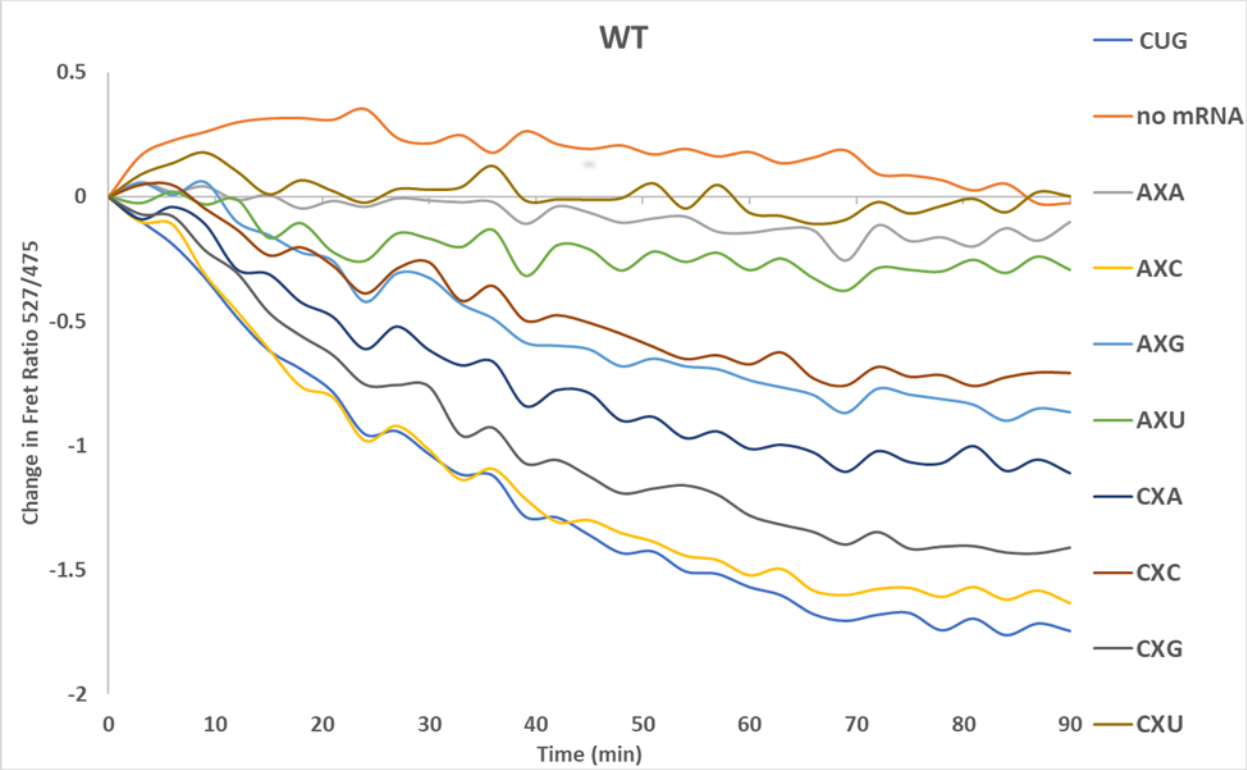


Figure S17. Affinity clamp fluorescence quenching of IVT using all NaM mRNAs 2-9 NXN and positive control mRNA 01 CUG without the presence of the conjugate 5SICS-tRNA NYN using WT ribosomes.

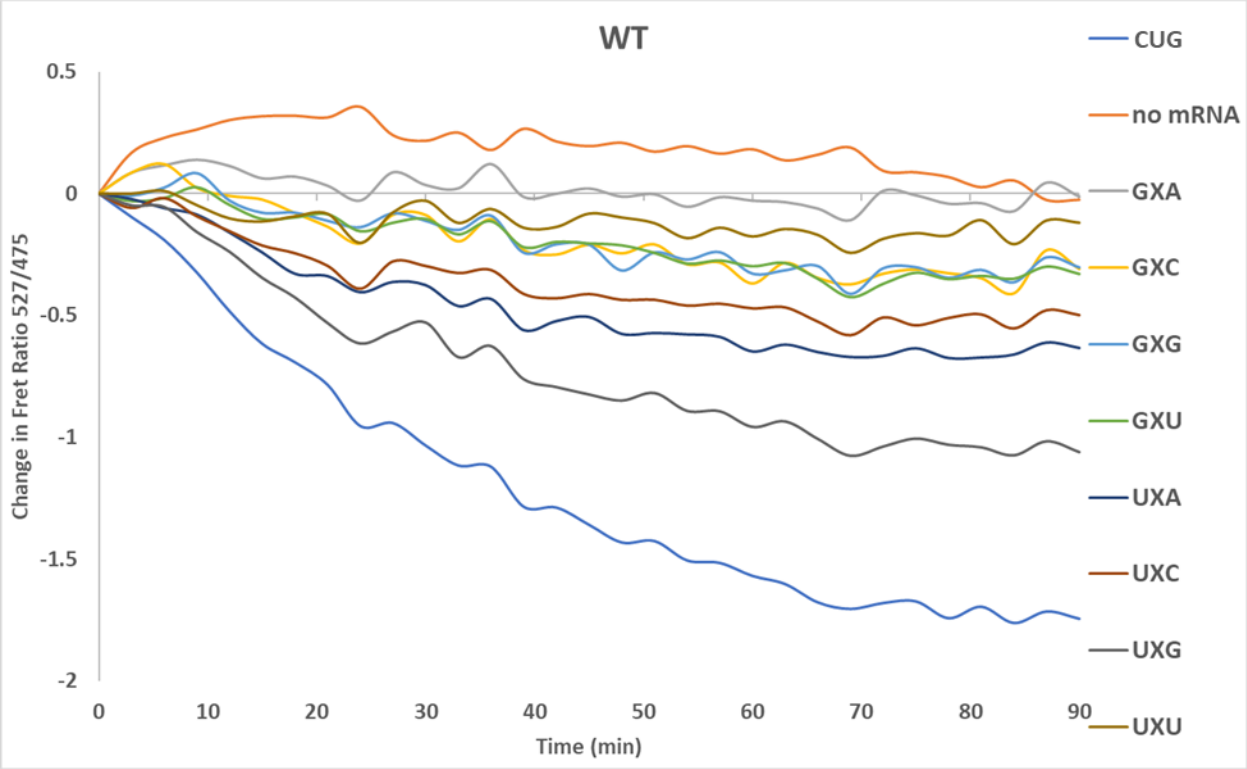


Figure S18 Affinity clamp fluorescence quenching of IVT using all NaM mRNAs 10-17 NXN without the presence of the conjugate 5SICS-tRNA NYN using WT ribosomes.

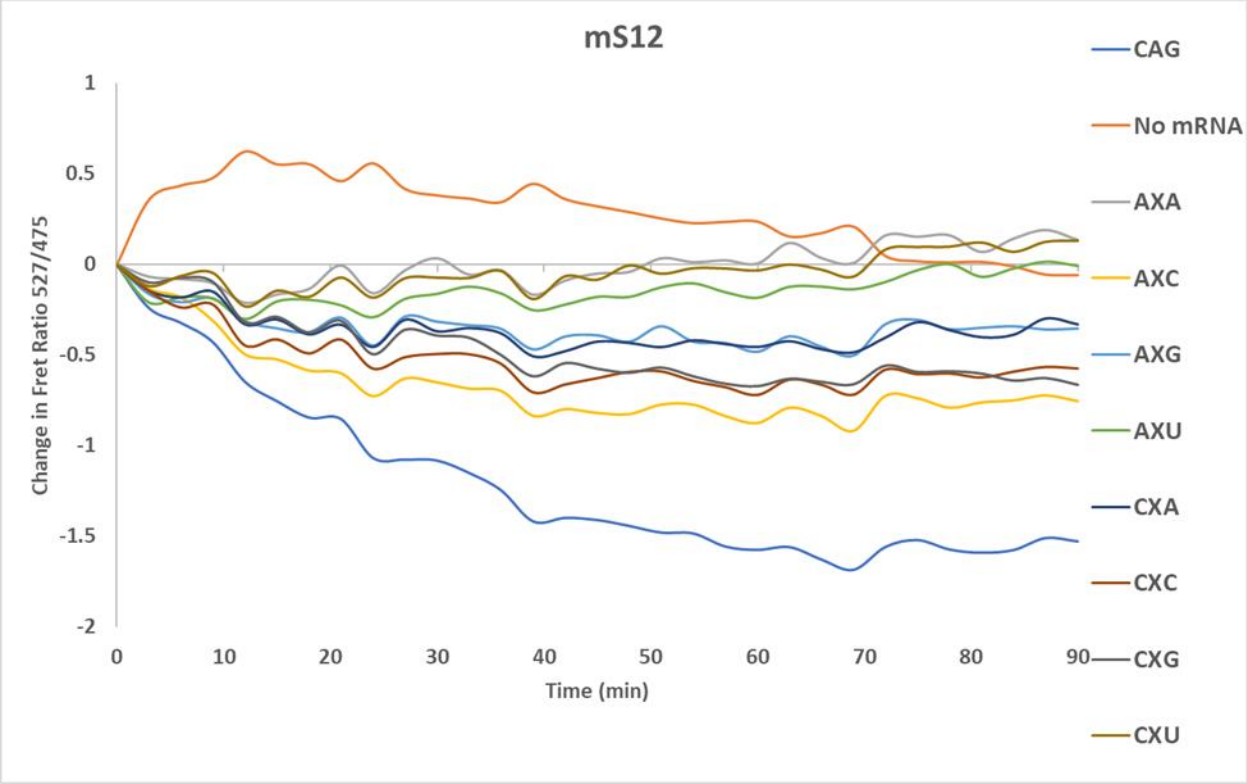


Figure S19. Affinity clamp fluorescence quenching of IVT using all NaM mRNAs 2-9 NXN without the presence of the conjugate 5SICS-tRNA NYN using mS12 Hyperaccurate ribosomes.

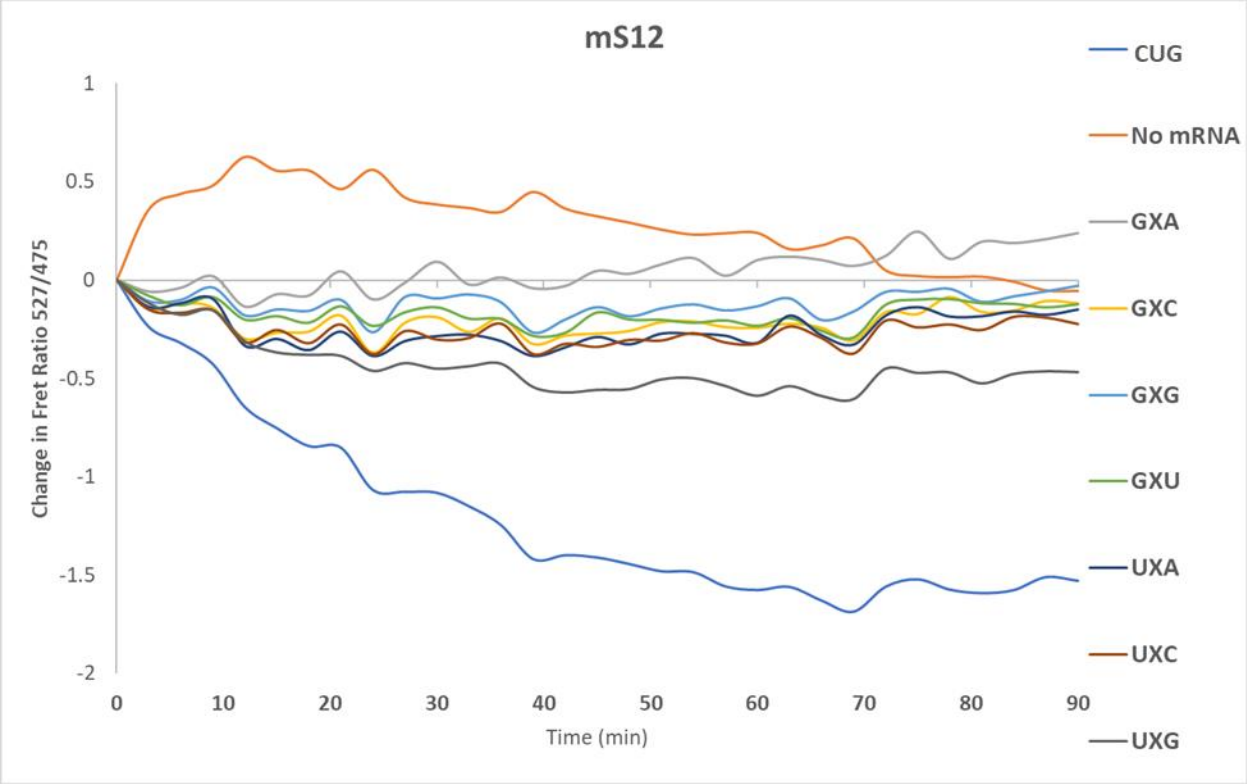


Figure S20. Affinity clamp fluorescence quenching of IVT using all NaM mRNAs 10-17 NXN without the presence of the conjugate 5SICS-tRNA NYN using mS12 Hyperaccurate ribosomes.

Mass Spec Translation of NaM Containing mRNAs in the absence of UBP tRNA

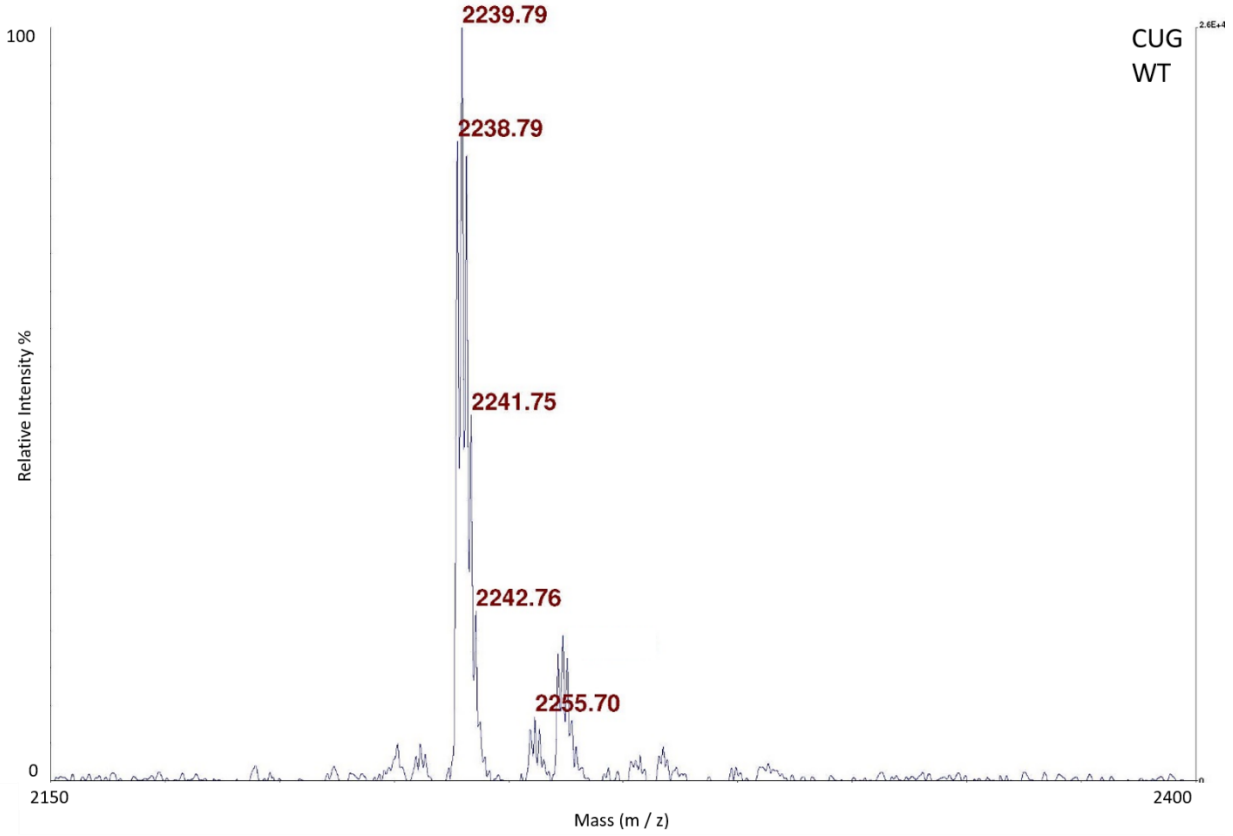


Figure S211. MALDI-MS spectra of mRNA-1 CUG using E. coli WT ribosome. [M+H] Calculated MH_6LMSPQPVDSWV: 2240.00; Observed: 2239.79.

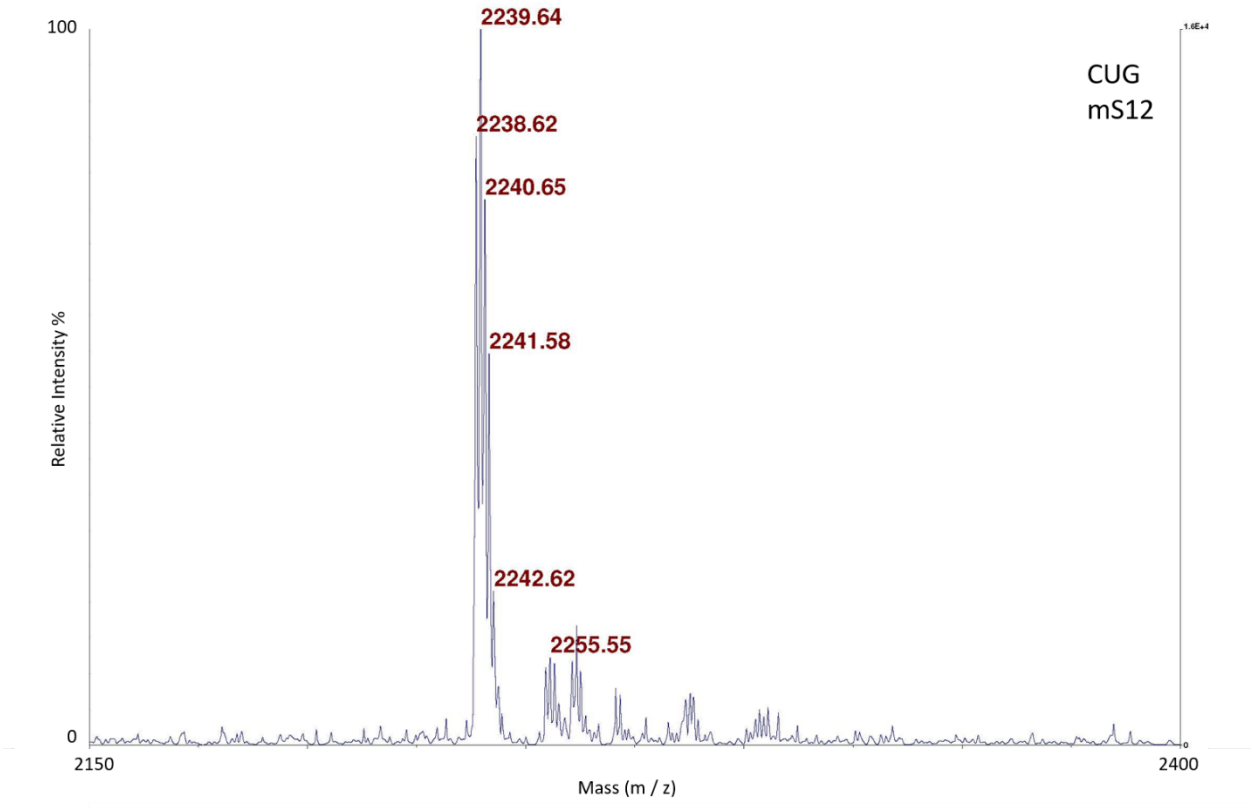


Figure S222. MALDI-MS spectra of mRNA-1 CUG using mS12 Hyperaccurate Ribosome. Calculated MH_6LMSPQPVDSWV: 2240.00; Observed: 2239.64.

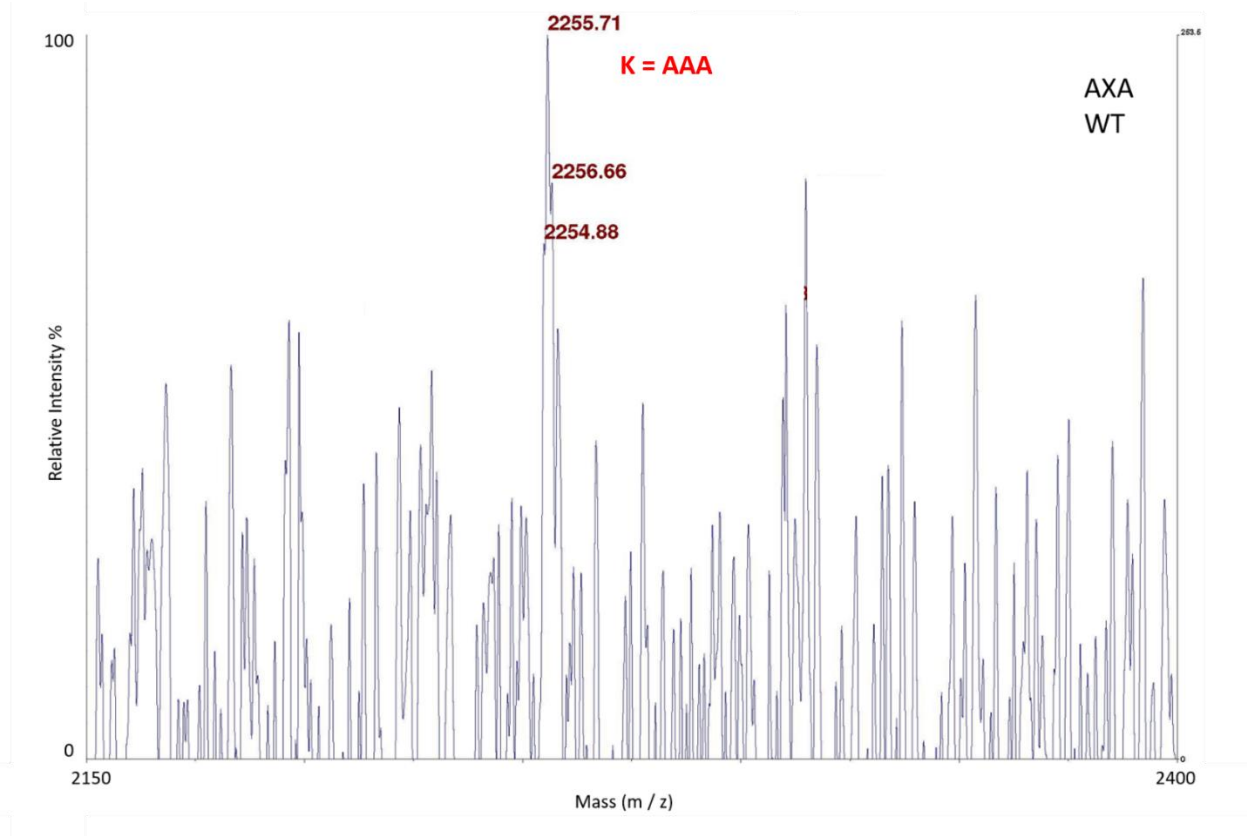


Figure S233. MALDI-MS spectra of mRNA-2 AXA using E. coli WT ribosome. Calculated MH_6KMSPQPVDSWV:2254.98; Observed:2254.88

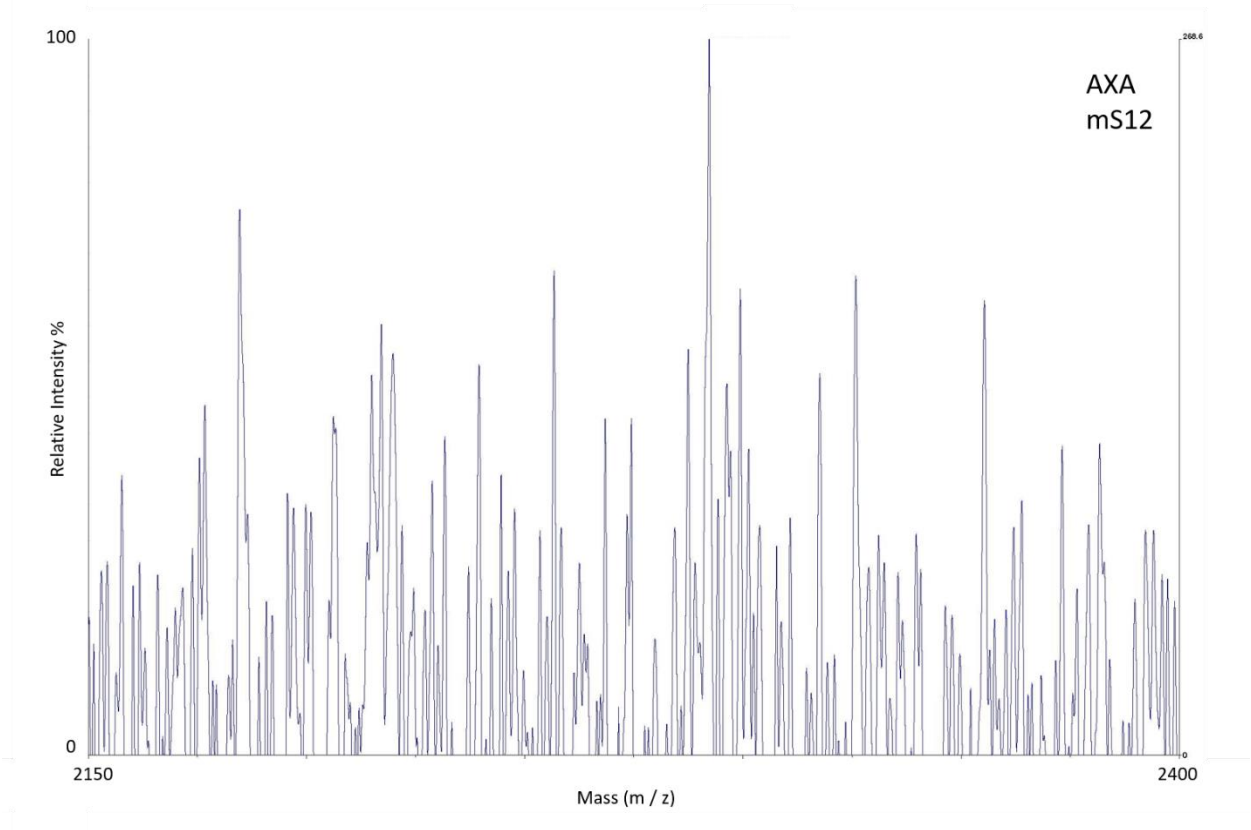


Figure S244. MALDI-MS zoomed spectra of mRNA-2 AXA using mS12 Hyperaccurate Ribosome. No assignable peaks in the mass range 2150-2400.

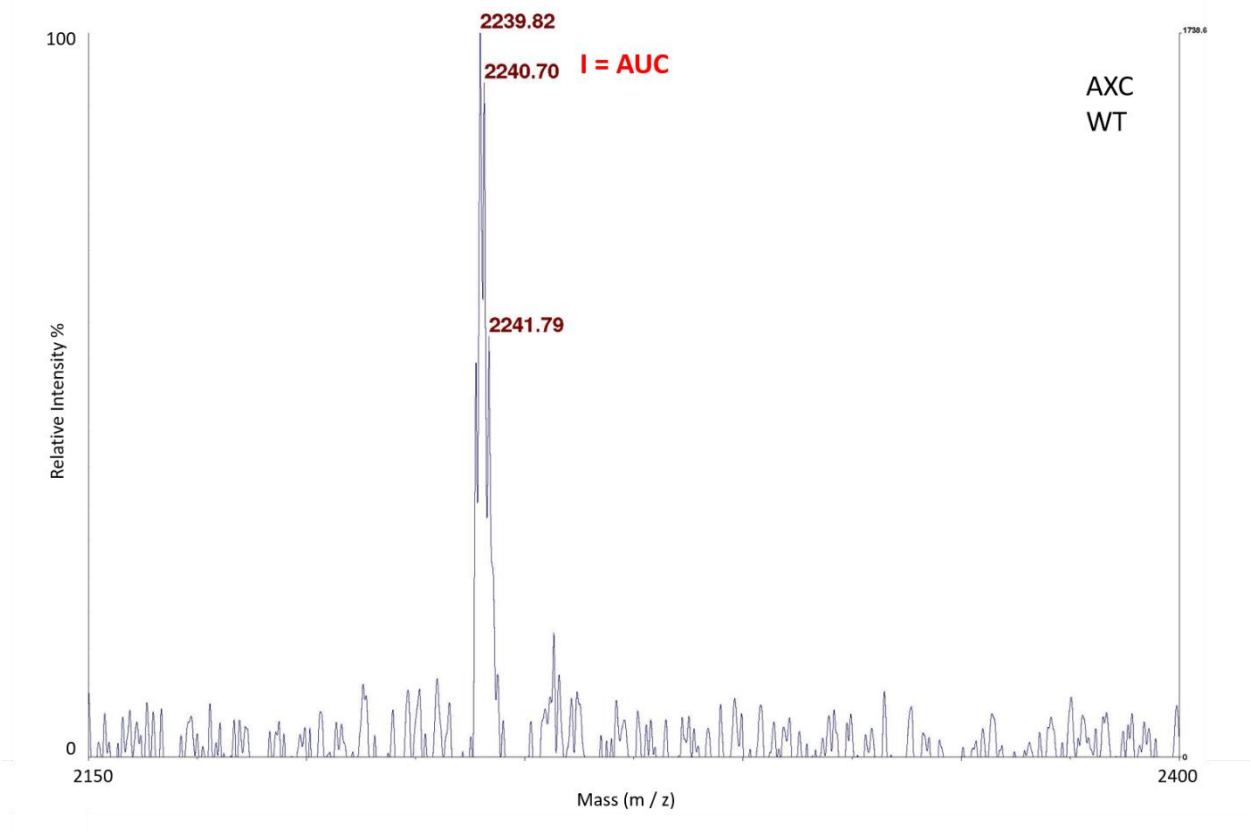


Figure S255. MALDI-MS spectra of mRNA-3 AXC using E. coli WT ribosome. Calculated MH_6IMSPQPVDSWV:2240.00; Observed:2239.82.

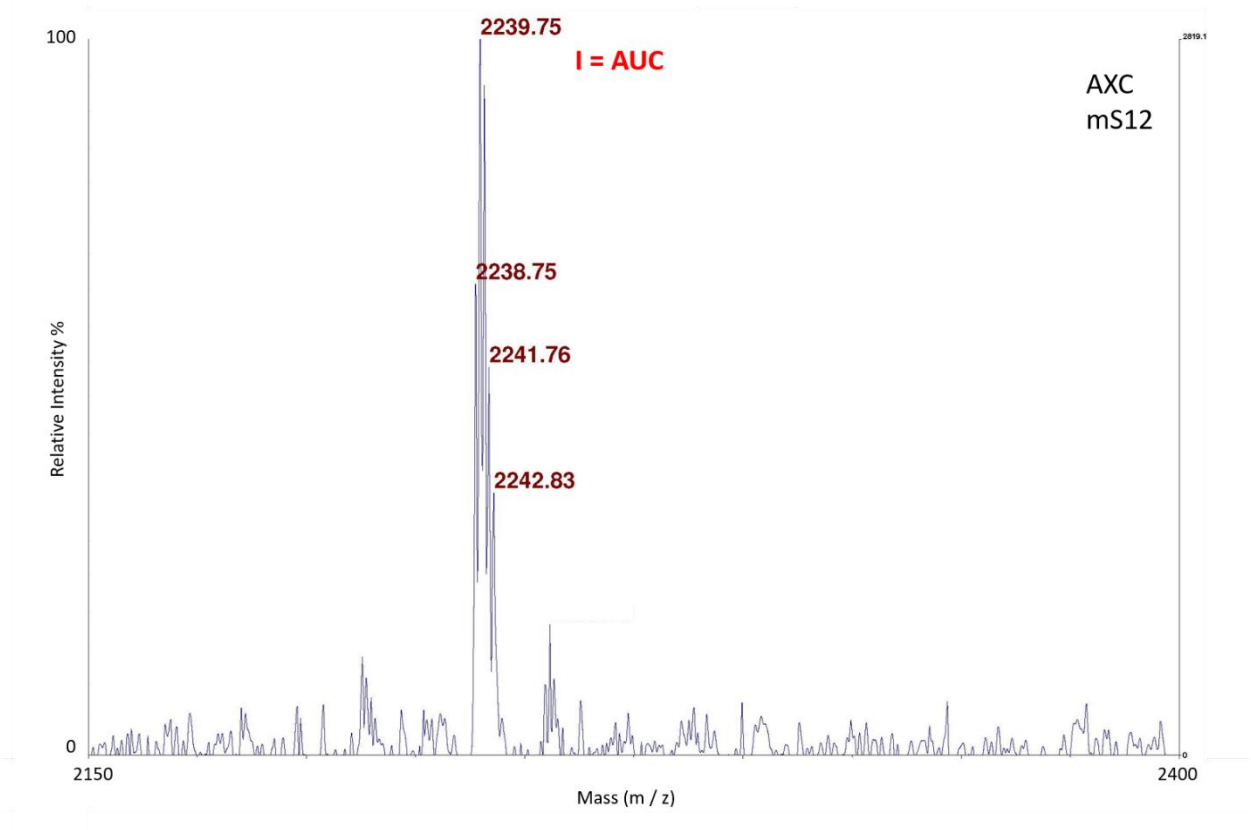


Figure S266. MALDI-MS zoomed spectra of mRNA-3 AXC using mS12 Hyperaccurate Ribosome. Calculated MH_6IMSPQPVDSWV: 2240.00; Observed: 2239.75.

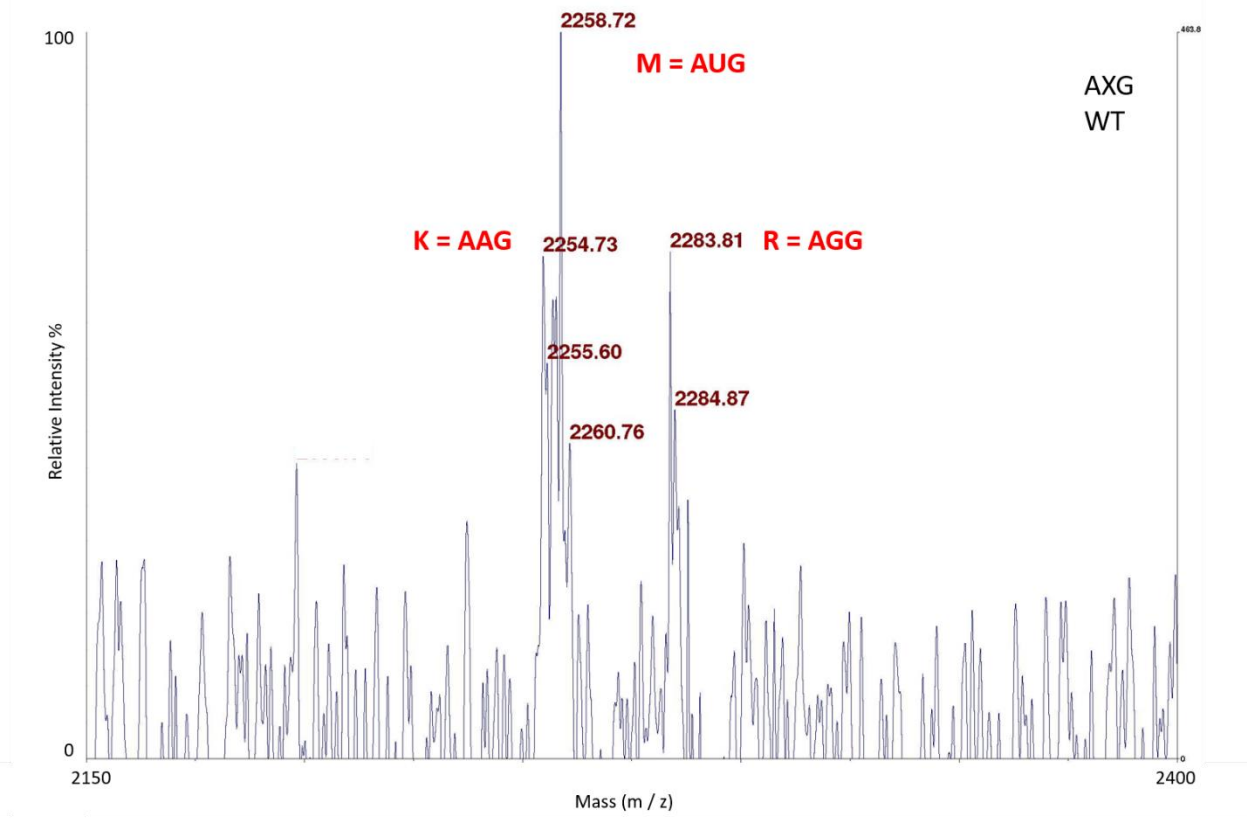


Figure S27. MALDI-MS spectra of mRNA-4 AXG using E. coli WT ribosome. Calculated MH_6KMSPQPVDSWV:2255.01; Observed:2254.73. Calculated MH_6MMSPQPVDSWV:2257.96; Observed:2258.72. Calculated MH_6RMSPQPVDSWV:2283.02; Observed:2283.81.

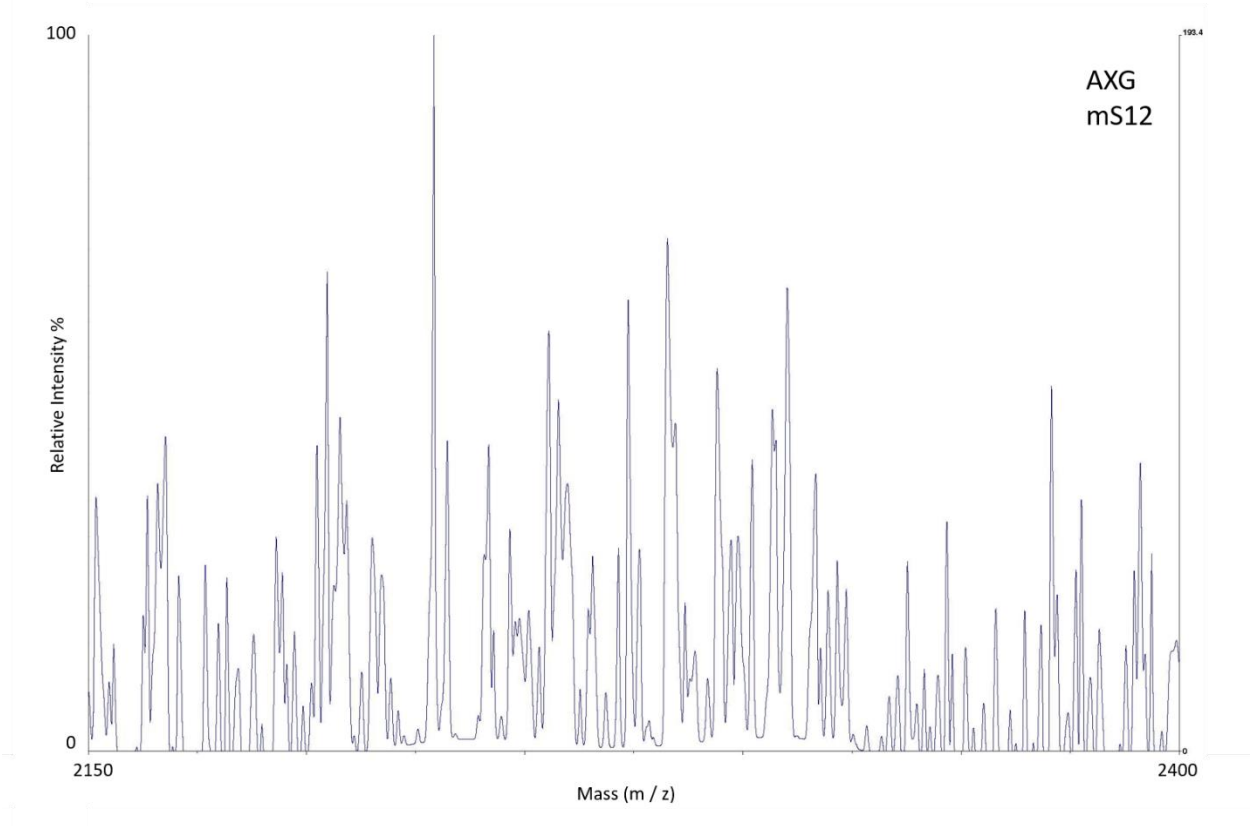


Figure S28. MALDI-MS spectra of mRNA-4 AXG using mS12 Hyperaccurate Ribosome. No assignable peaks were detected in mass range 2150-2400.

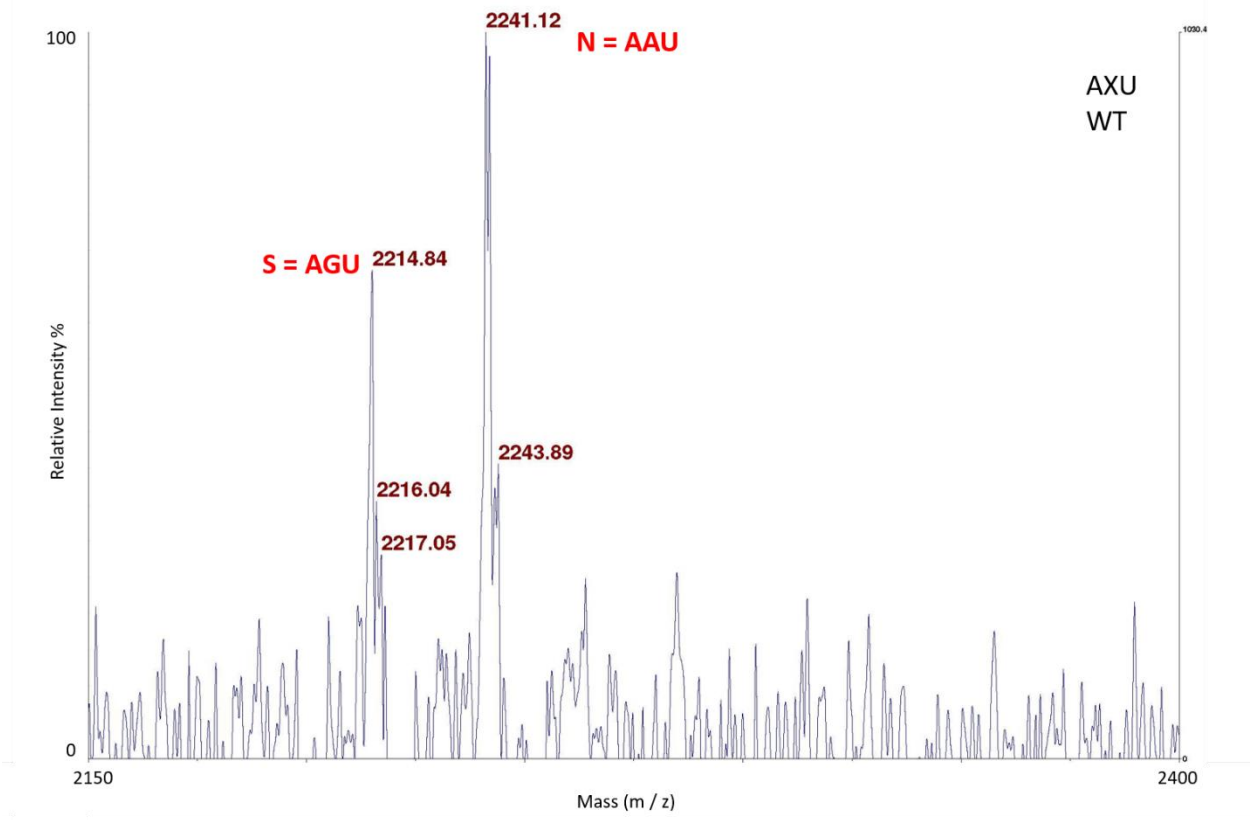


Figure S29. MALDI-MS spectra of mRNA-5 AXU using E. coli WT ribosome. Calculated MH_6SMSPQPVDSWV: 2213.95; Observed: 2214.84. Calculated MH_6NMSPQPVDSWV: 2240.96; Observed: 2241.12.

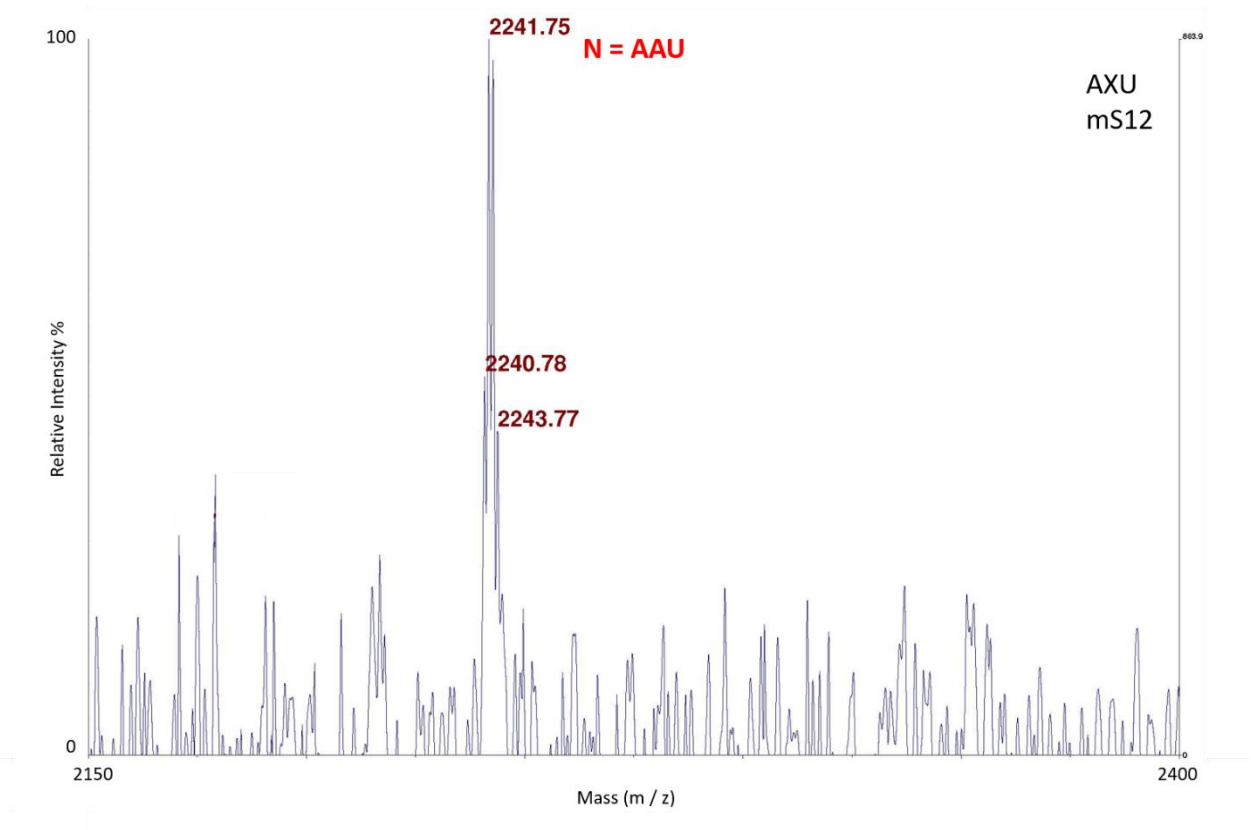


Figure S30. MALDI-MS spectra of mRNA-5 AXU using mS12 Hyperaccurate Ribosome. Calculated MH_6NMSPQPVDSWV: 2240.96; Observed: 2240.78.

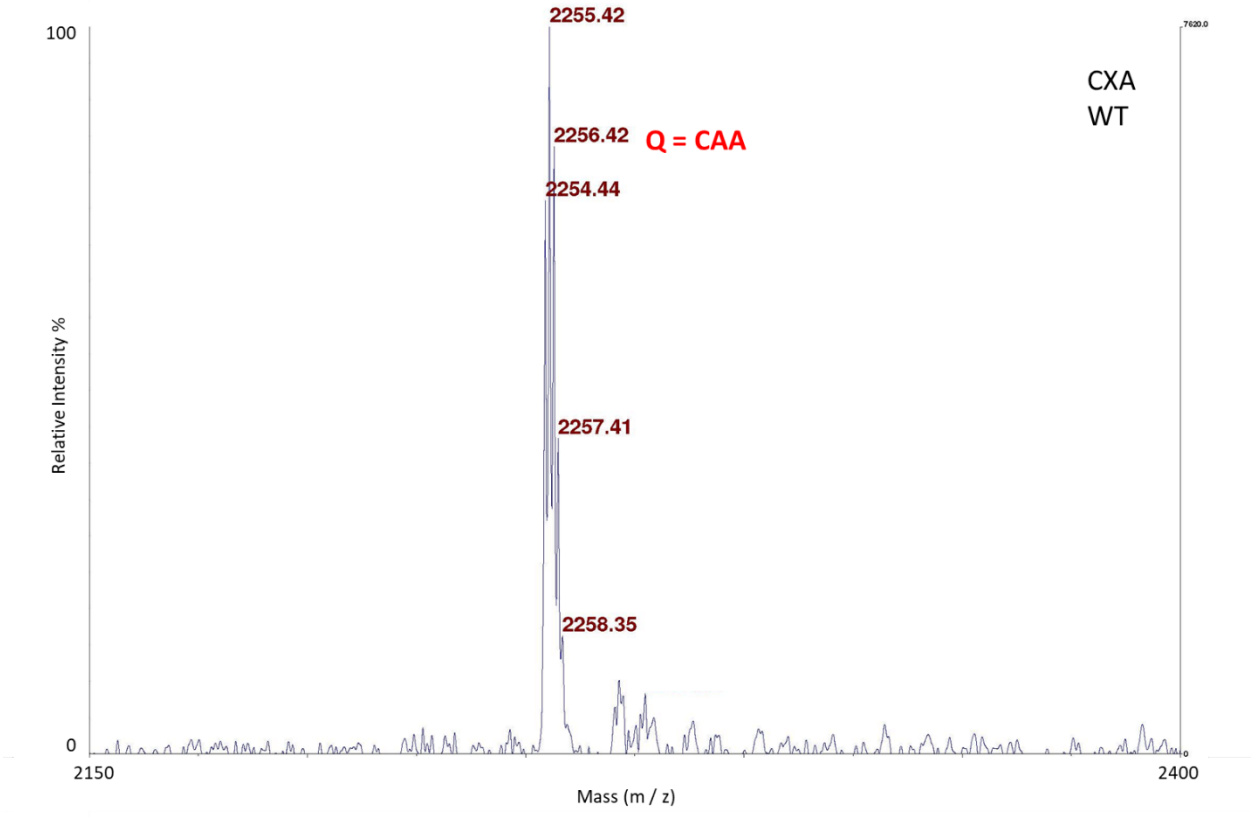


Figure S27. MALDI-MS spectra of mRNA-6 CXA using E. coli WT ribosome. Calculated MH_6QMSPQPVDSWV:2254.98; Observed:2255.27.

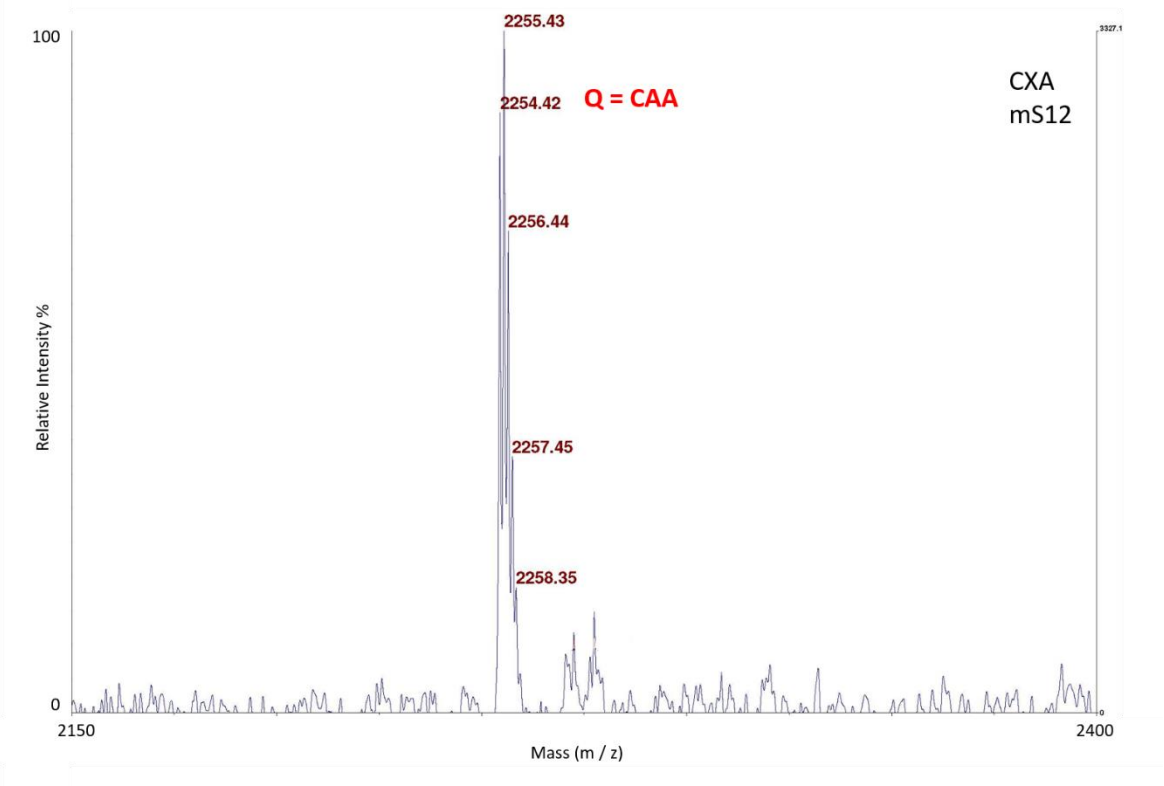


Figure S32. MALDI-MS zoomed spectra of mRNA-6 CXA using mS12 Hyperaccurate Ribosome. Calculated MH_6QMSPQPVDSWV: 2254.98; Observed: 2254.42.

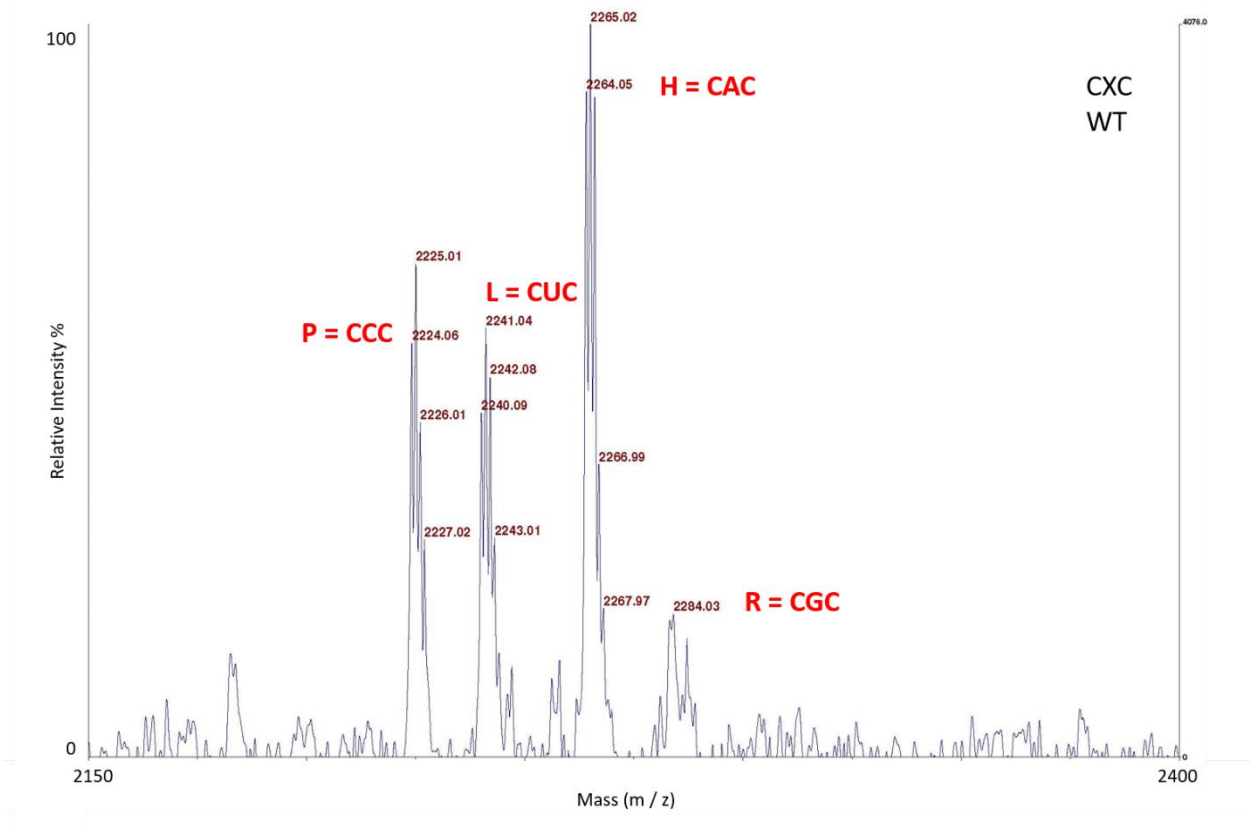


Figure S33. MALDI-MS spectra of mRNA-7 CXC using E. coli WT ribosome. Calculated MH_6PMSPQPVDSWV:2223.97; Observed: 2224.06. MH_6LMSPQPVDSWV:2240.00; Observed: 2240.09. MH_7MSPQPVDSWV:2263.98; Observed: 2264.05. MH_6RMSPQPVDSWV: 2283.02; Observed: 2284.03.

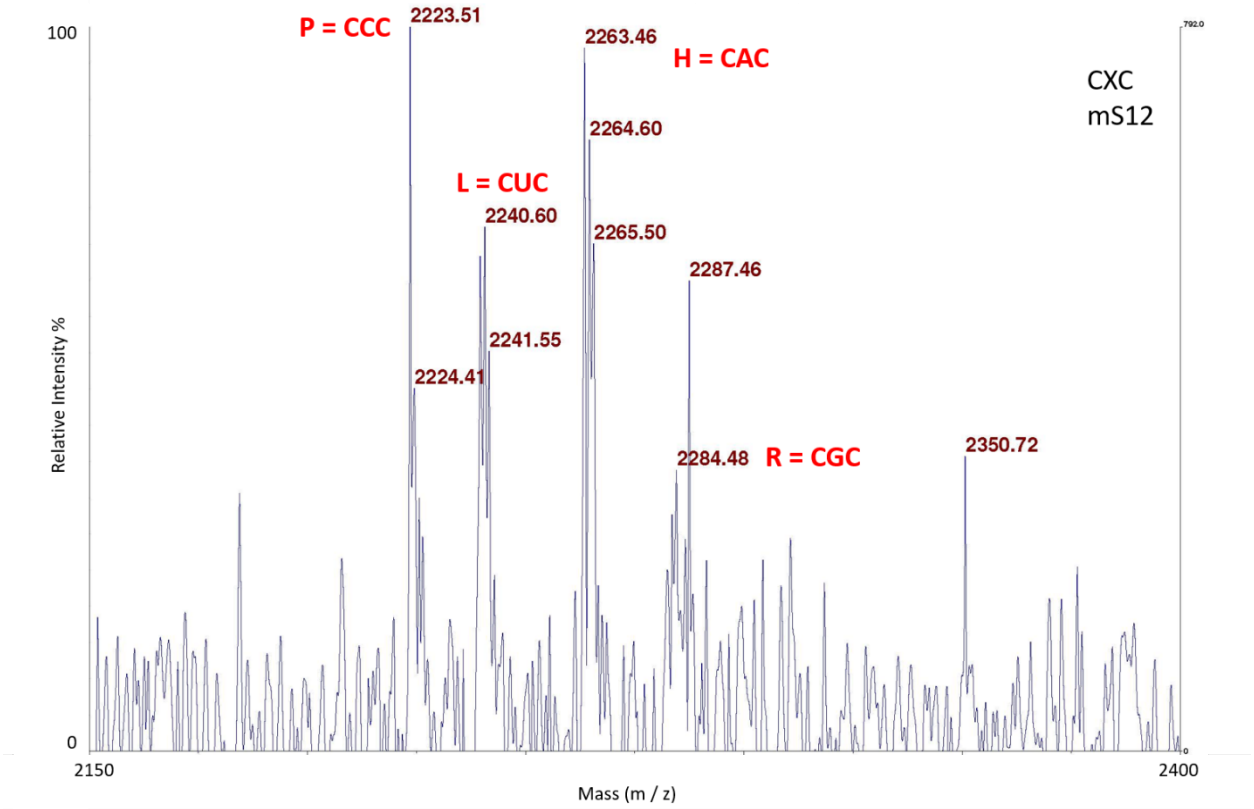


Figure S28. MALDI-MS zoomed spectra of mRNA-7 CXC using mS12 Hyperaccurate Ribosome. Calculated MH_6PMSPQPVDSWV: 2223.97; Observed: 2223.51. Calculated MH_6LMSPQPVDSWV: 2240.00; Observed: 2241.67. Calculated MH_7MSPQPVDSWV: 2263.98; Observed: 2263.46. Calculated MH_6RMSPQPVDSWV: 2283.02; Observed: 2284.48.

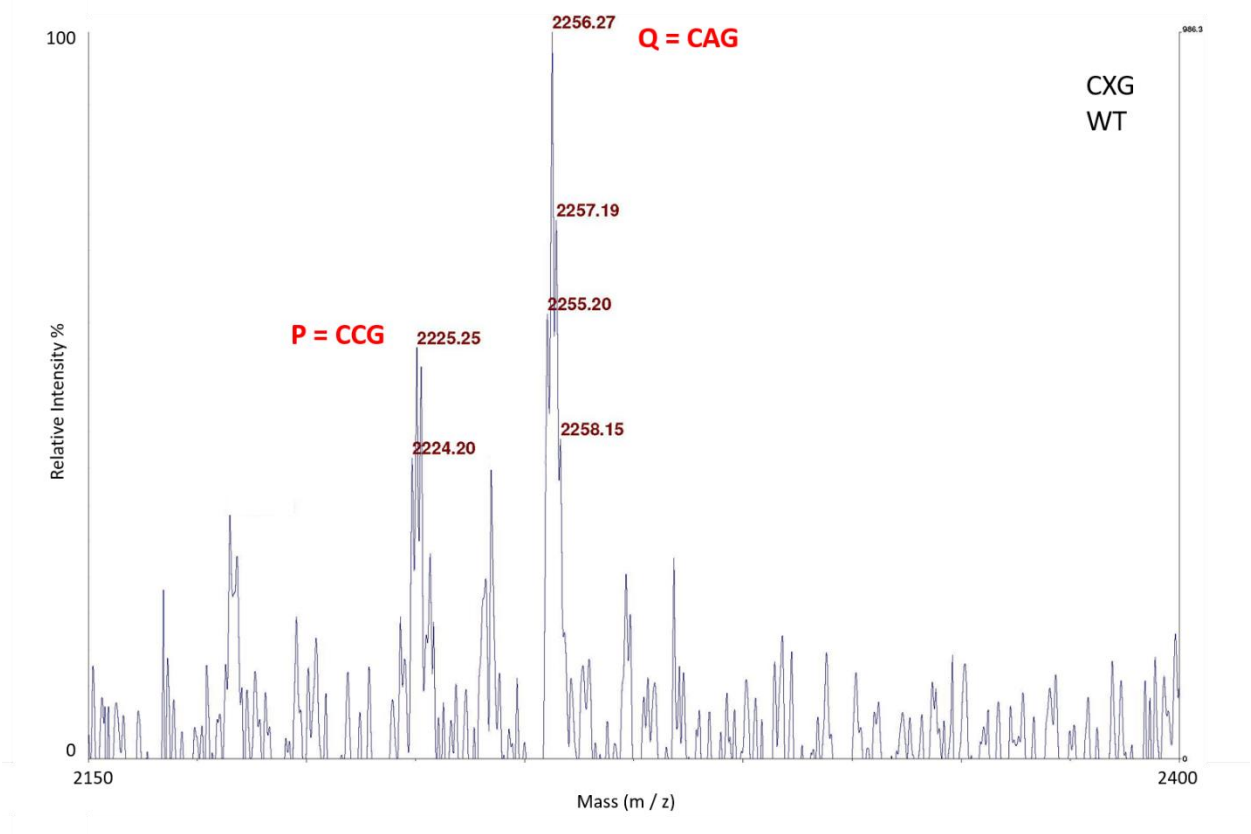


Figure S295. MALDI-MS spectra of mRNA-8 CXG using E. coli WT ribosome. Calculated MH_6PMSPQPVDSWV: 2223.97; Observed: 2224.20. Calculated MH_6QMSPQPVDSWV: 2254.98; Observed: 2255.20.

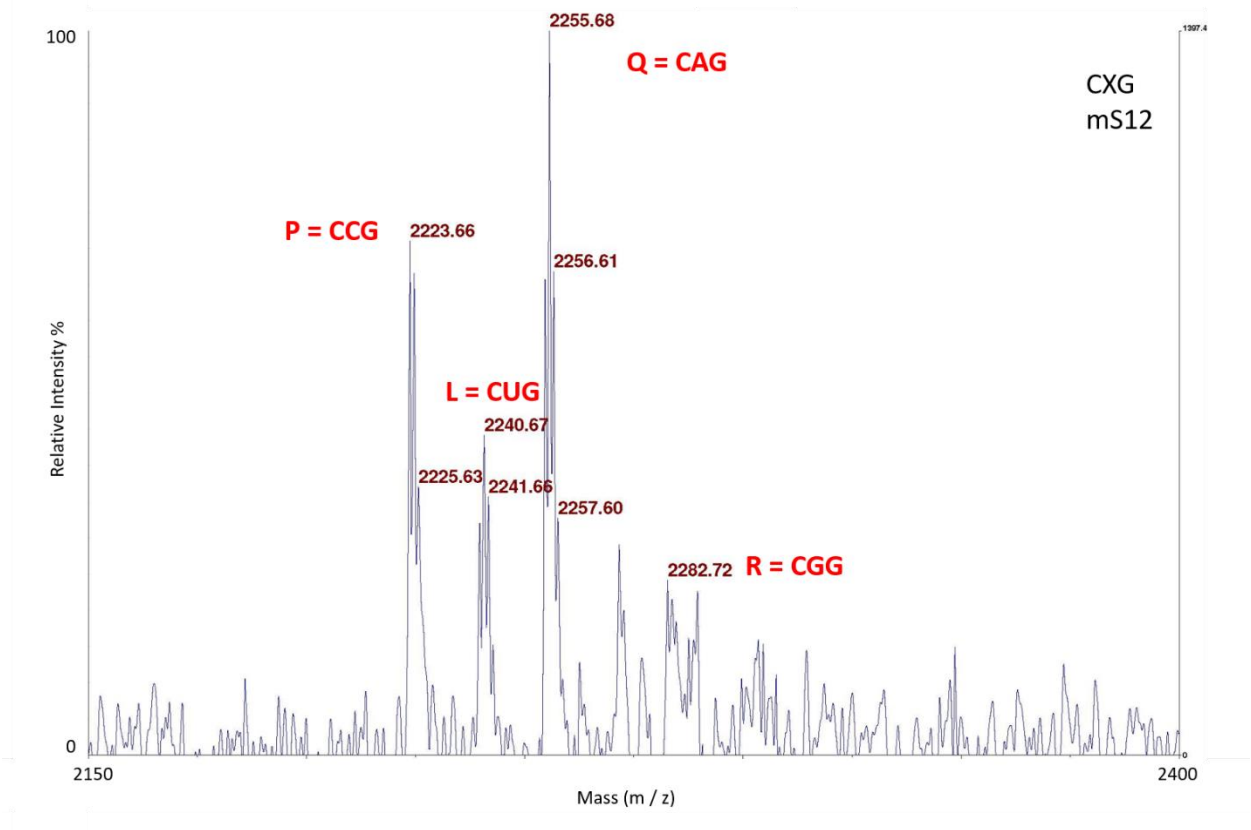


Figure S36. MALDI-MS spectra of mRNA-8 CXG using mS12 Hyperaccurate Ribosome. Calculated MH_6PMSPQPVDSWV: 2223.97; Observed: 2223.66. Calculated MH_6LMSPQPVDSWV: 2240.00; Observed: 2240.67. Calculated MH_6QMSPQPVDSWV: 2254.98; Observed: 2255.68. Calculated MH_6RMSPQPVDSWV: 2283.20; Observed: 2282.72.

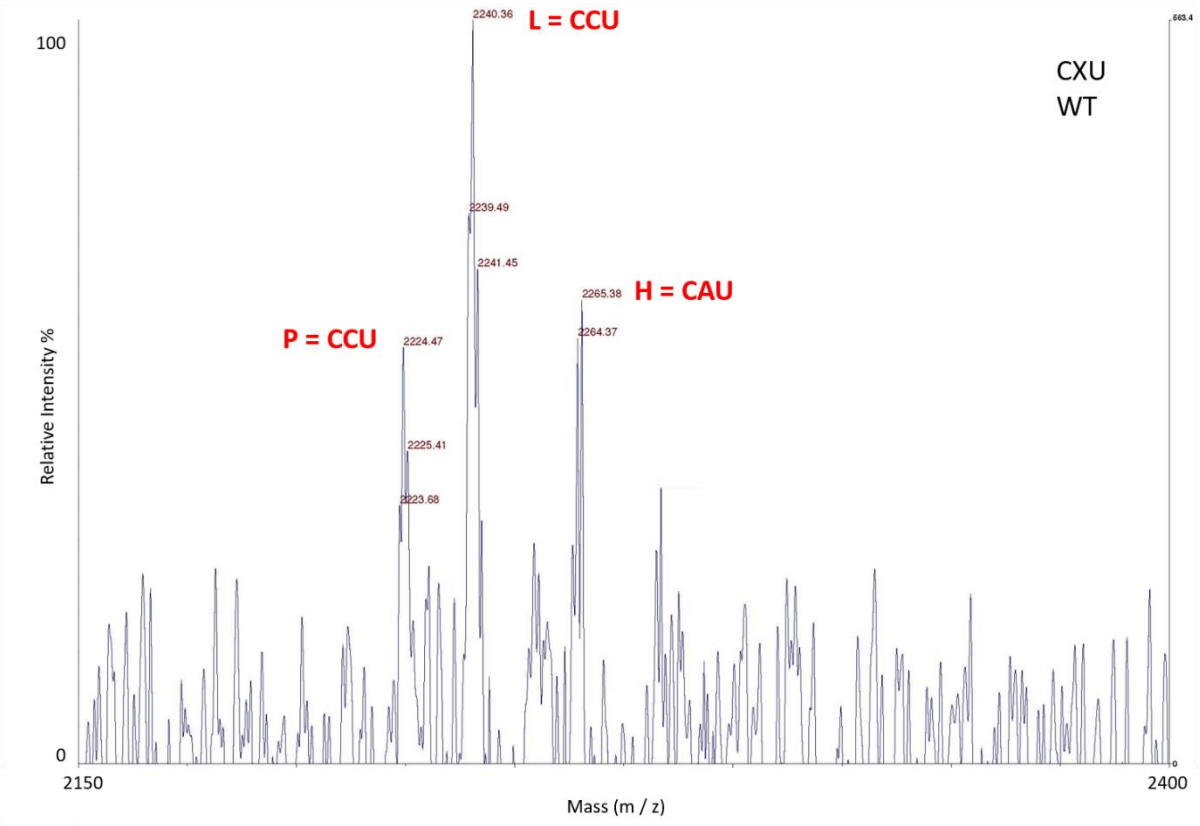


Figure S37. MALDI-MS spectra of mRNA-9 CXU using E. coli WT ribosome. Calculated MH_6PMSPQPVDSWV: 2223.97; Observed: 2223.68. Calculated MH_6LMSPQPVDSWV: 2240.00; Observed: 2239.49. Calculated MH_7MSPQPVDSWV: 2263.98; Observed: 2264.37.

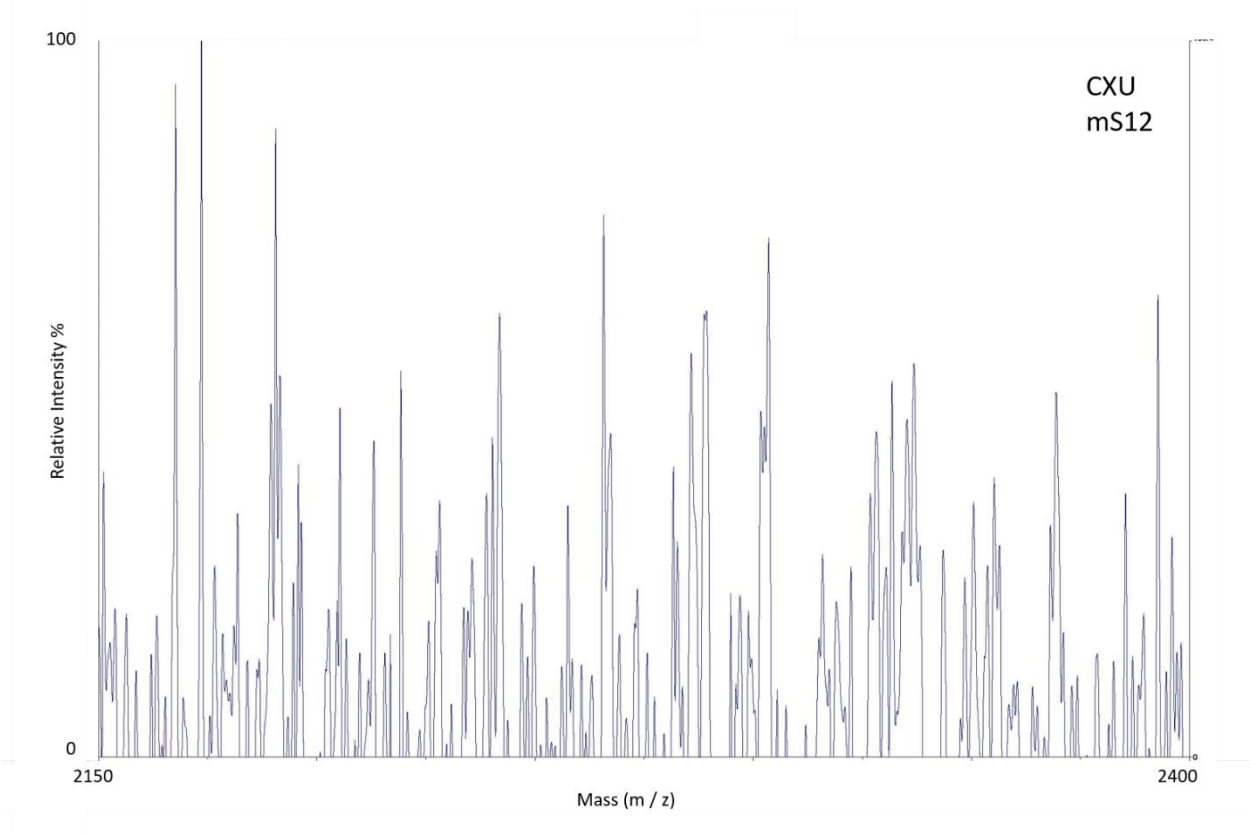


Figure S38.. MALDI-MS spectra of mRNA-9 CXU using mS12 Hyperaccurate Ribosome. No assignable peaks detected in mass range 2150 to 2400.

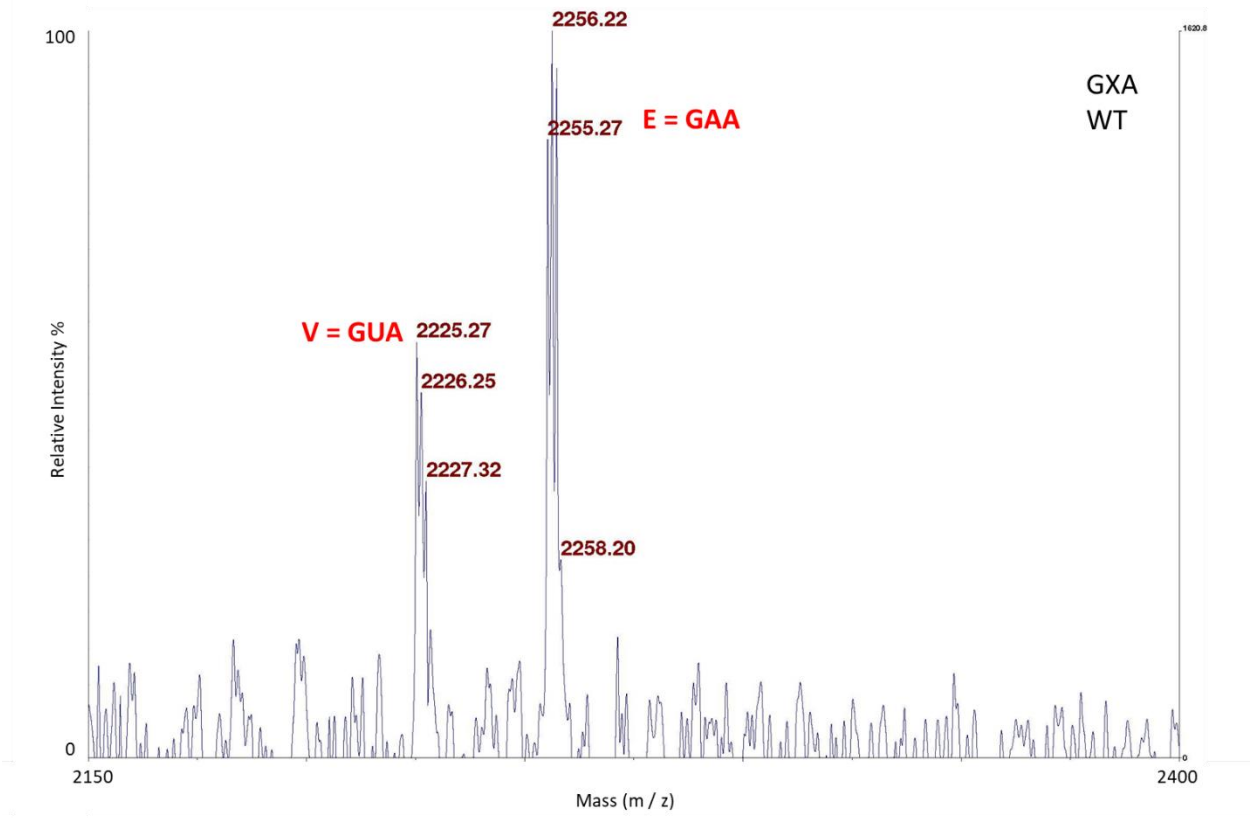


Figure S30. MALDI-MS spectra of mRNA-10 GXA using E. coli WT ribosome. Calculated MH_6EMSPQPVDSWV:2255.96; Observed:2255.42.

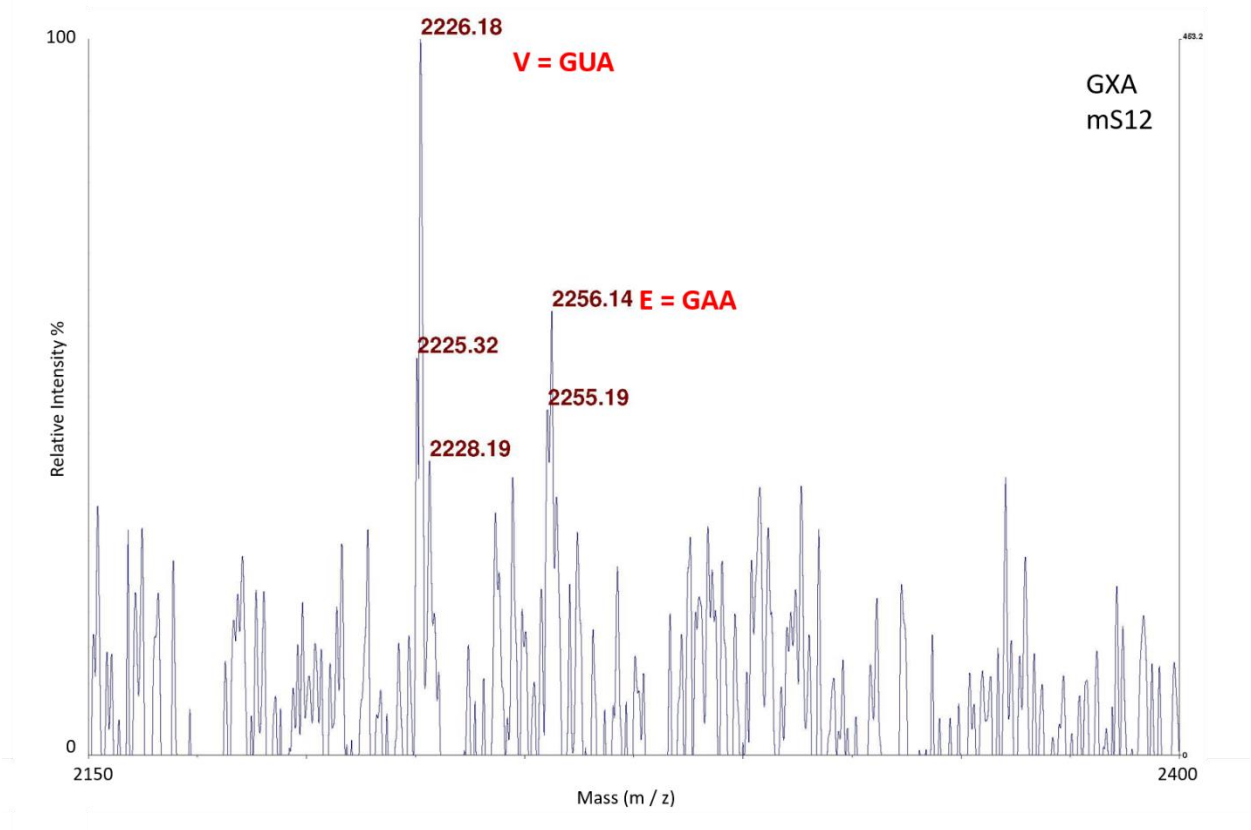


Figure S31. MALDI-MS zoomed spectra of mRNA-10 GXA using mS12 Hyperaccurate Ribosome. Calculated MH_6VMSPQPVDSWV: 2225.99; Observed: 2226.18. Calculated MH_6EMSPQPVDSWV: 2255.96; Observed: 2256.14

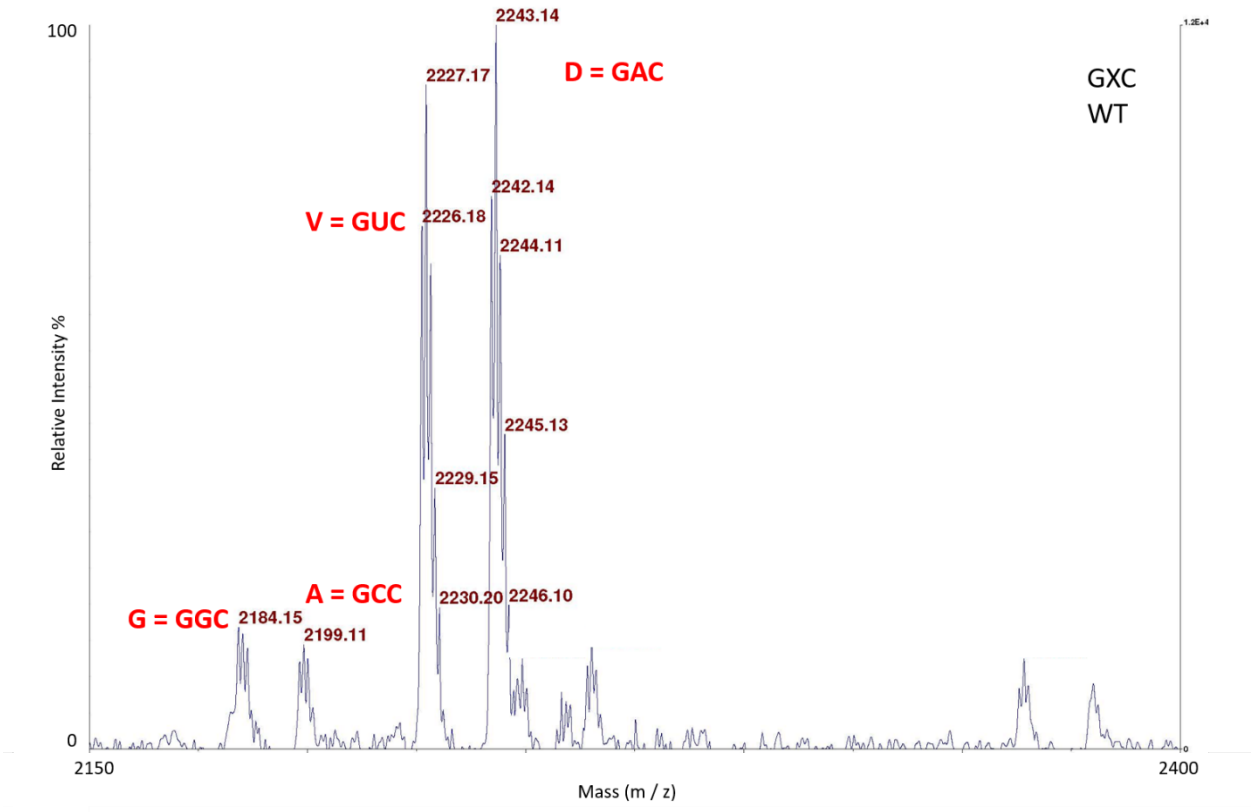


Figure S41. MALDI-MS zoomed spectra of mRNA-11 GXC using E. coli WT ribosome. Calculated MH_6GMSPQPVDSWV:2183.94; Observed:2184.13. MH_6AMSPQPVDSWV:2197.96; Observed: 2199.11. MH_6VMSPQPVDSWV:2225.99; Observed: 2226.18. MH_6DMSPQPVDSWV:2241.95; Observed: 2242.14.

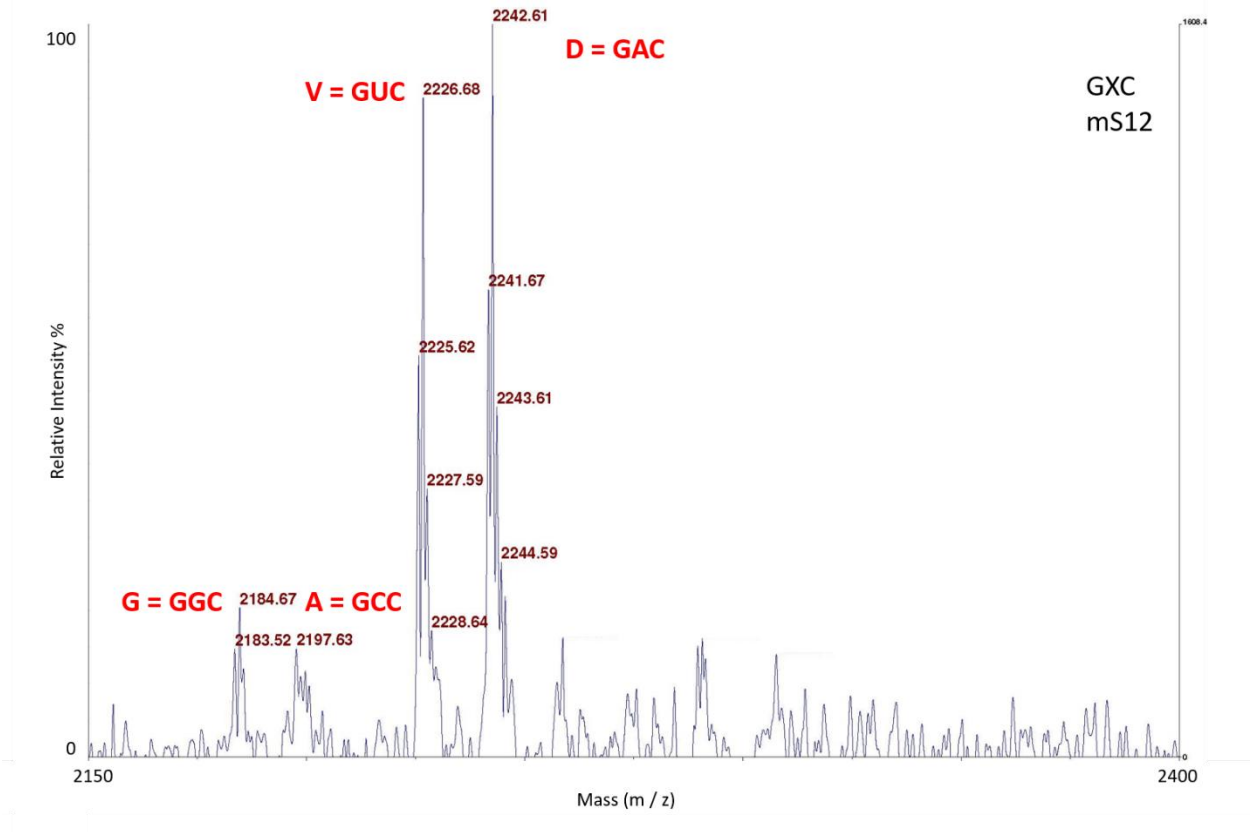


Figure S42. MALDI-MS spectra of mRNA-11 GXC using mS12 Hyperaccurate Ribosome. Calculated MH_6GMSPQPVDSWV: 2183.94 Observed: 2183.52. Calculated MH_6AMSPQPVDSWV: 2197.96; Observed: 2197.63. Calculated MH_6VMSPQPVDSWV: 2225.99; Observed: 2226.68. Calculated MH_6DMSPQPVDSWV: 2241.95; Observed: 2241.67.

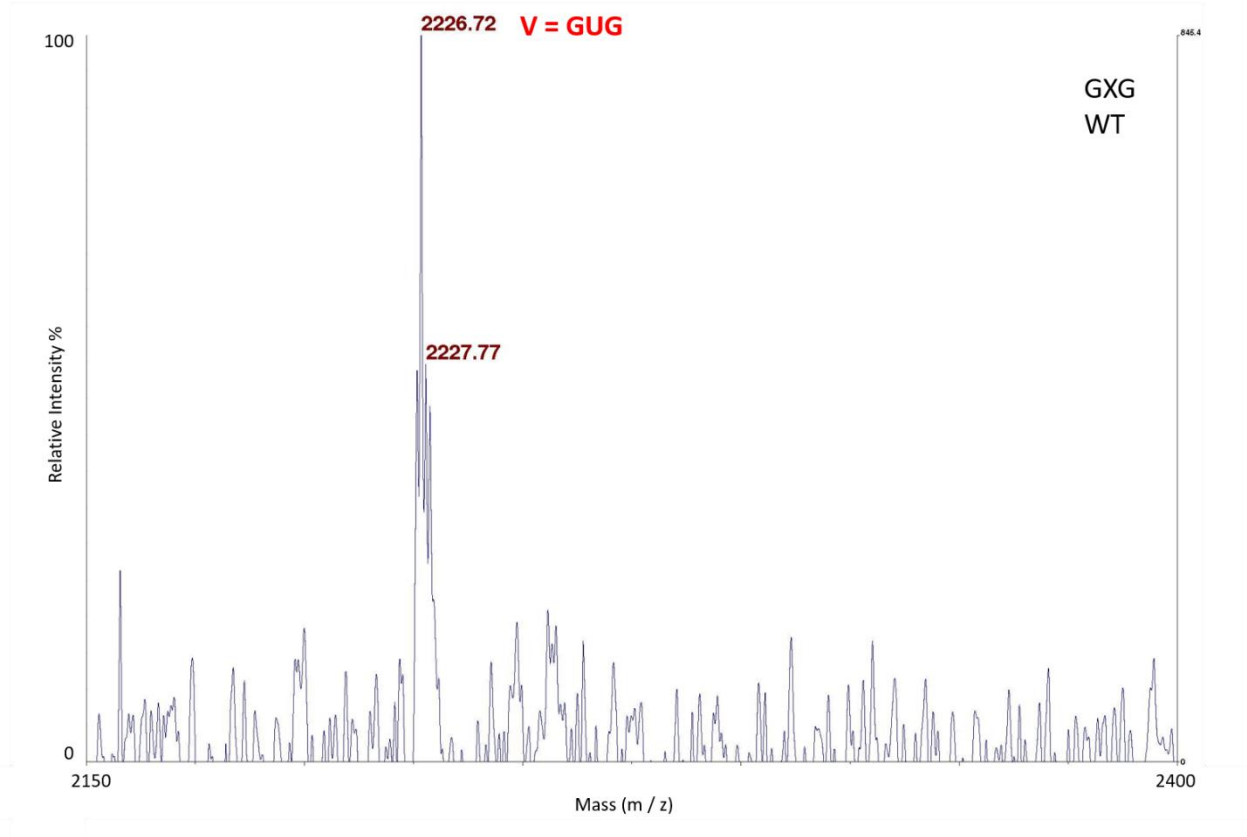


Figure S32. MALDI-MS spectra of mRNA-12 GXG using E. coli WT ribosome. Calculated MH_6VMSPQPVDSWV: 2225.99; Observed: 2226.72.

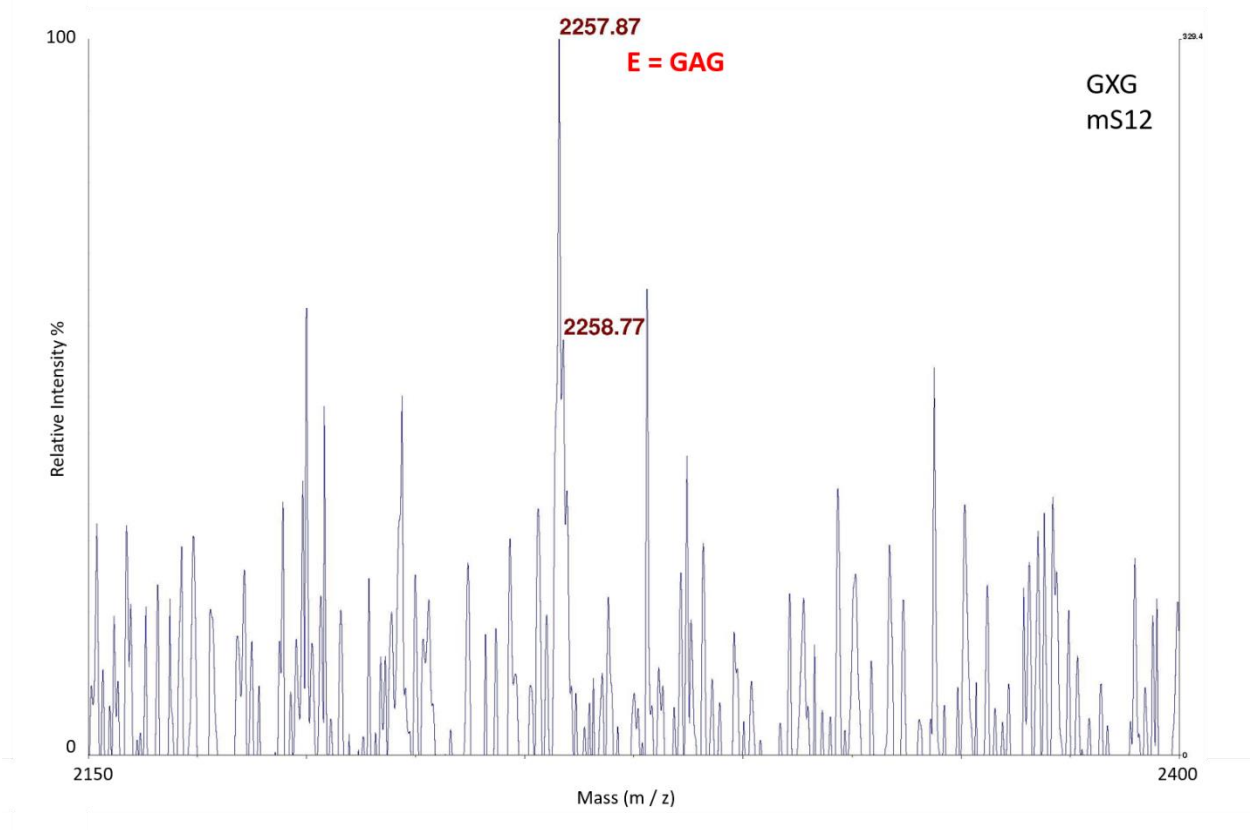


Figure S44. MALDI-MS spectra of mRNA-12 GXG using mS12 Hyperaccurate Ribosome. Calculated MH_6EMSPQPVDSWV: 2255.95; Observed: 2257.87.

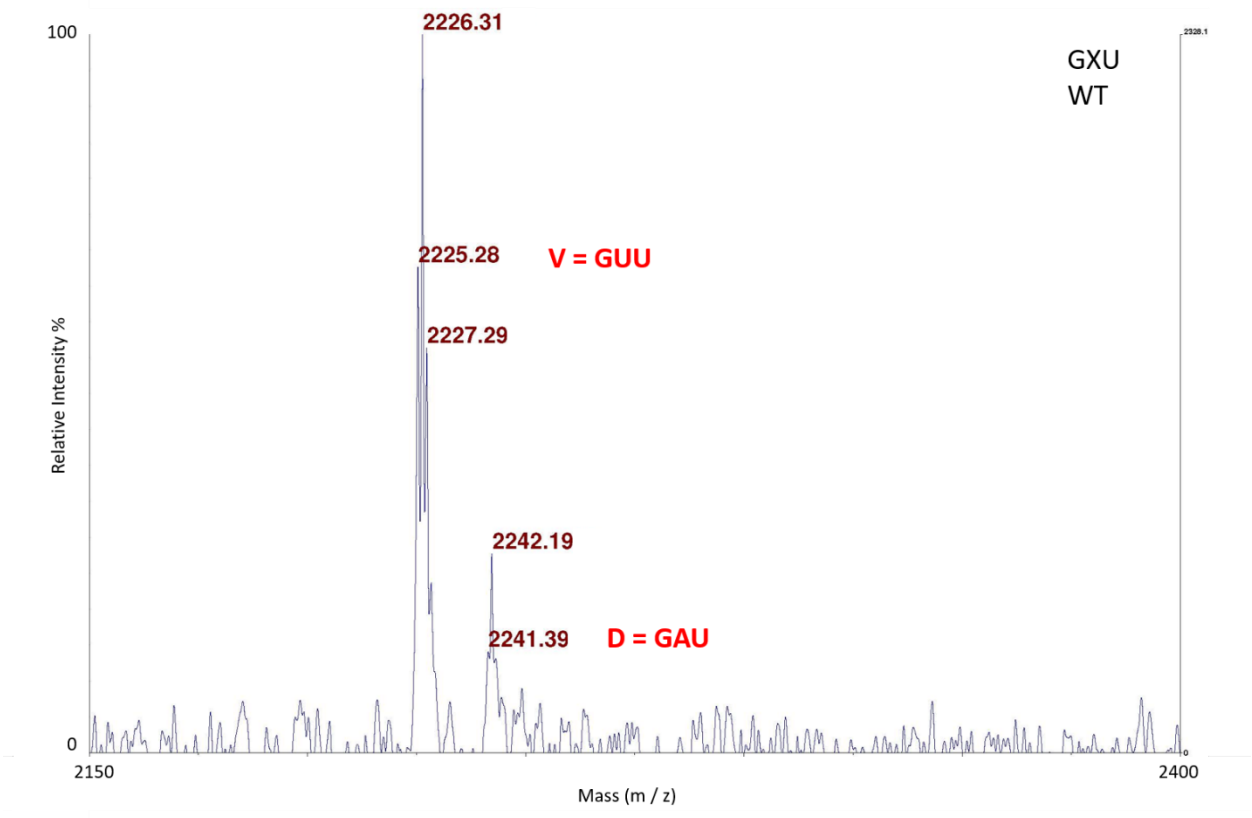


Figure S45. MALDI-MS spectra of mRNA-13 GXU using E. coli WT ribosome. Calculated MH_6VMSPQPVDSWV: 2225.99; Observed: 2225.28. Calculated MH_6DMSPQPVDSWV: 2241.95; Observed: 2241.19.

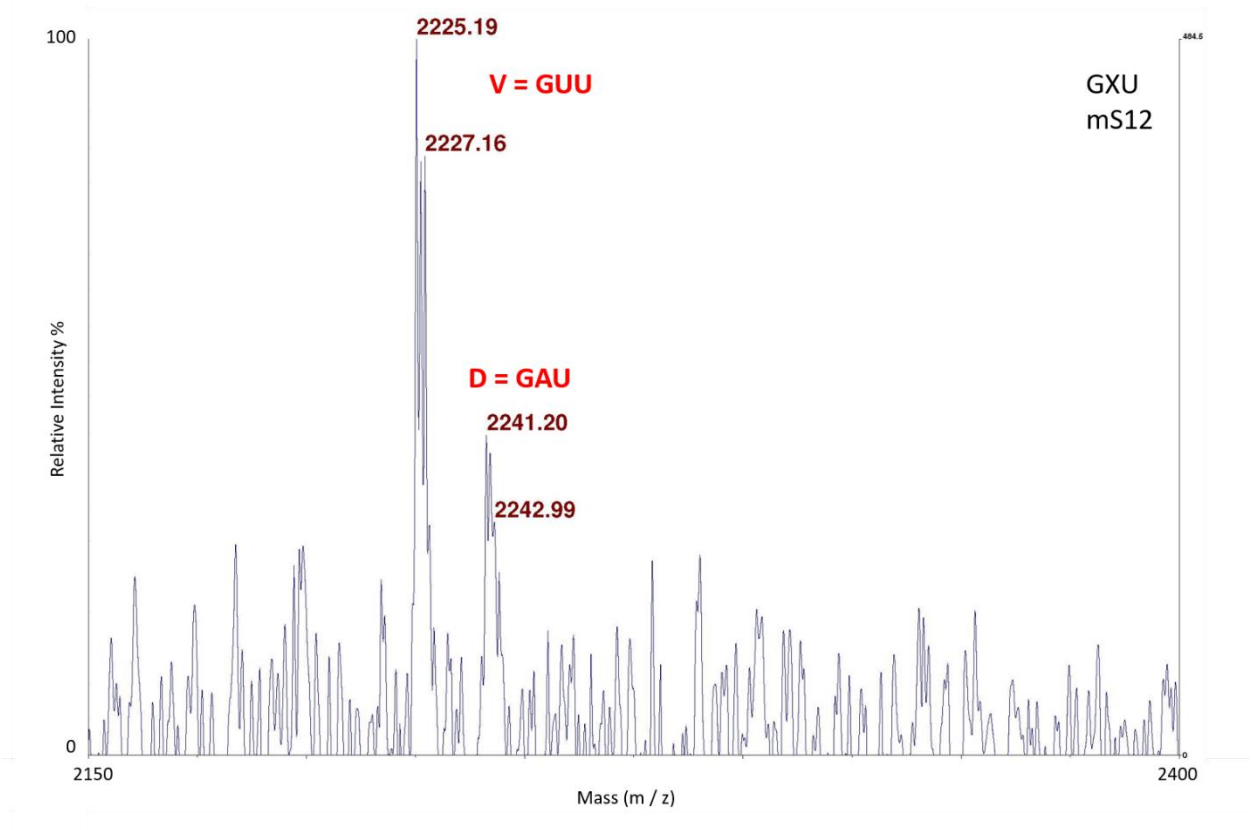


Figure S46. MALDI-MS spectra of mRNA-13 GXU using mS12 Hyperaccurate Ribosome. Calculated MH_6VMSPQPVDSWV: 2225.99; Observed: 2225.19. Calculated MH_6DMSPQPVDSWV: 2241.95; Observed: 2241.20

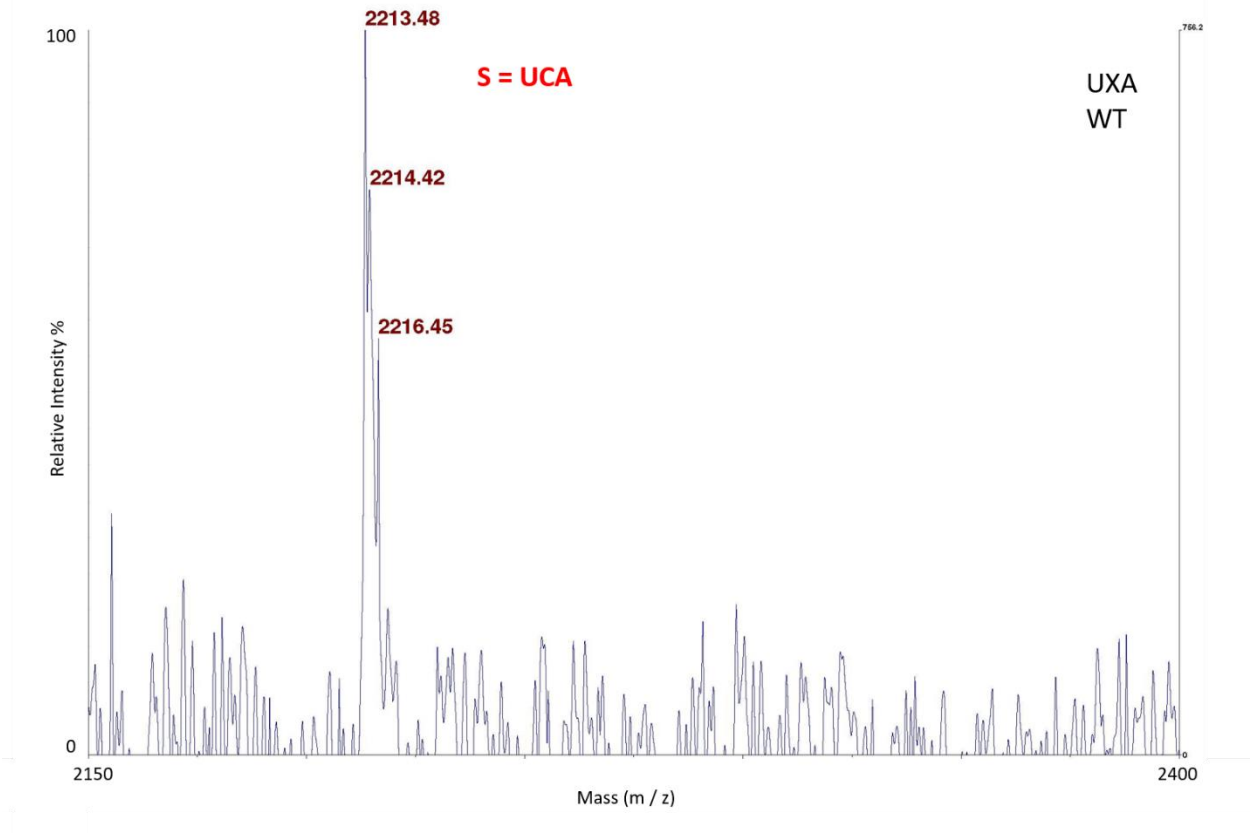


Figure S47. MALDI-MS spectra of mRNA-14 UXA using E. coli WT ribosome. Calculated MH_6SMSPQPVDSWV:2213.95; Observed:2213.48.

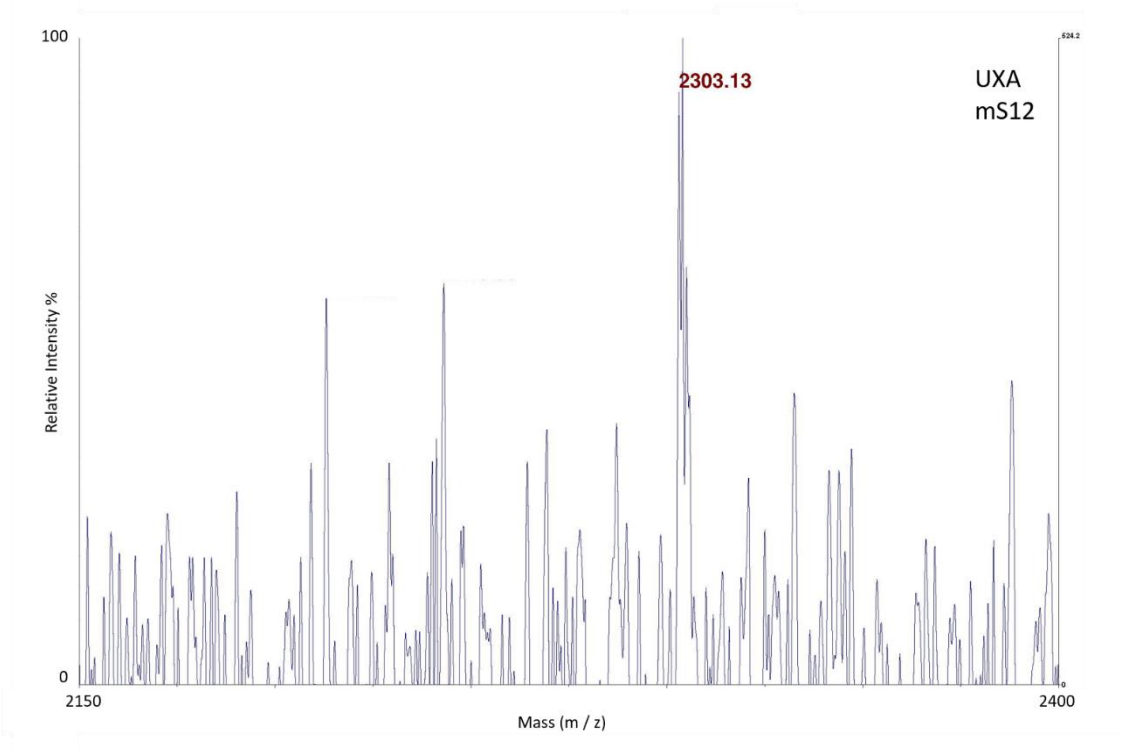


Figure S48. MALDI-MS zoomed spectra of mRNA-14 UXA using mS12 Hyperaccurate Ribosome. No ASSIANgable peaks were detected in the mass range 2150-2400.

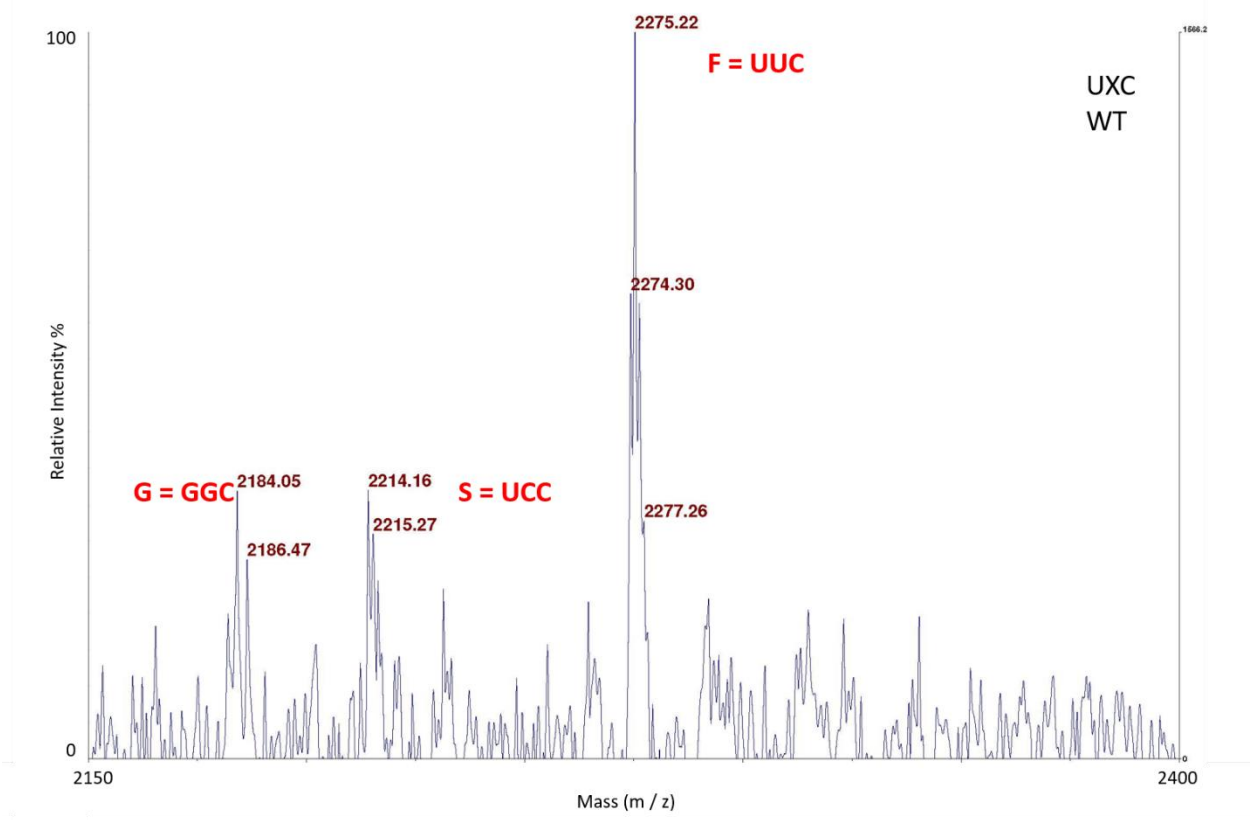


Figure S49. MALDI-MS spectra of mRNA-15 UXC using E. coli WT ribosome. Calculated MH_6SMSPQPVDSWV: 2183.94; Observed: 2184.05. Calculated MH_6SMSPQPVDSWV: 2213.95; Observed: 2214.16. Calculated MH_6FMSPQPVDSWV: 2273.99; Observed: 2274.30.

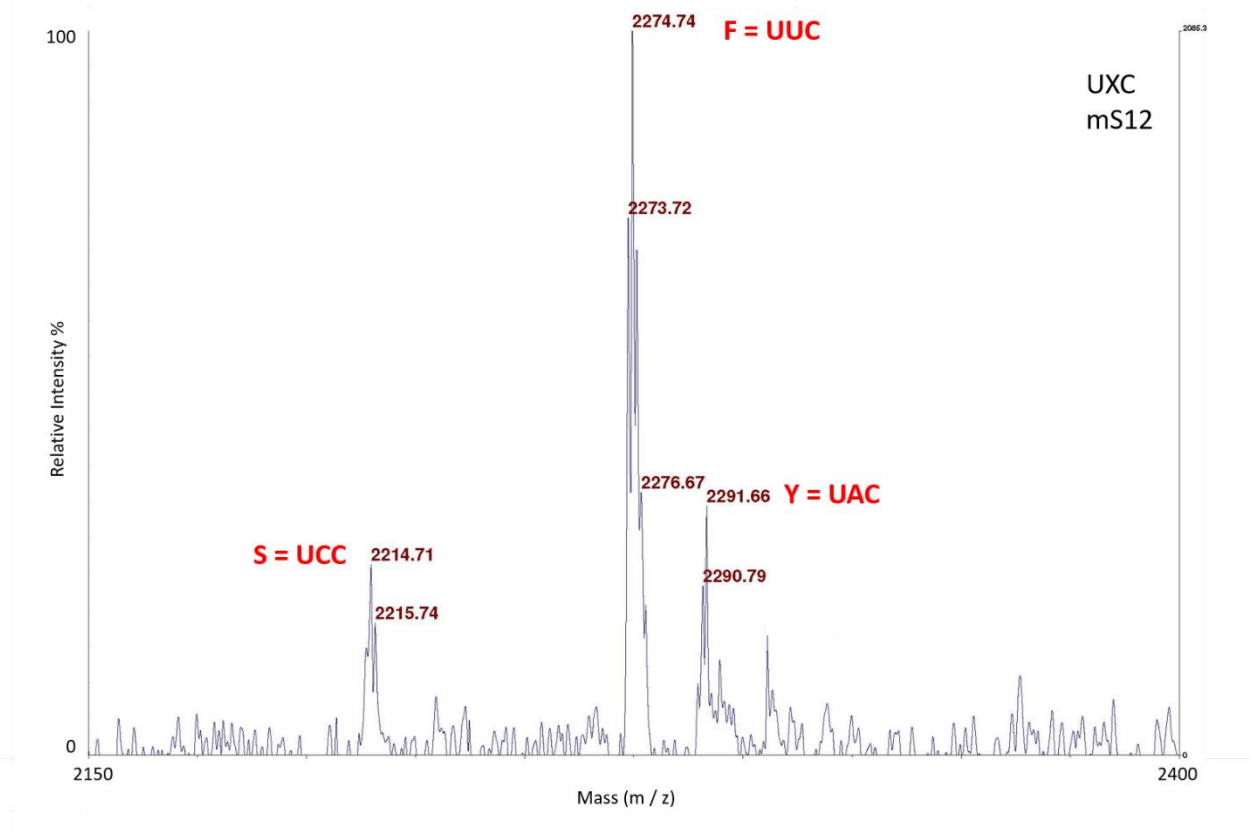


Figure S50. MALDI-MS spectra of mRNA-15 UXC using mS12 Hyperaccurate Ribosome. Calculated MH_6SMSPQPVDSWV: 2213.95; Observed: 2214.71. Calculated MH_6FMSPQPVDSWV: 2273.99; Observed: 2273.72. MH_6YMSPQPVDSWV: 2289.98; Observed: 2290.79

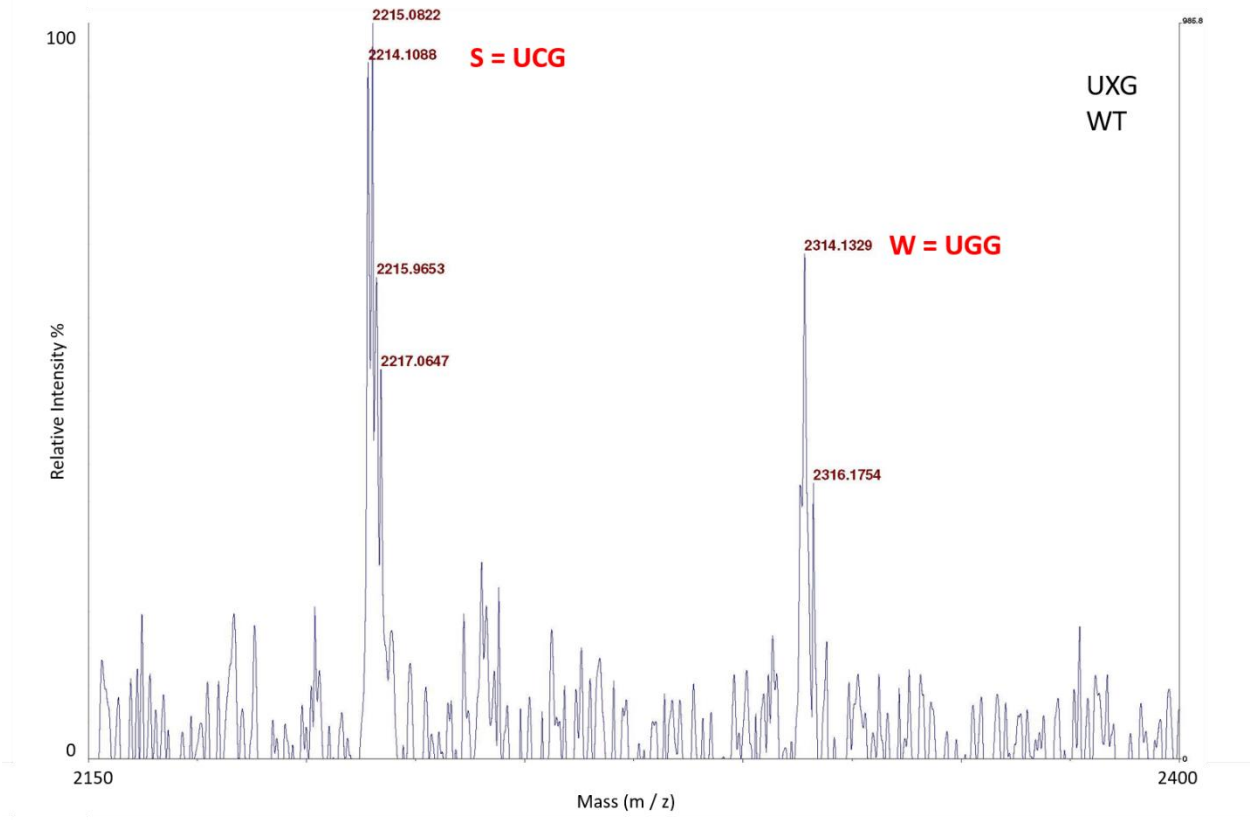


Figure S51. MALDI-MS spectra of mRNA-16 UXG using E. coli WT ribosome. Calculated MH_6SMSPQPVDSWV: 2213.95; Observed: 2214.71. MH_6WMSPQPVDSWV: 2313.00; Observed: 2314.13

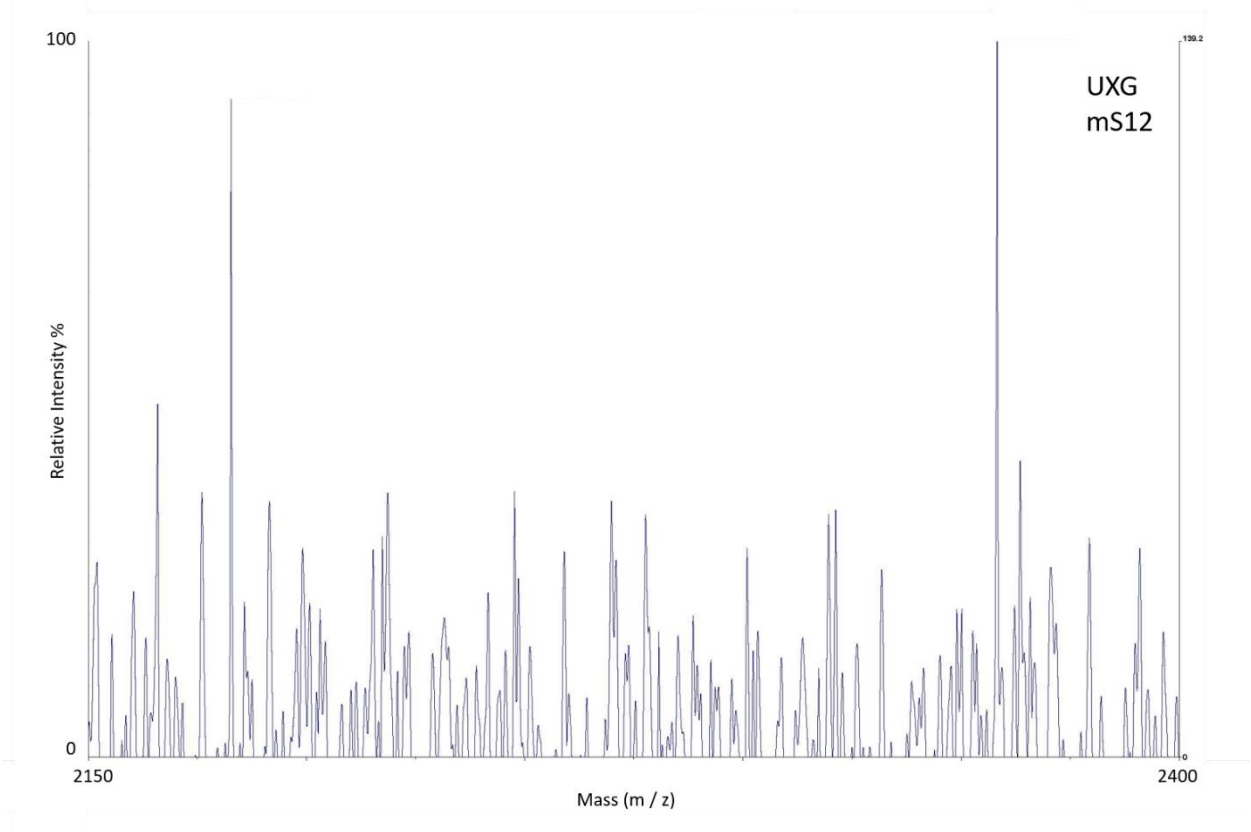


Figure S52. MALDI-MS spectra of mRNA-16 UXG using mS12 Hyperaccurate Ribosome. No assignable peaks were detected in mass range 2150-2400.

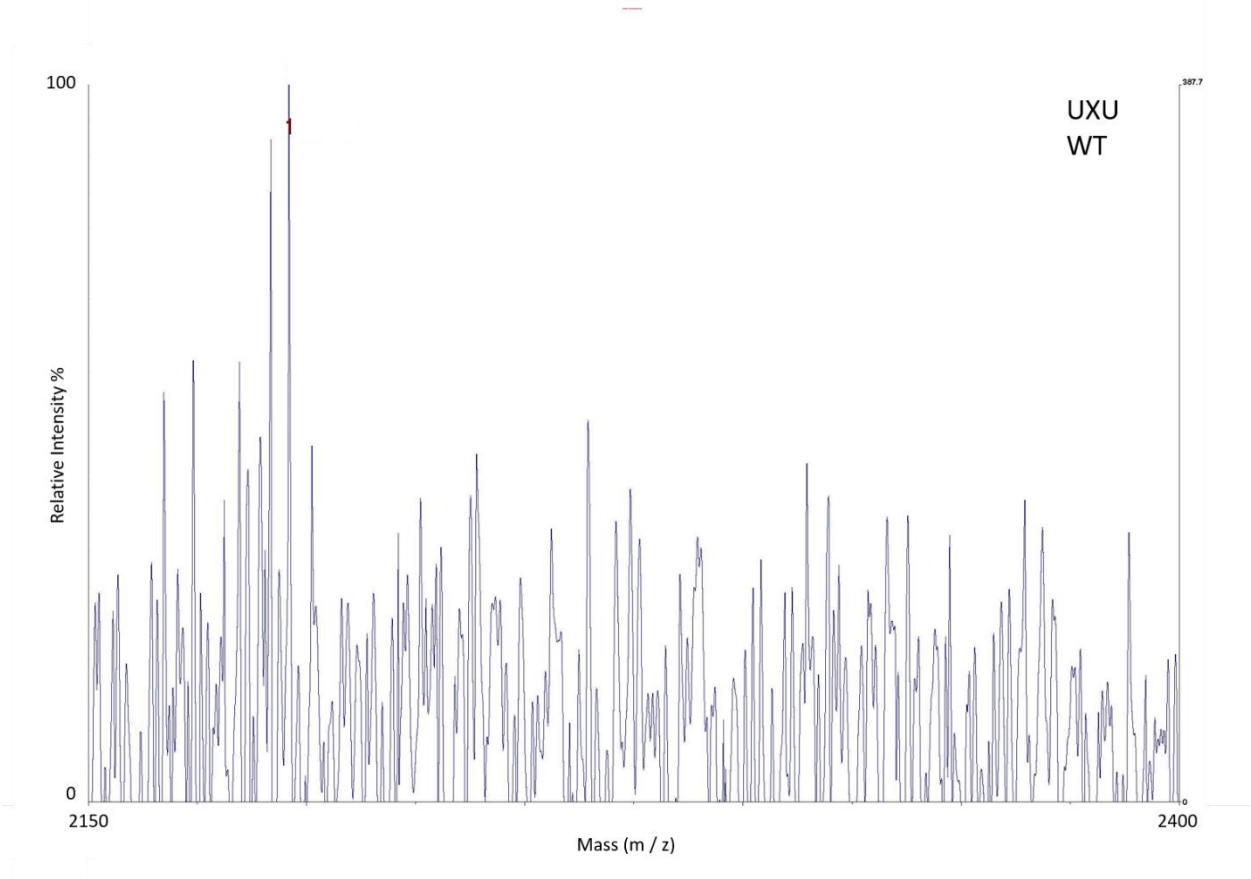


Figure S33. MALDI-MS spectra of mRNA-17 UXU using E.coli WT ribosome. No assignable peaks were detected in mass range 2150-2400.

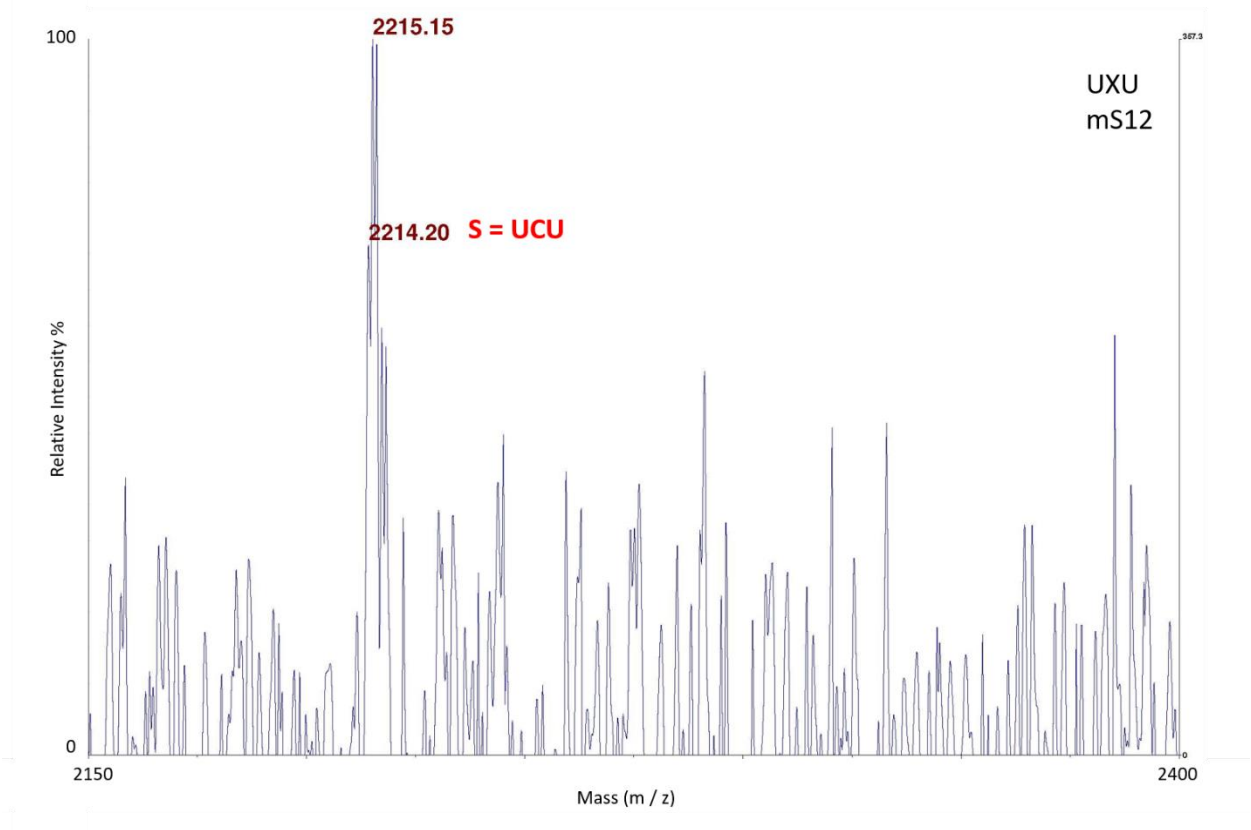


Figure S34. MALDI-MS spectra of mRNA-17 UXU using mS12 Hyperaccurate Ribosome. Calculated MH_6SMSPQPVDSWV: 2213.95; Observed: 2214.20.

Leu-tRNA Containing 5SICS Pre-Charging After Reductive Amination and Nuclease Digestion S58-S60

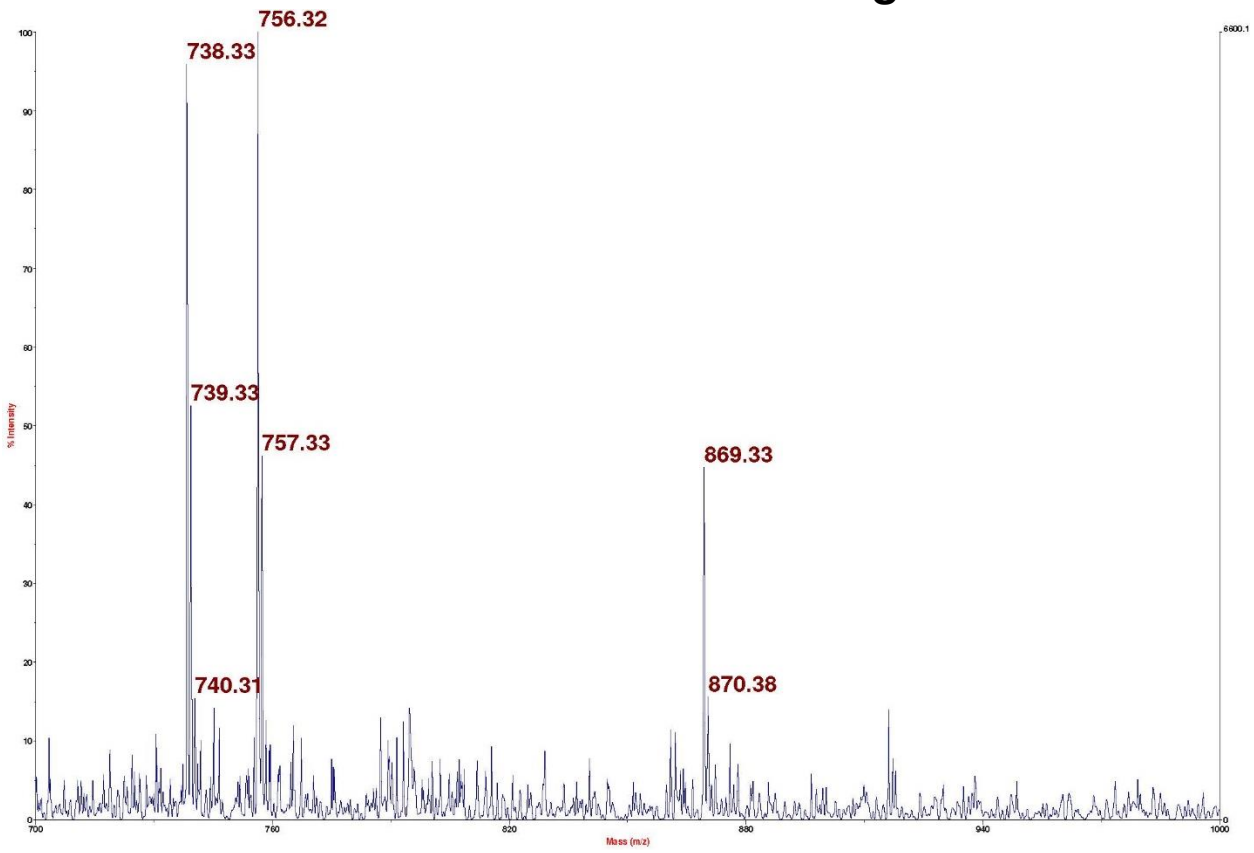


Figure S35. MALDI-MS of precharged Leu-tRNA-2 GYU after reductive amination and nuclease digestion. EM [M+H]: 869.32, observed: 869.33

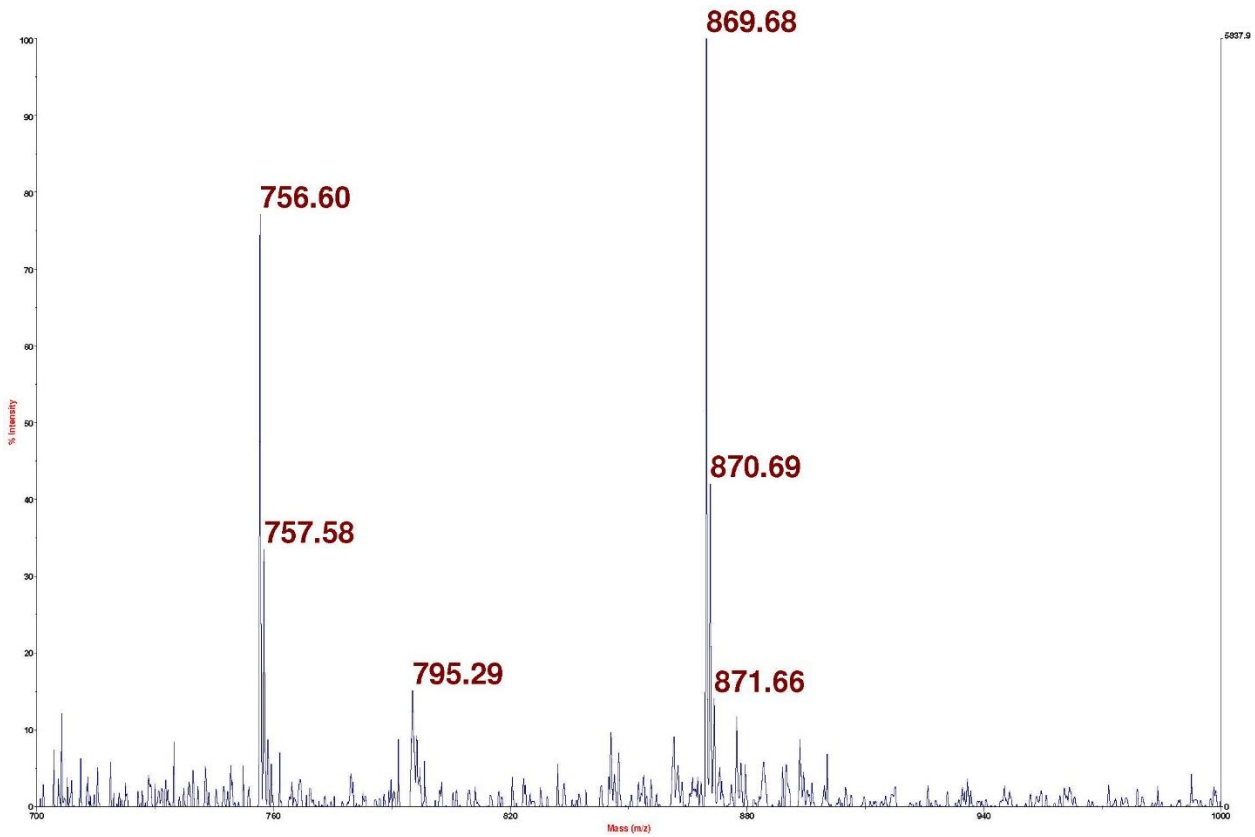


Figure S366. MALDI-MS of precharged Leu-tRNA-1 GYC after reductive amination and nuclease digestion. EM [M+H]: 869.32, observed: 869.68

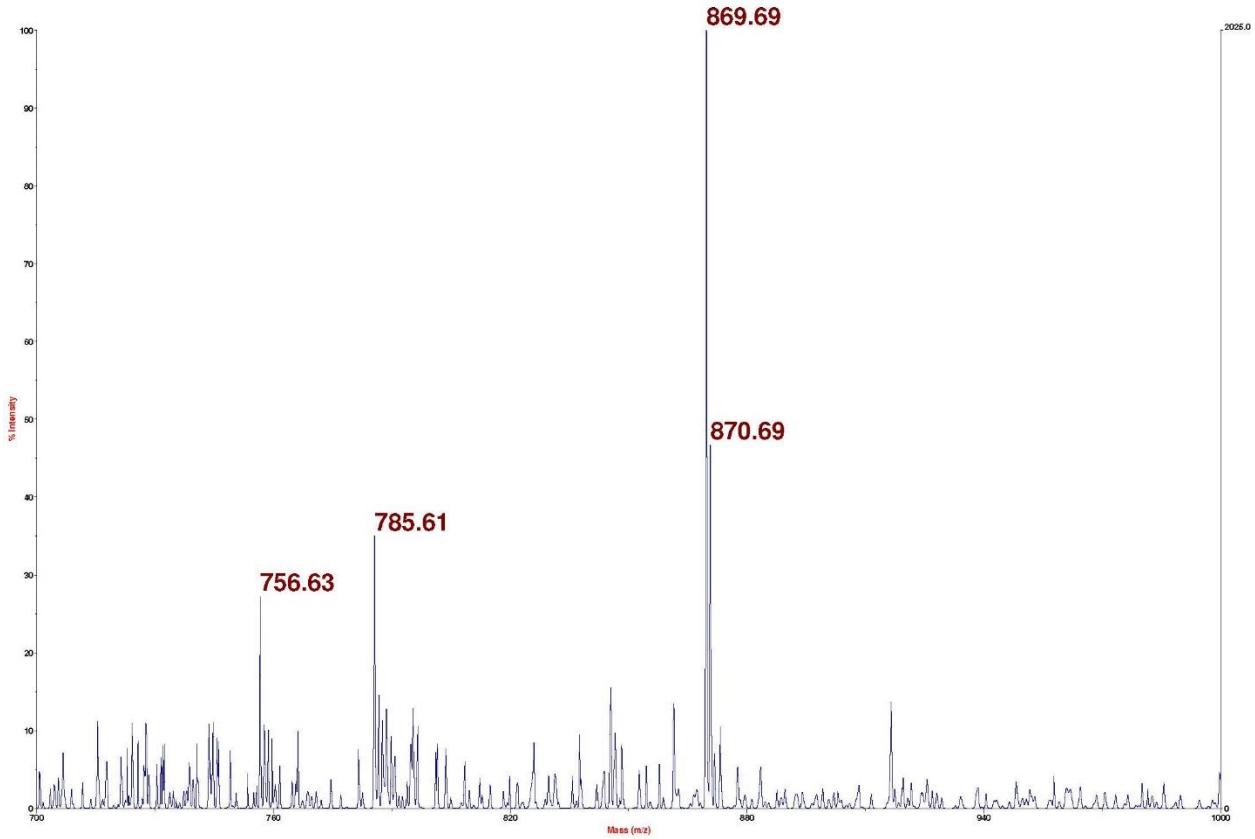


Figure S57. MALDI-MS of precharged Leu-tRNA-3 AYC after reductive amination and nuclease digestion. EM [M+H]: 869.32, observed: 869.69

UBP Translation Mass Spec with Conjugate 5SICS-tRNA S61-S63

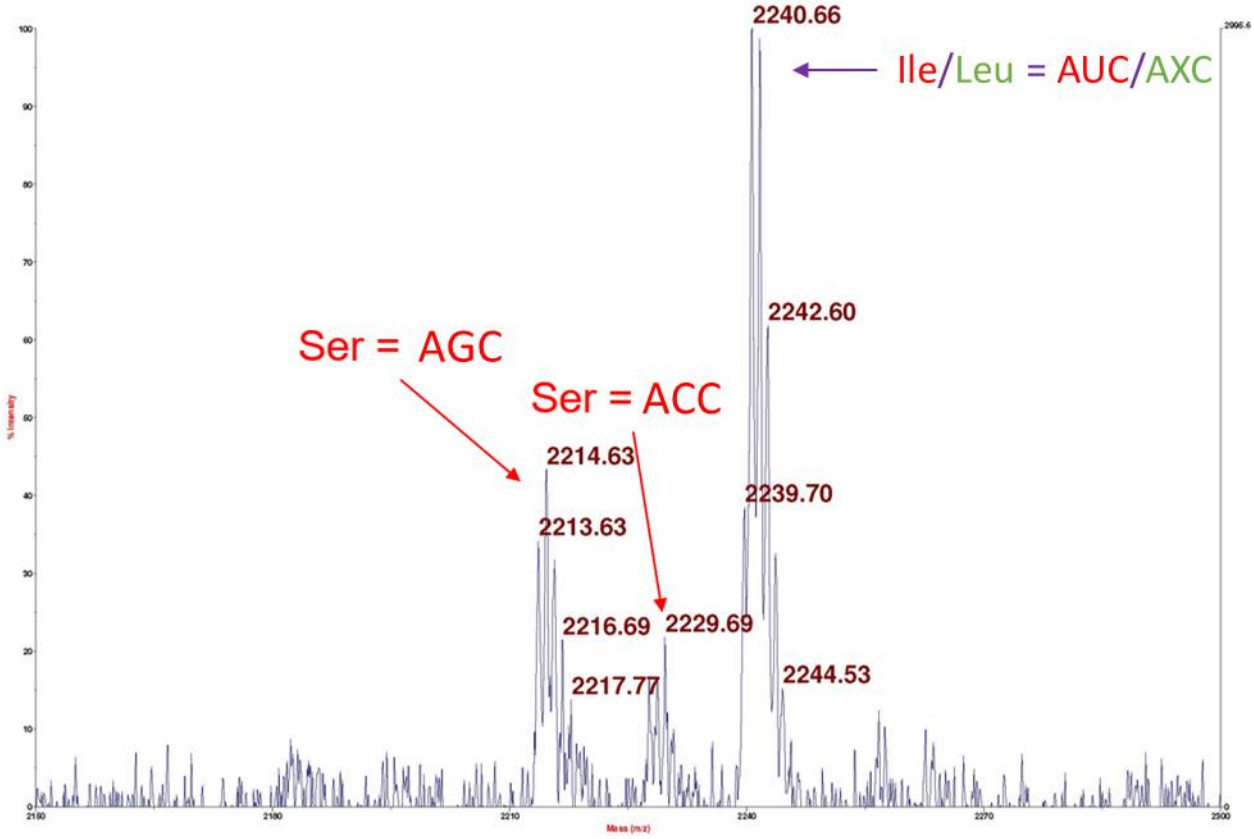


Figure S58. MALDI-MS of the translation product using WT ribosome and template mRNA-3 AXC with 40 uM of Leu-tRNA-2 GYU.

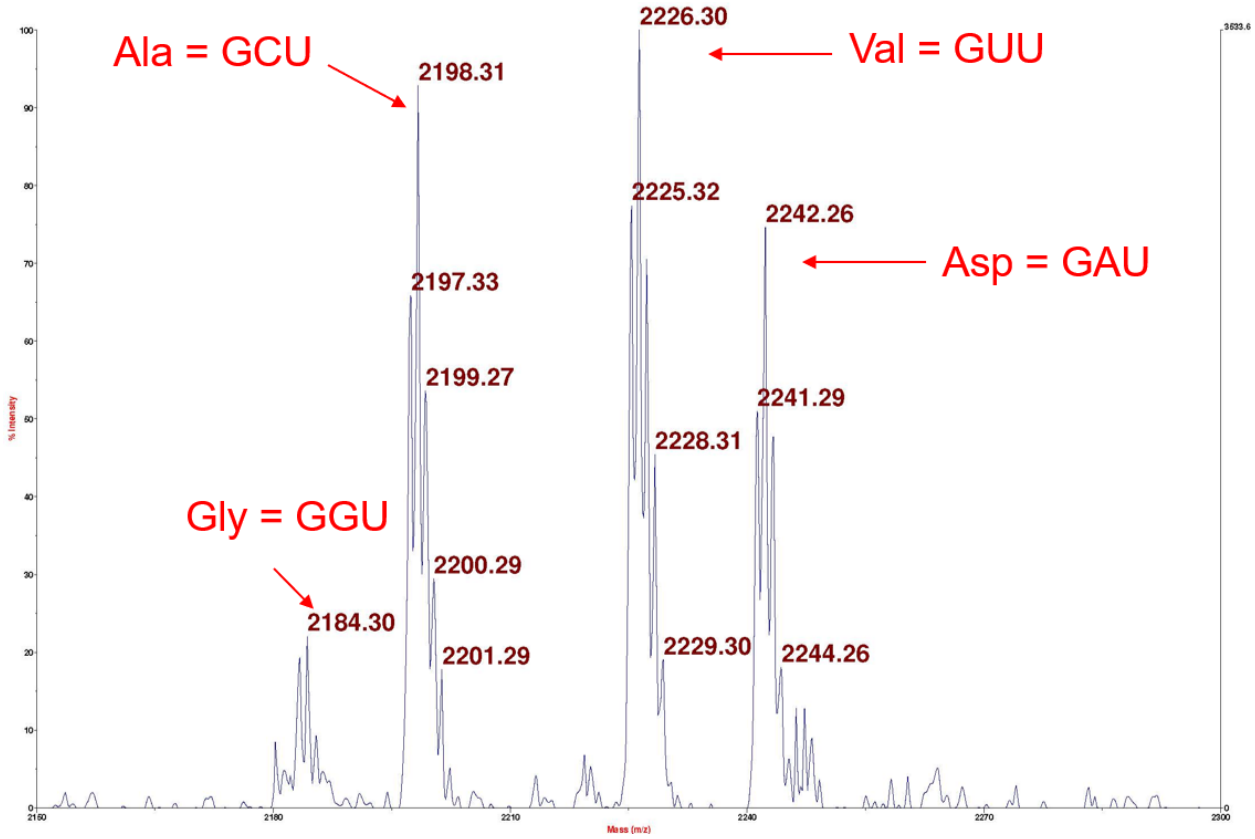


Figure S37. MALDI-MS of the translation product using WT ribosome and template mRNA-13 GXU with 40 uM of Leu-tRNA-3 AYC.

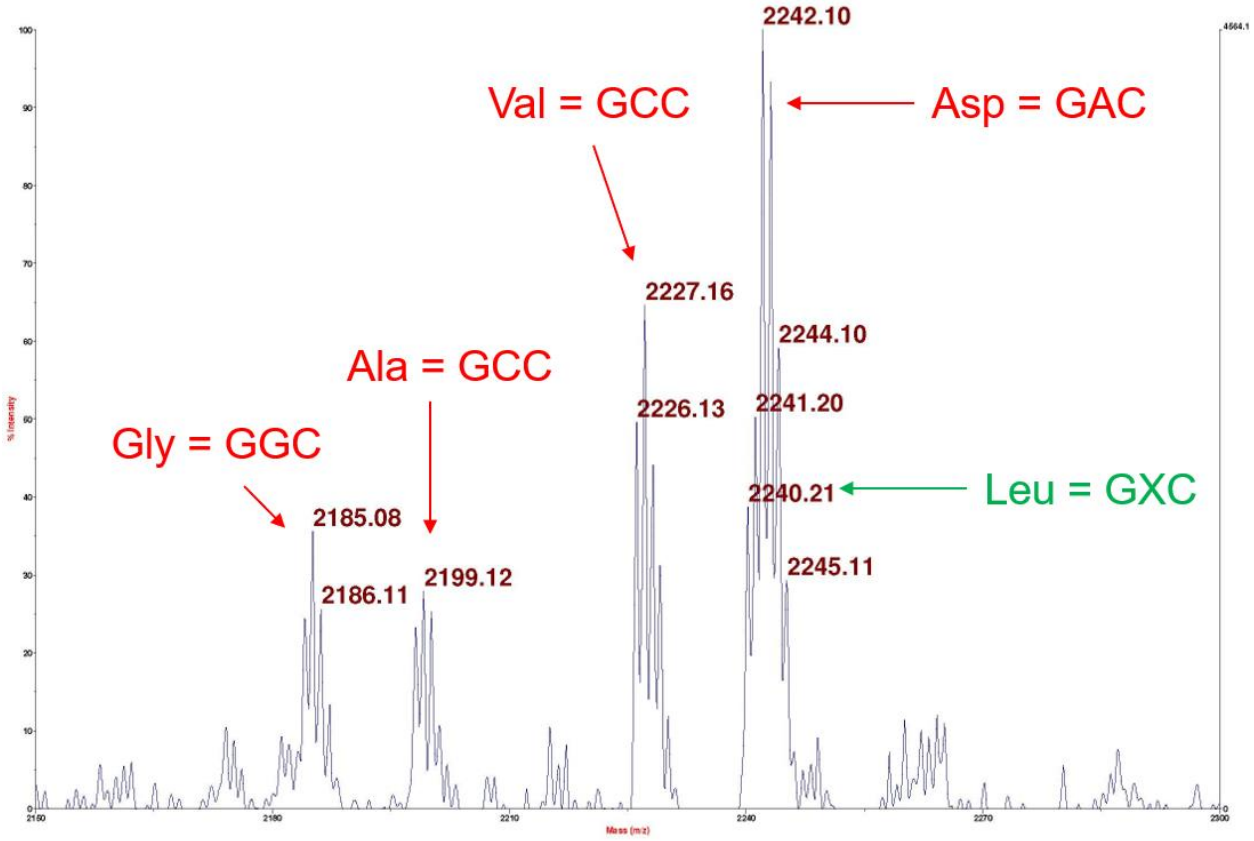


Figure S380. MALDI-MS of the translation product using WT ribosome and template mRNA-11 GXC with 40 uM of Leu-tRNA-1 GYC.

NMR of Synthesized Compounds

NaM Synthetic series

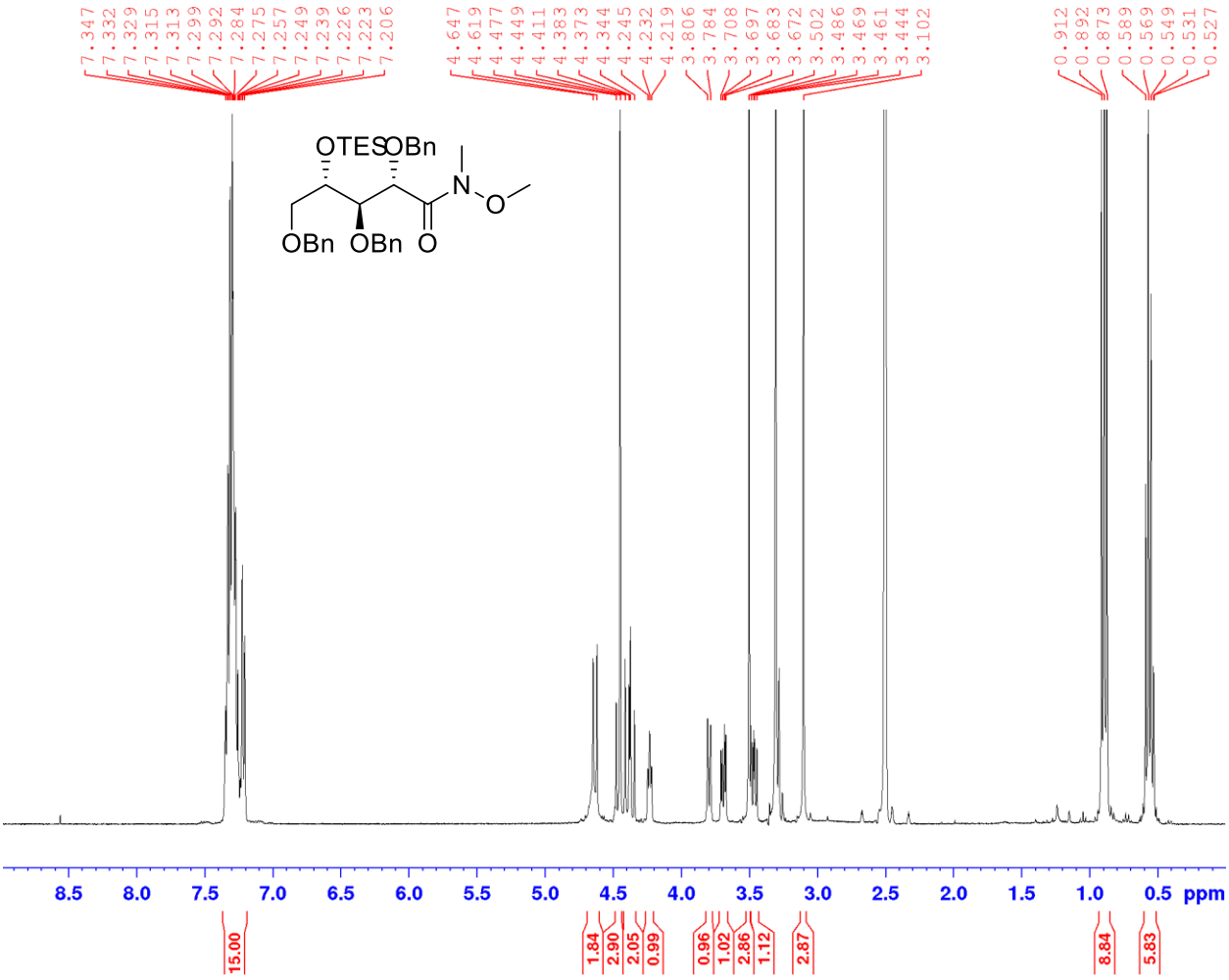


Figure S391. ^{1}H NMR of Compound 3a.

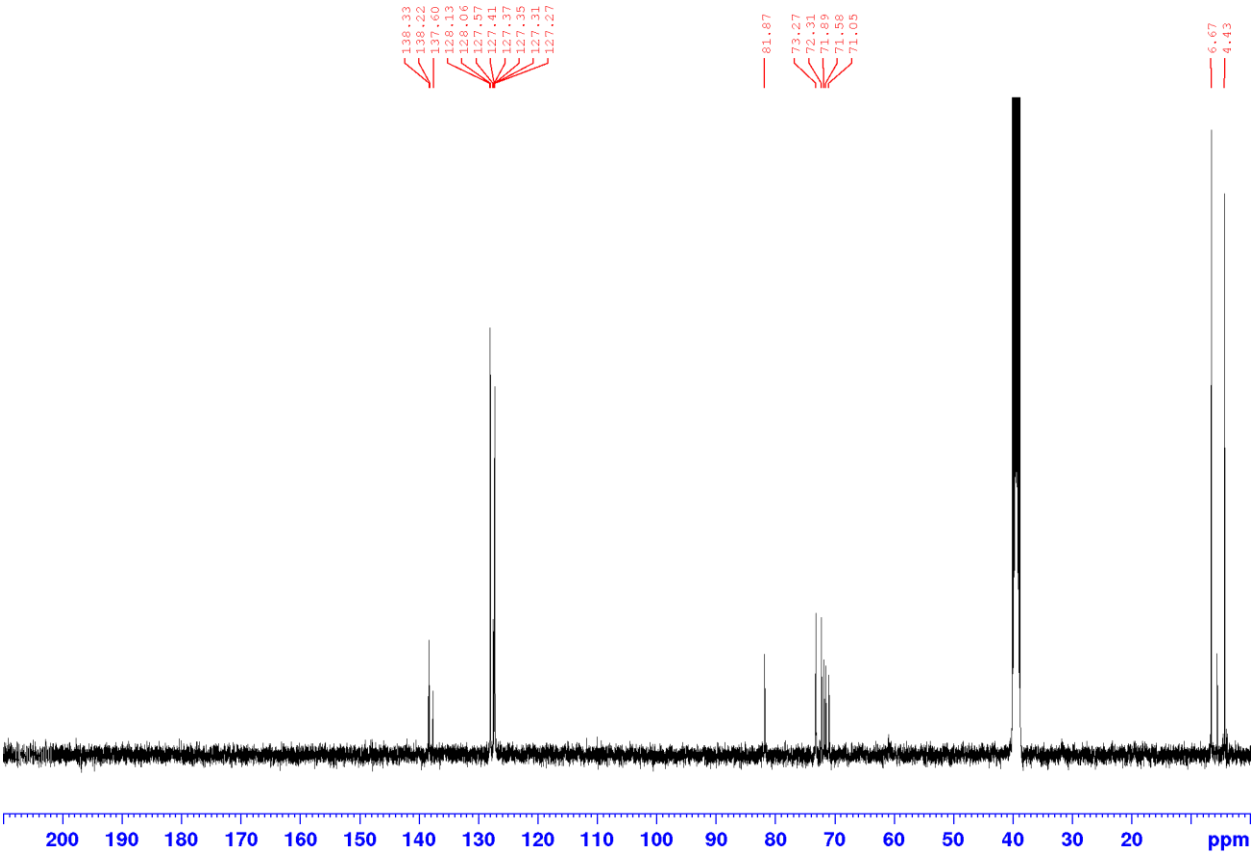


Figure S402. ^{13}C NMR of compound 3a.

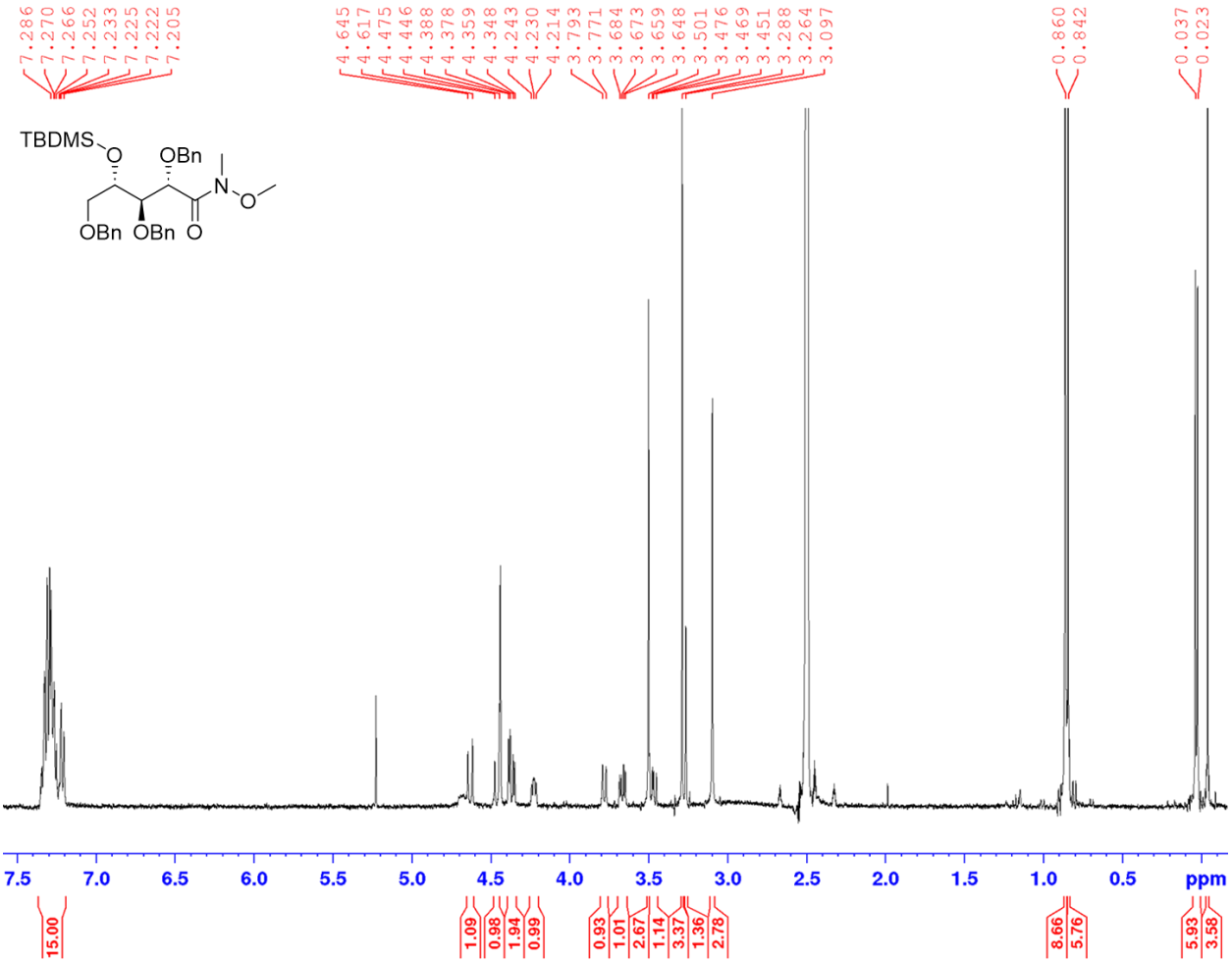


Figure S413. ^{1}H NMR of compound 3b.

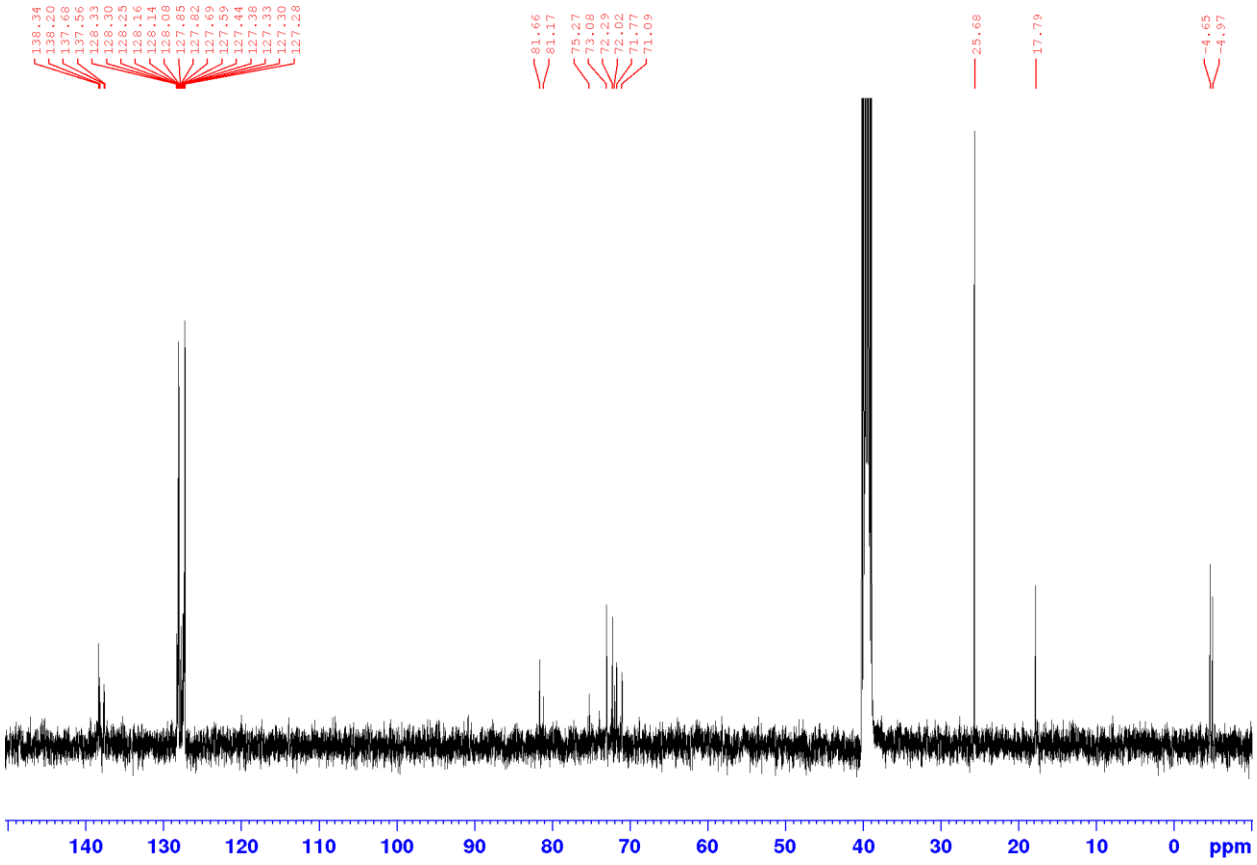


Figure S424. ^{13}C NMR of compound 3b.

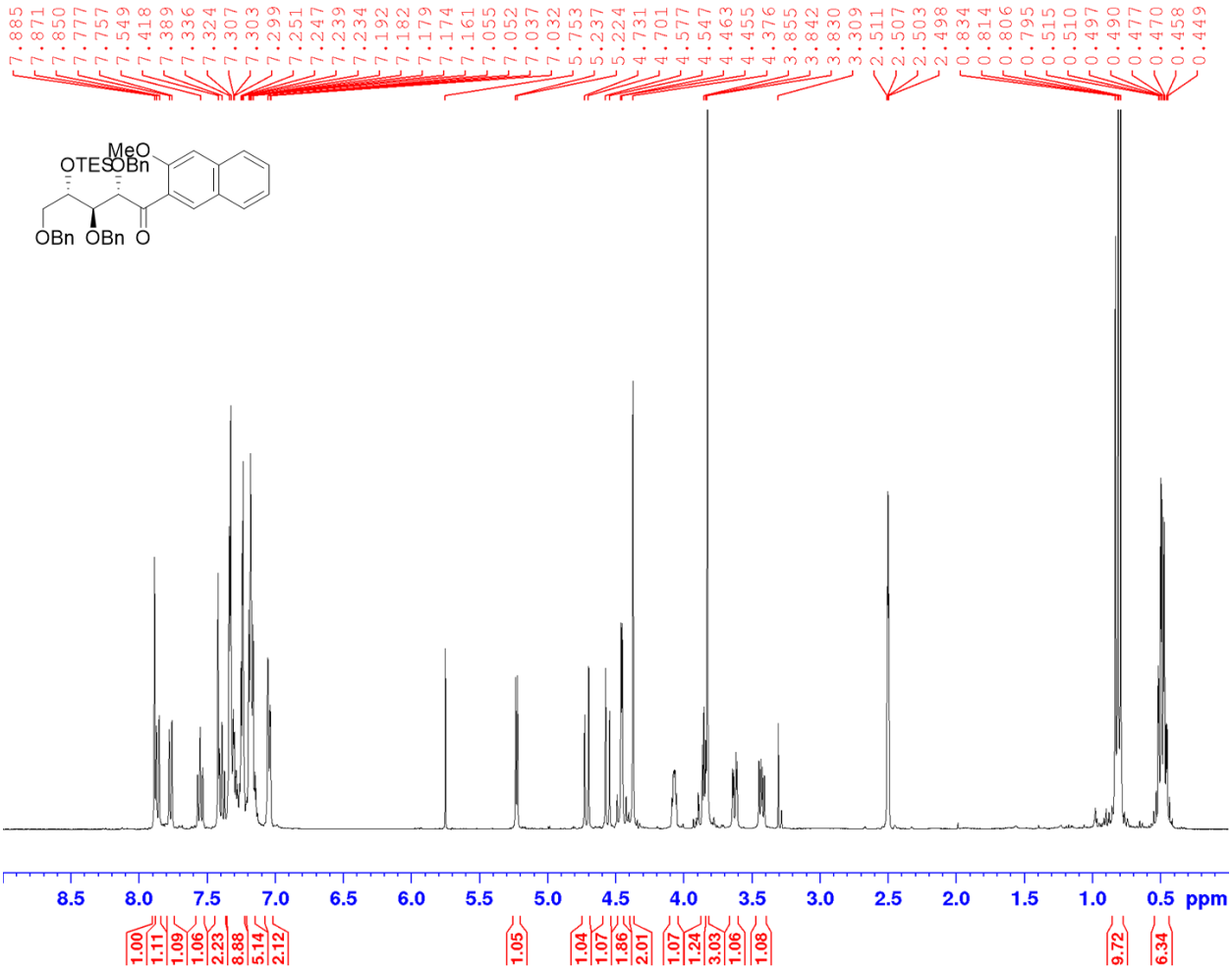


Figure S435. ^{1}H NMR of compound 4a.

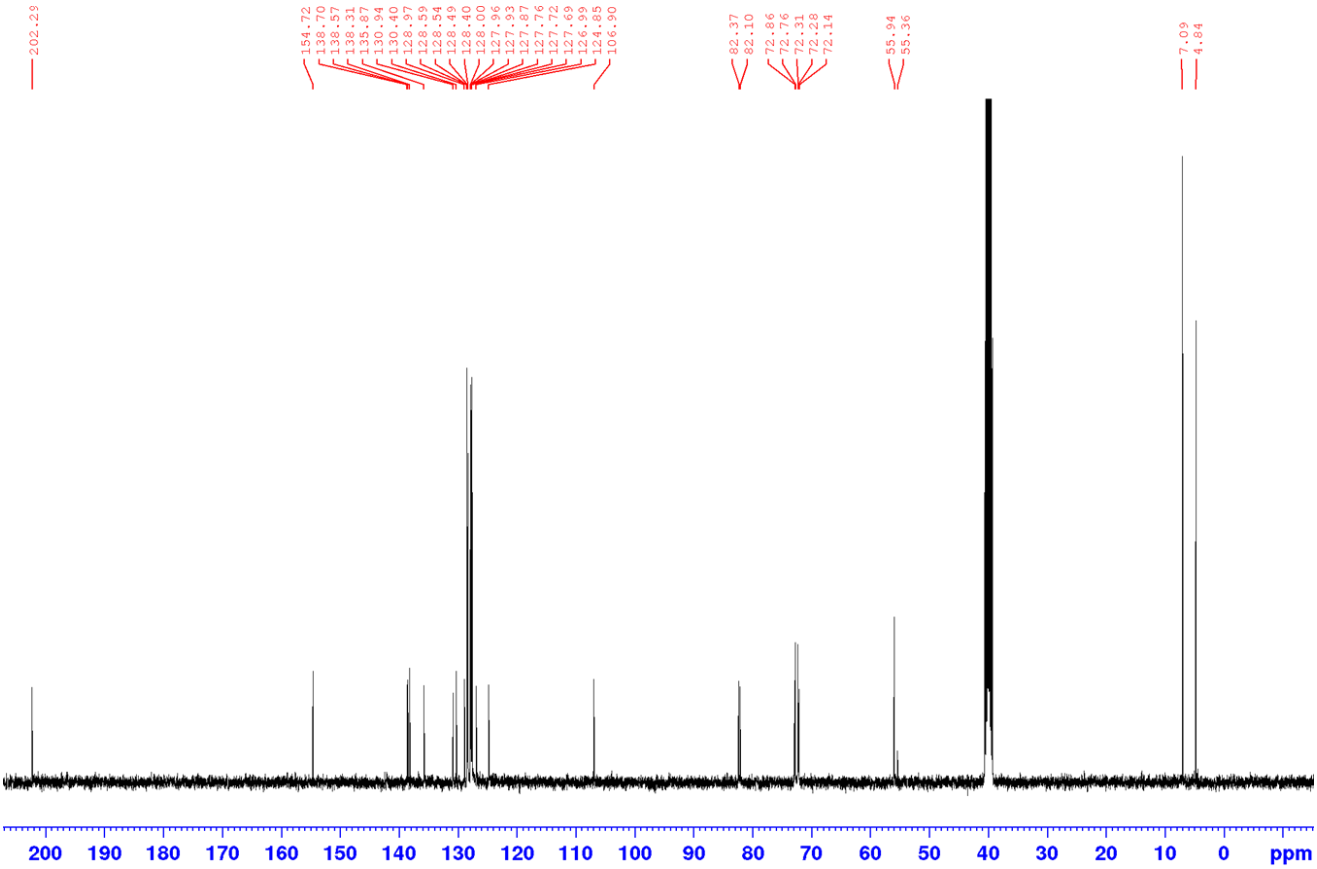


Figure S446. ^{13}C NMR of compound 4a.

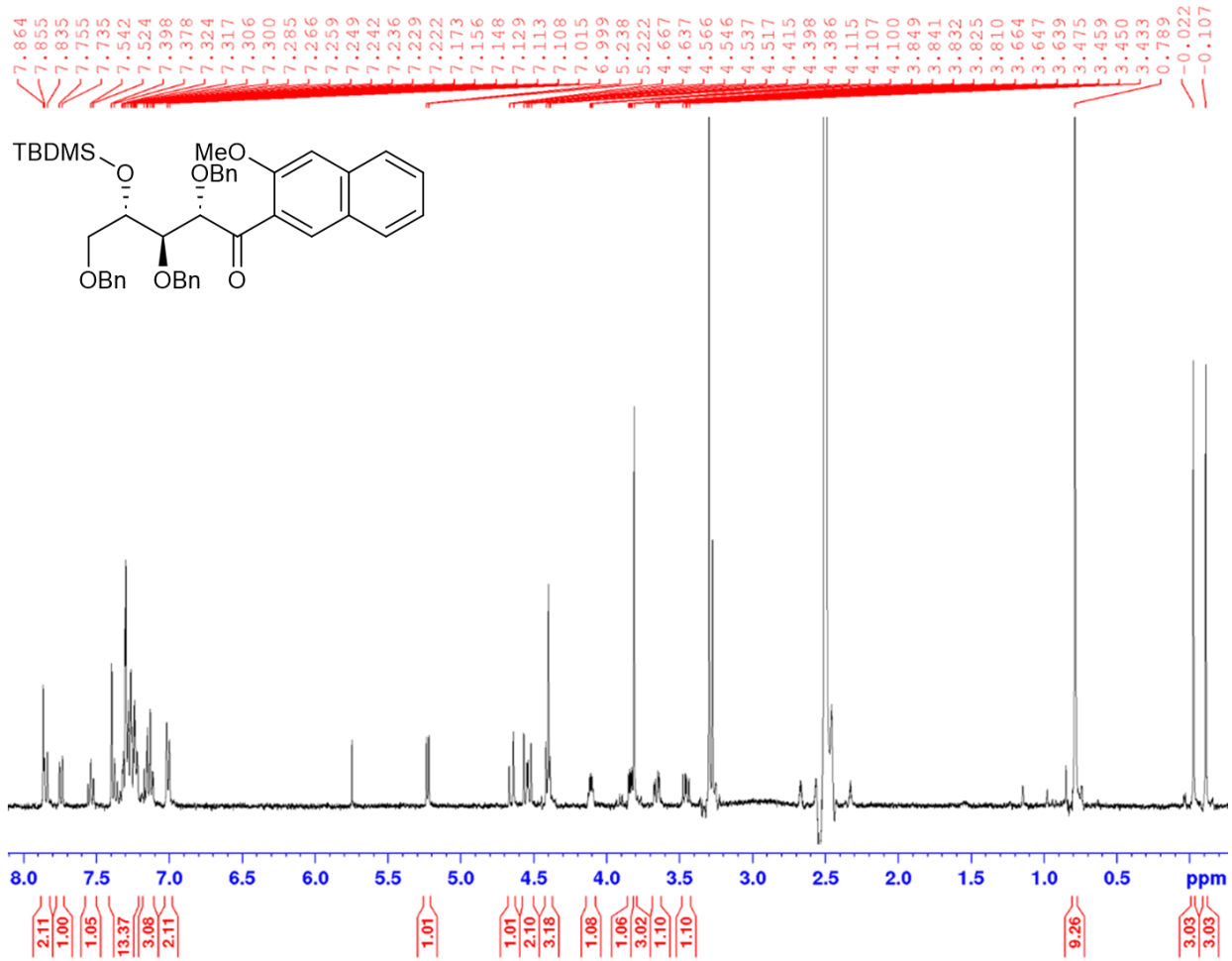


Figure S67. ^{1}H NMR of compound 4b.

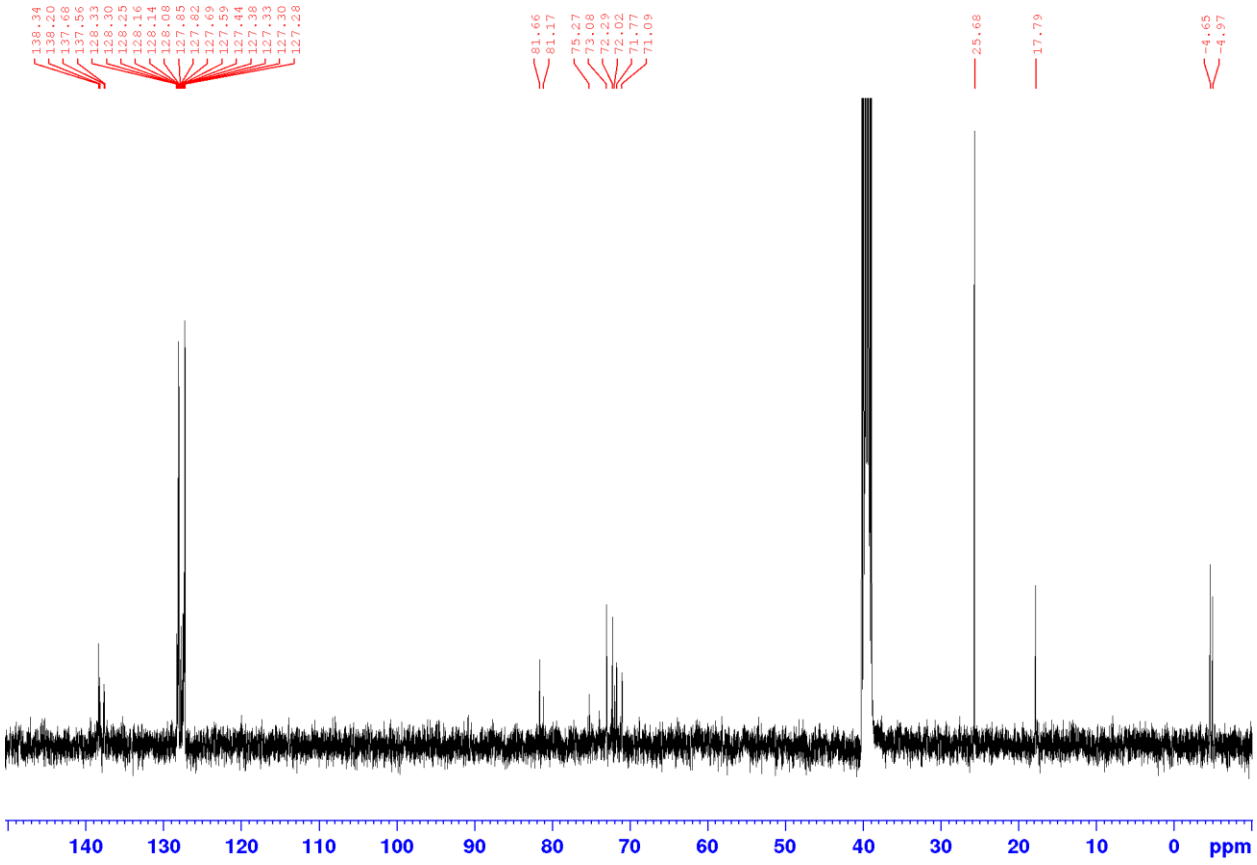


Figure S68. ^{13}C NMR of compound 4b.

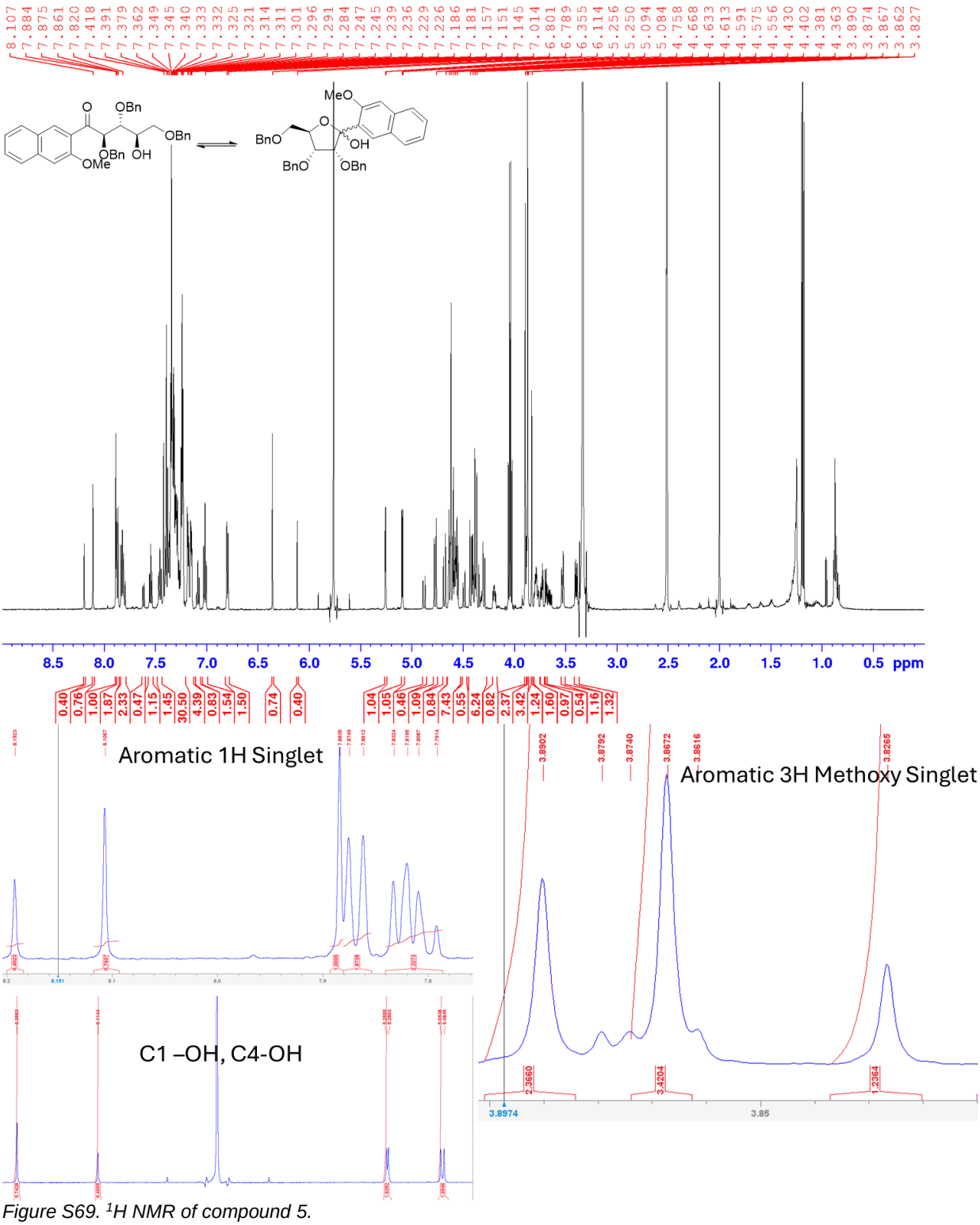


Figure S69. ^{1}H NMR of compound 5.

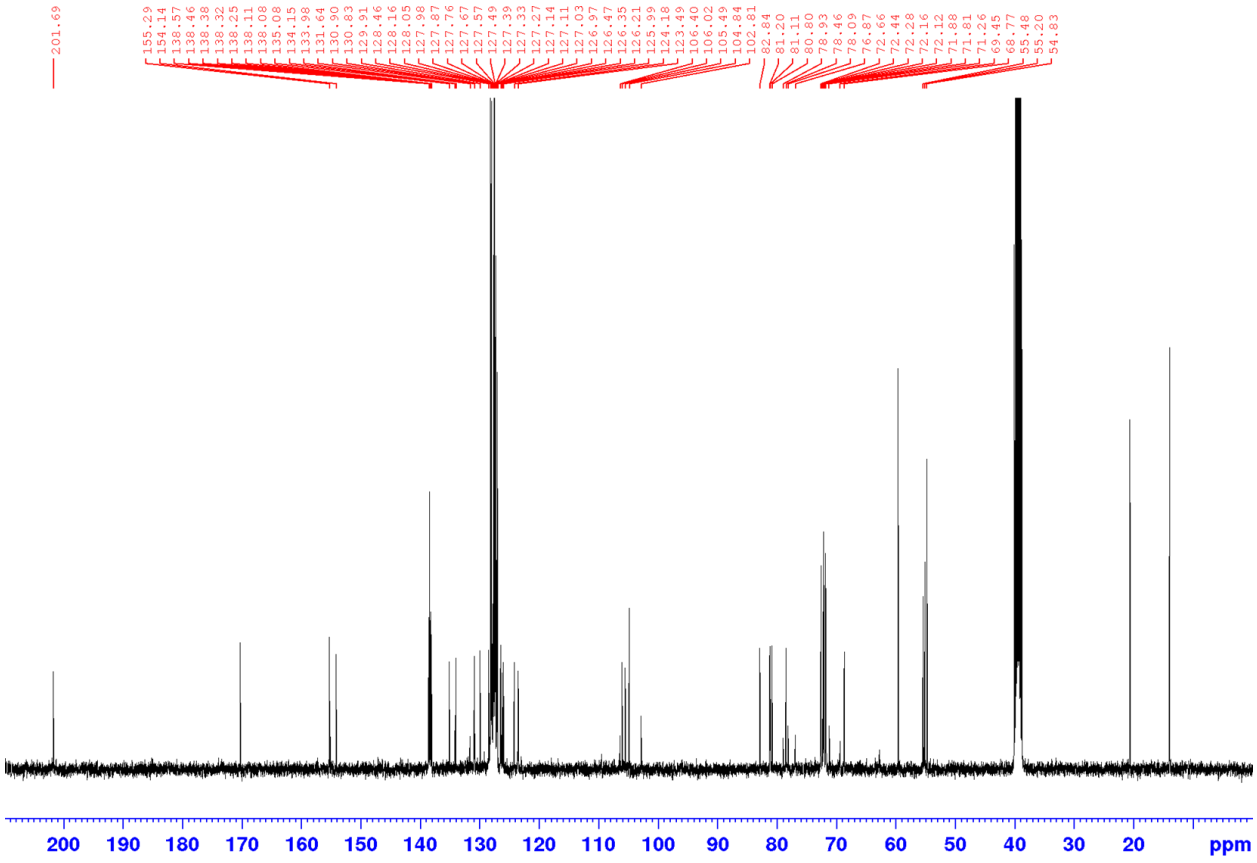


Figure S70. ^{13}C NMR of compound 5

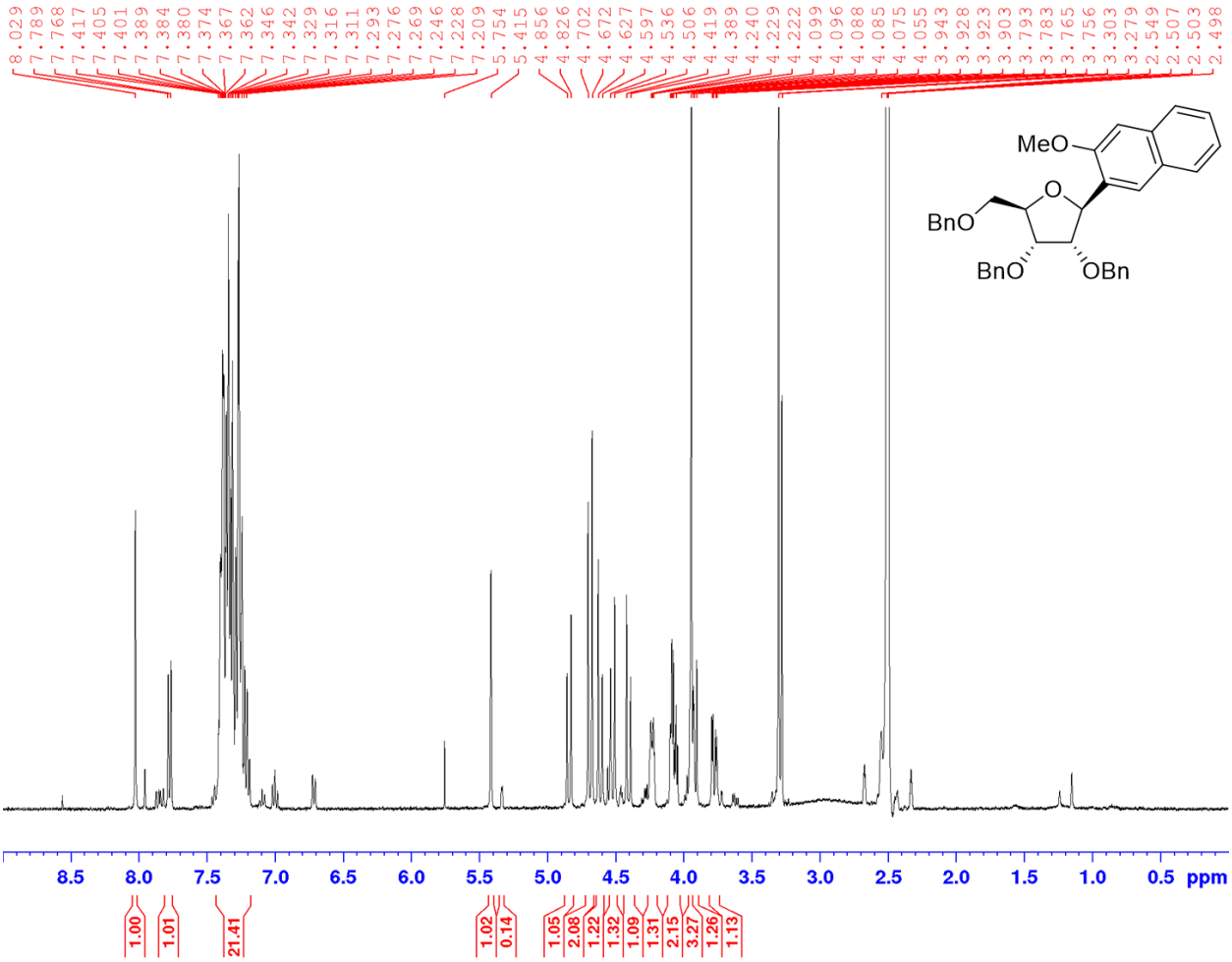


Figure S71. ^{1}H NMR of compound 6.

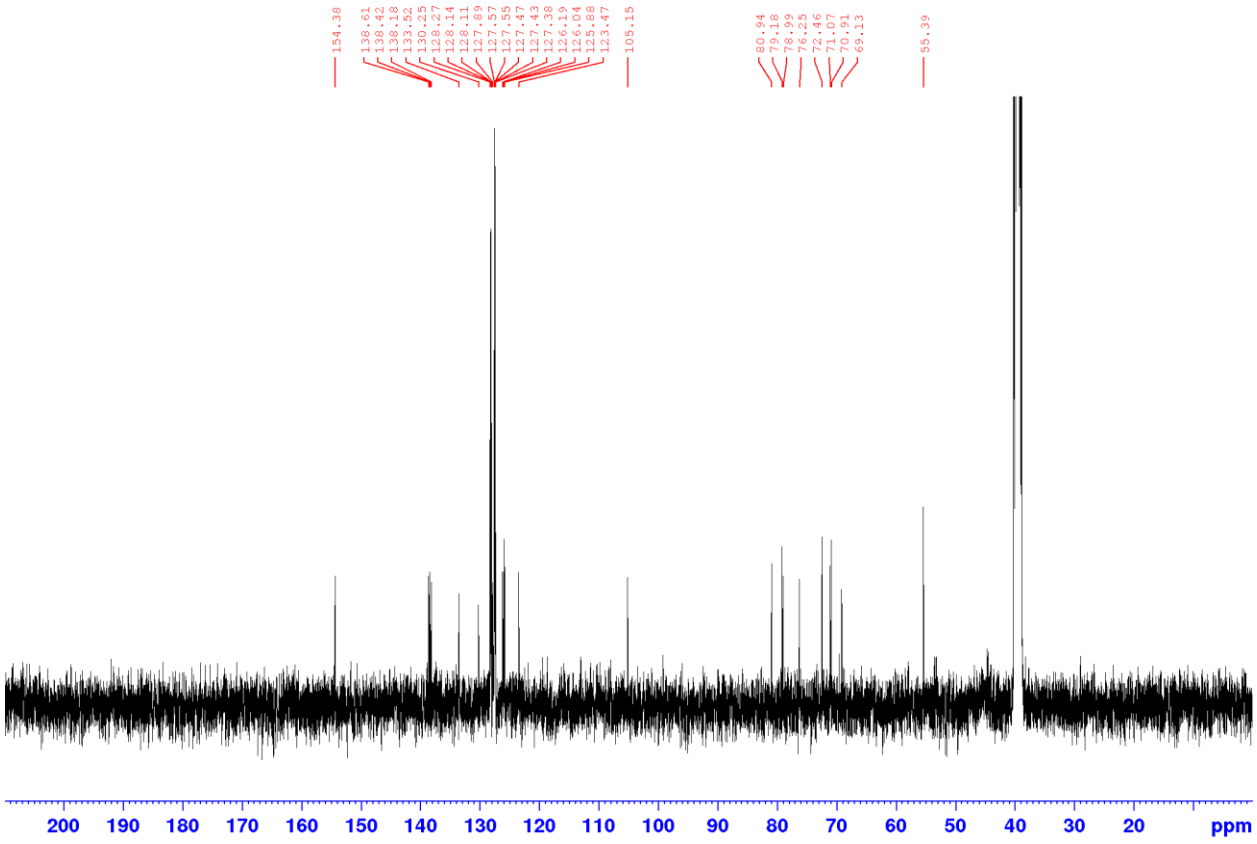


Figure S72. ^{13}C NMR of compound 6.

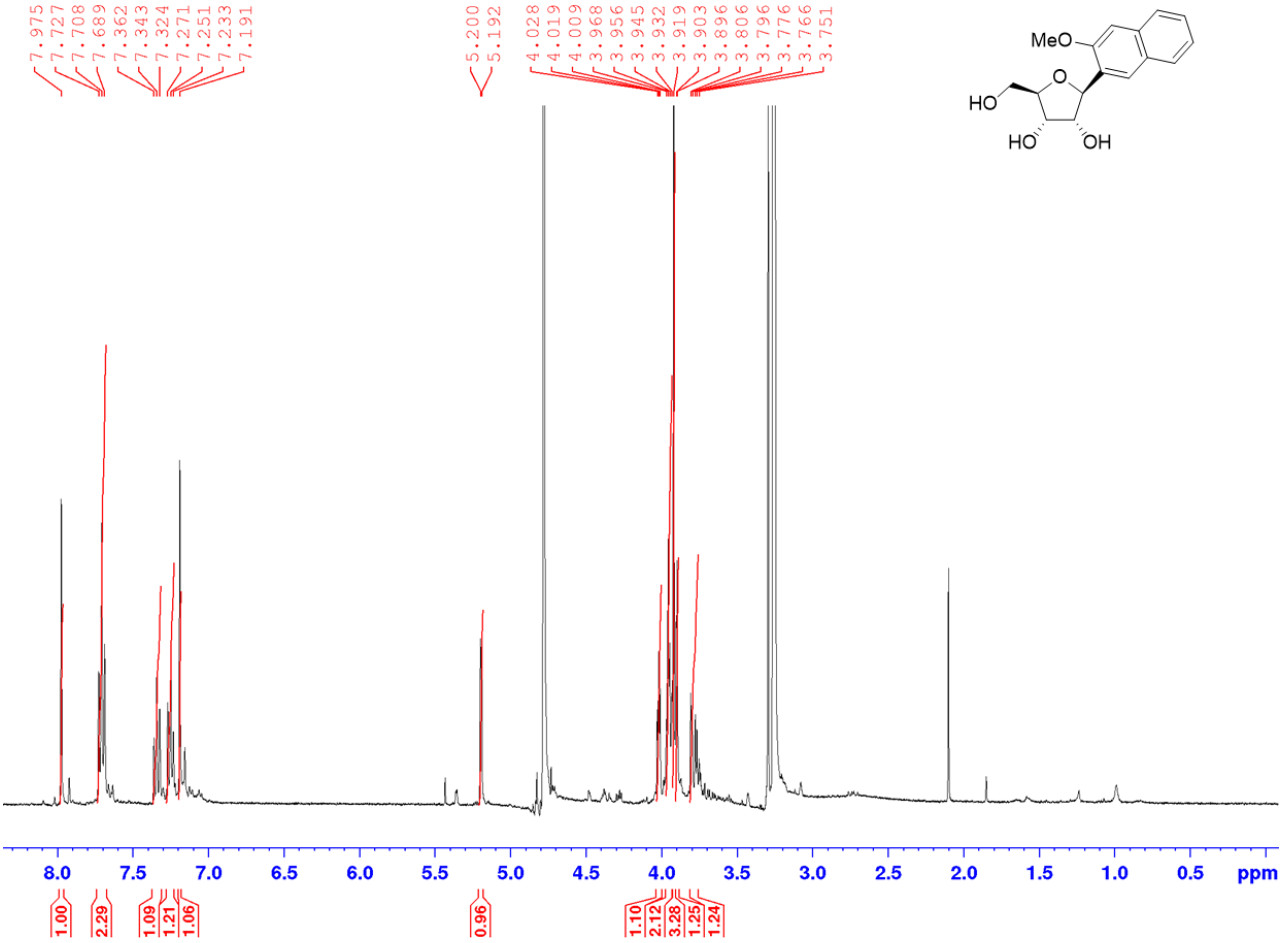


Figure S453. ^{1}H NMR of compound 7.

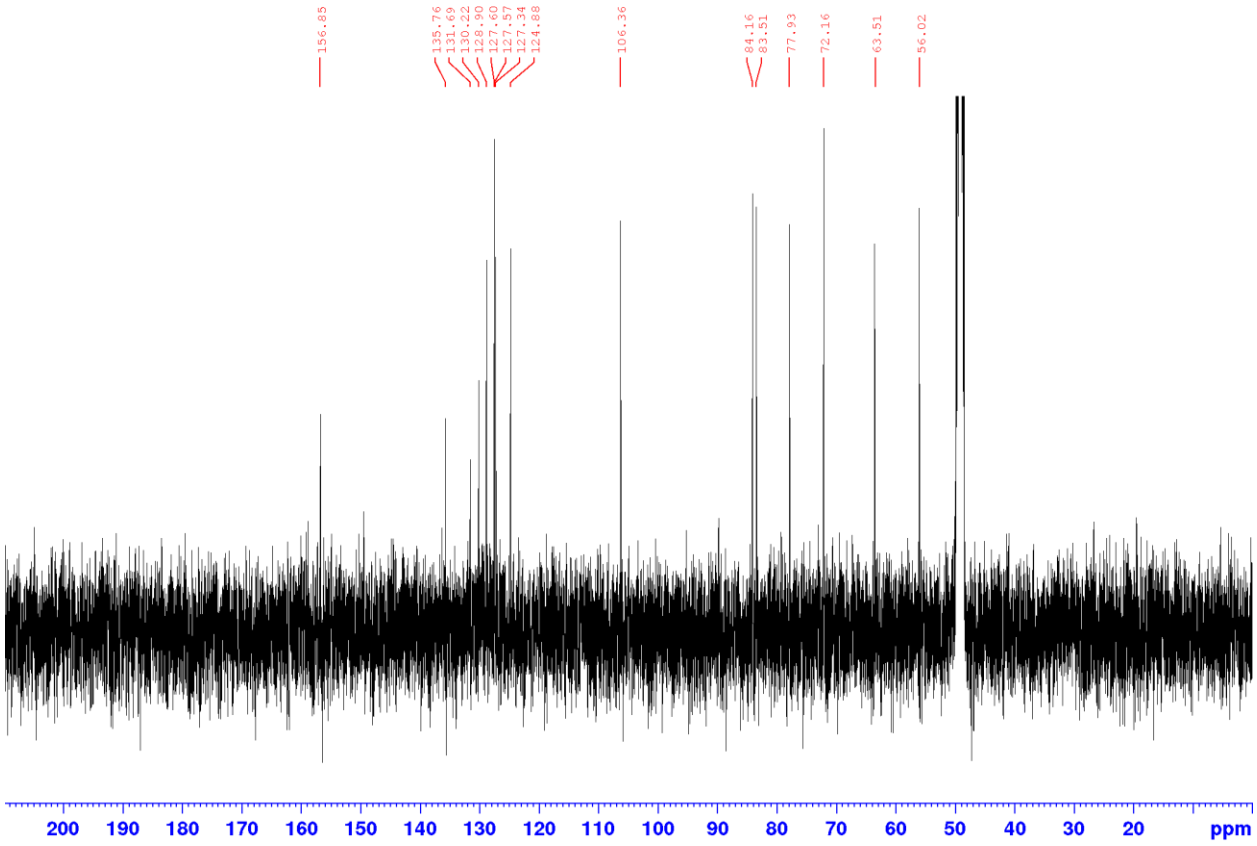


Figure S464. ^{13}C NMR of compound 7.

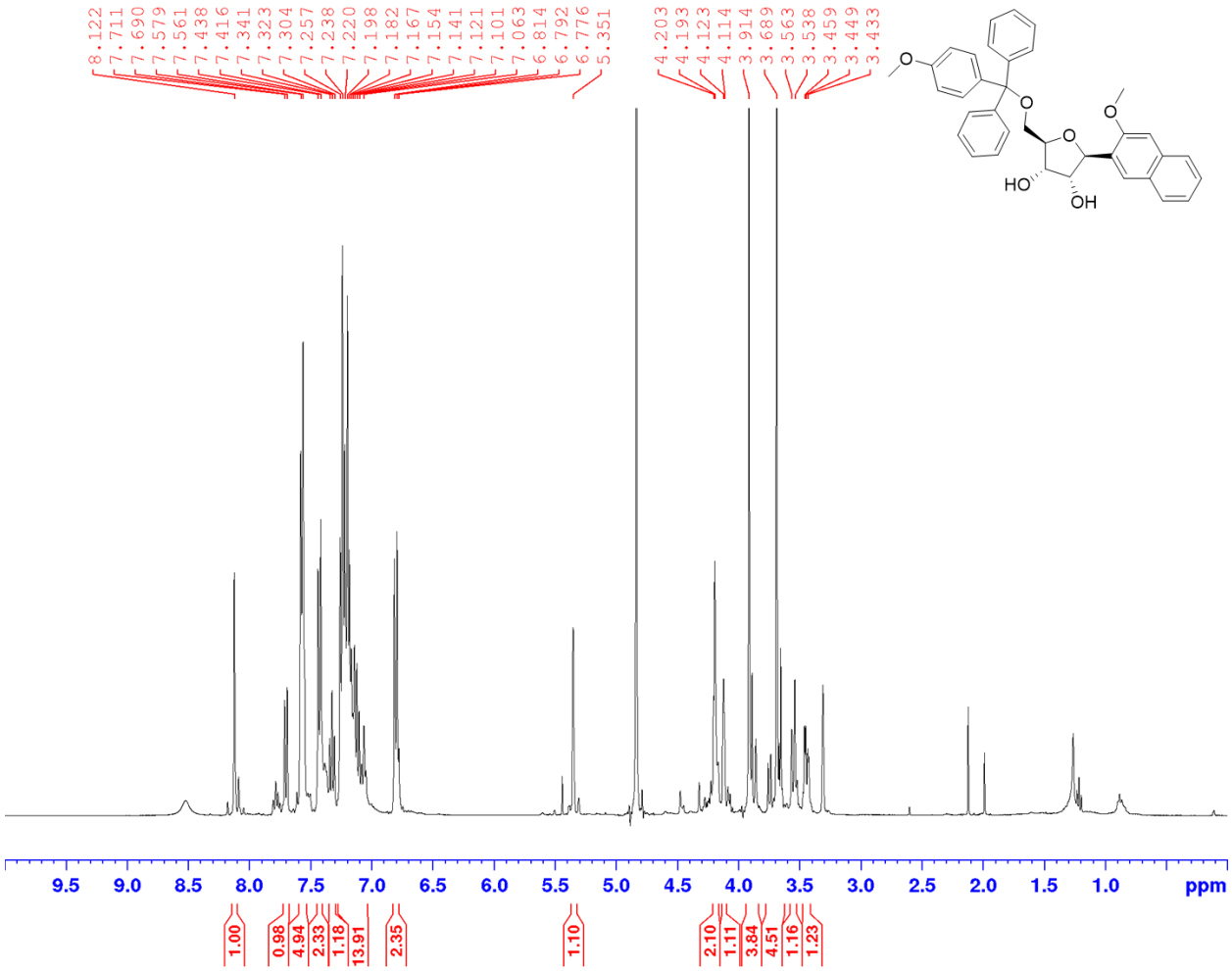


Figure S75. ^{1}H NMR of compound 8a.

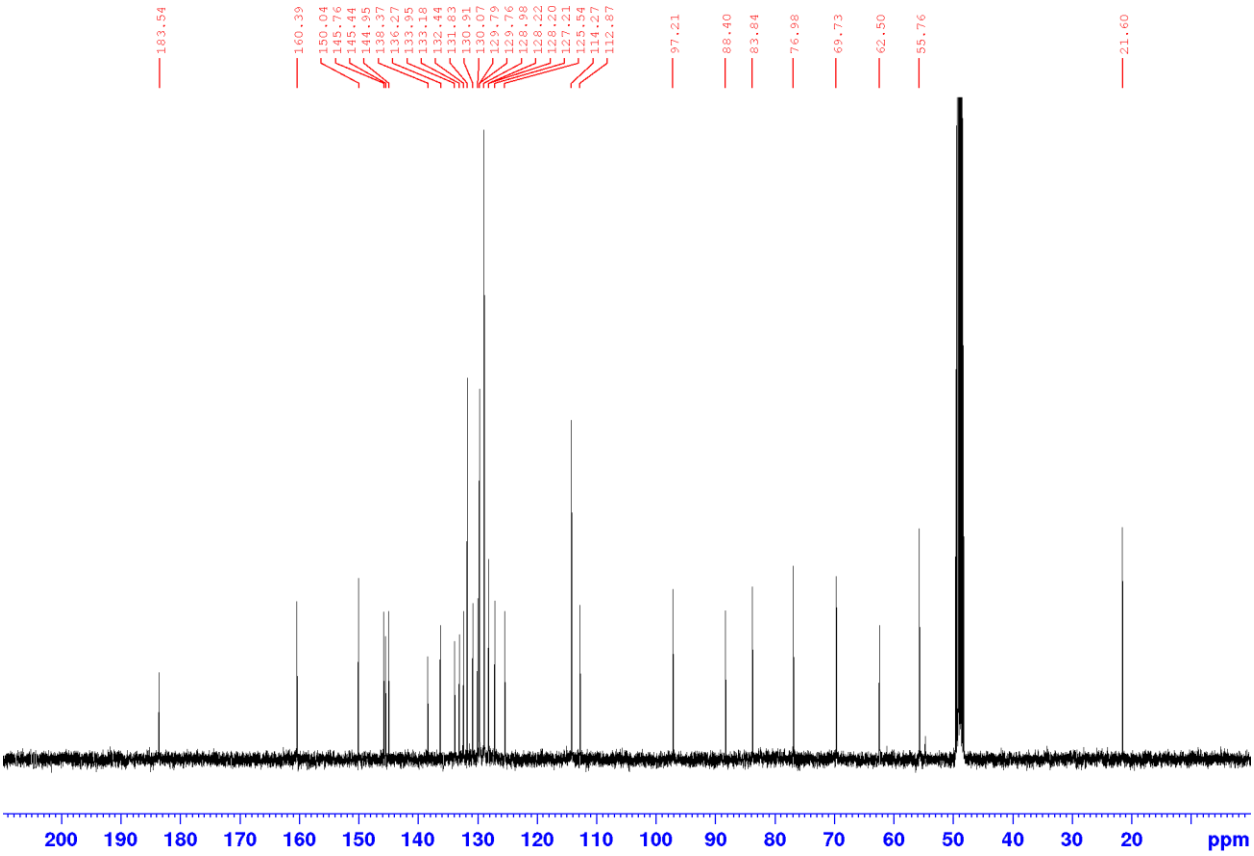


Figure S476. ^{13}C NMR of compound 8a.

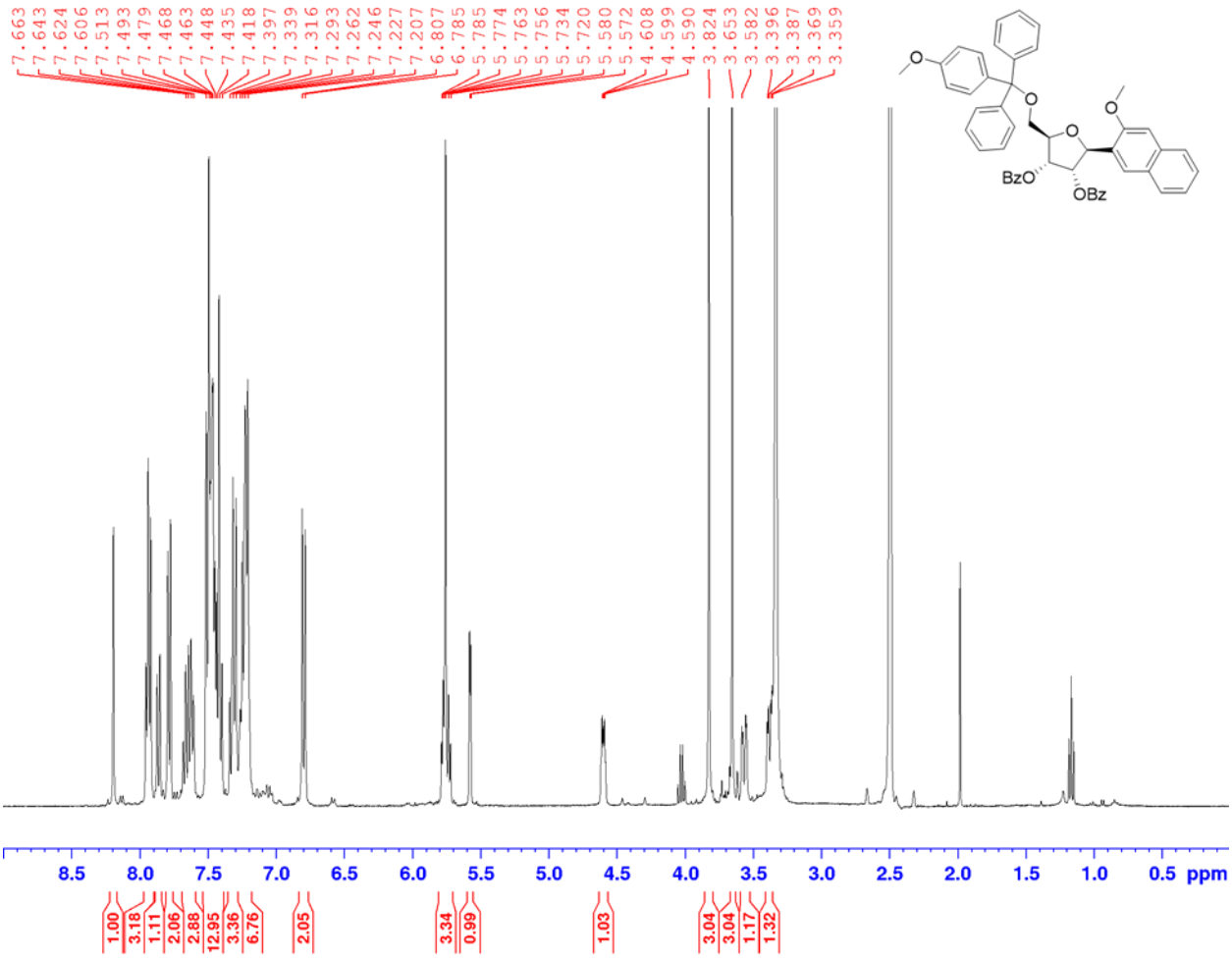


Figure S7748. ^{1}H NMR of compound 9a.

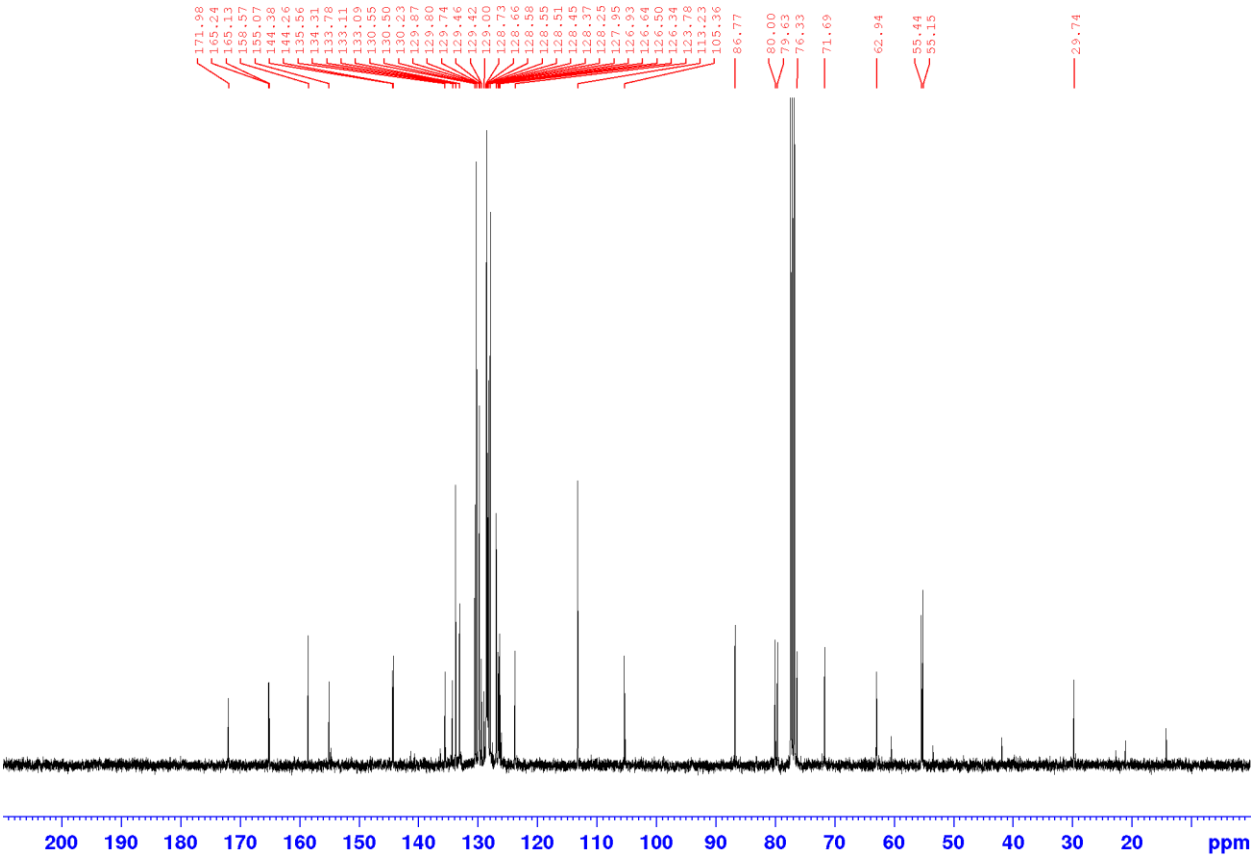


Figure S78. ^{13}C NMR of compound 9a.

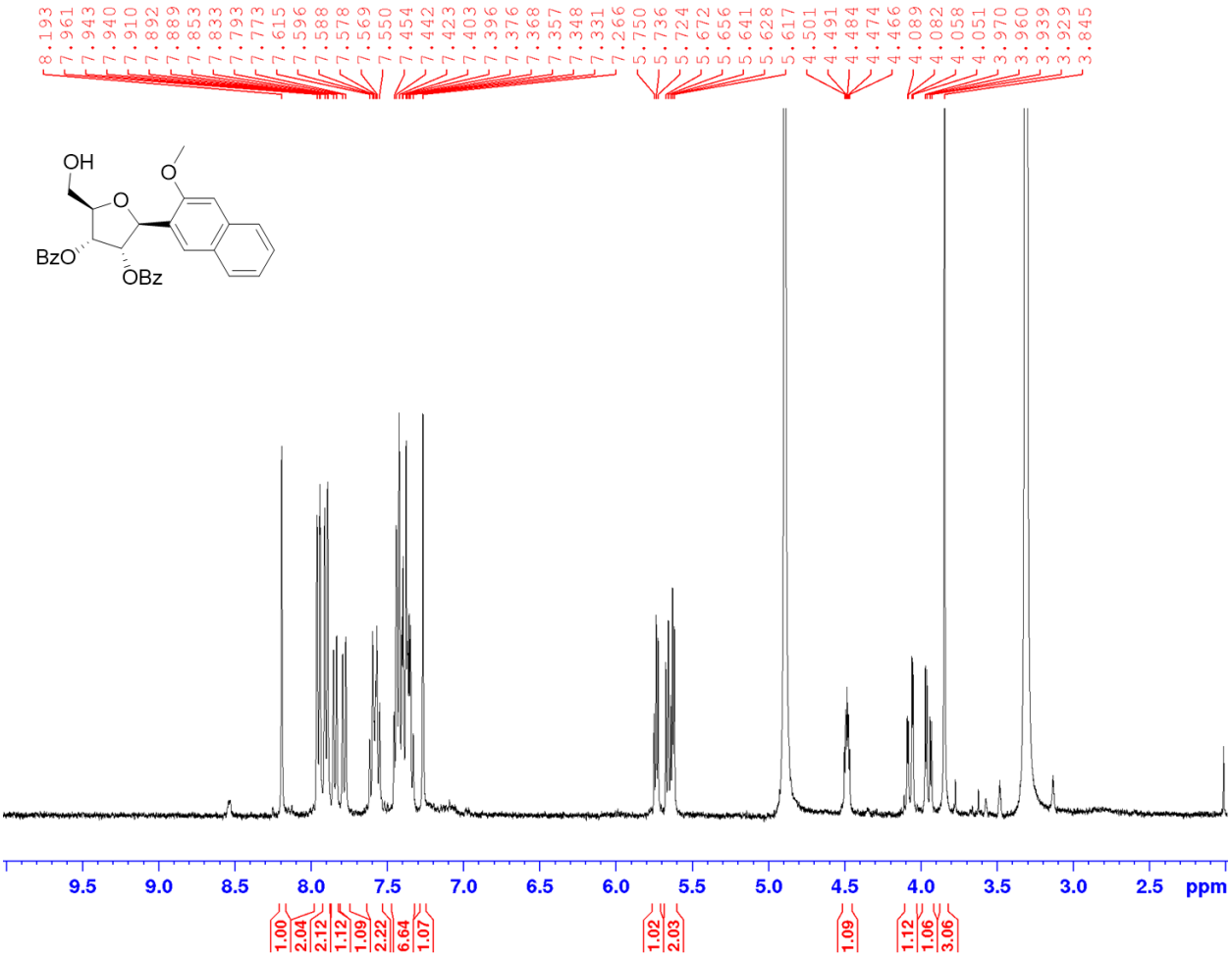


Figure S79. ^{1}H NMR of compound 10a.

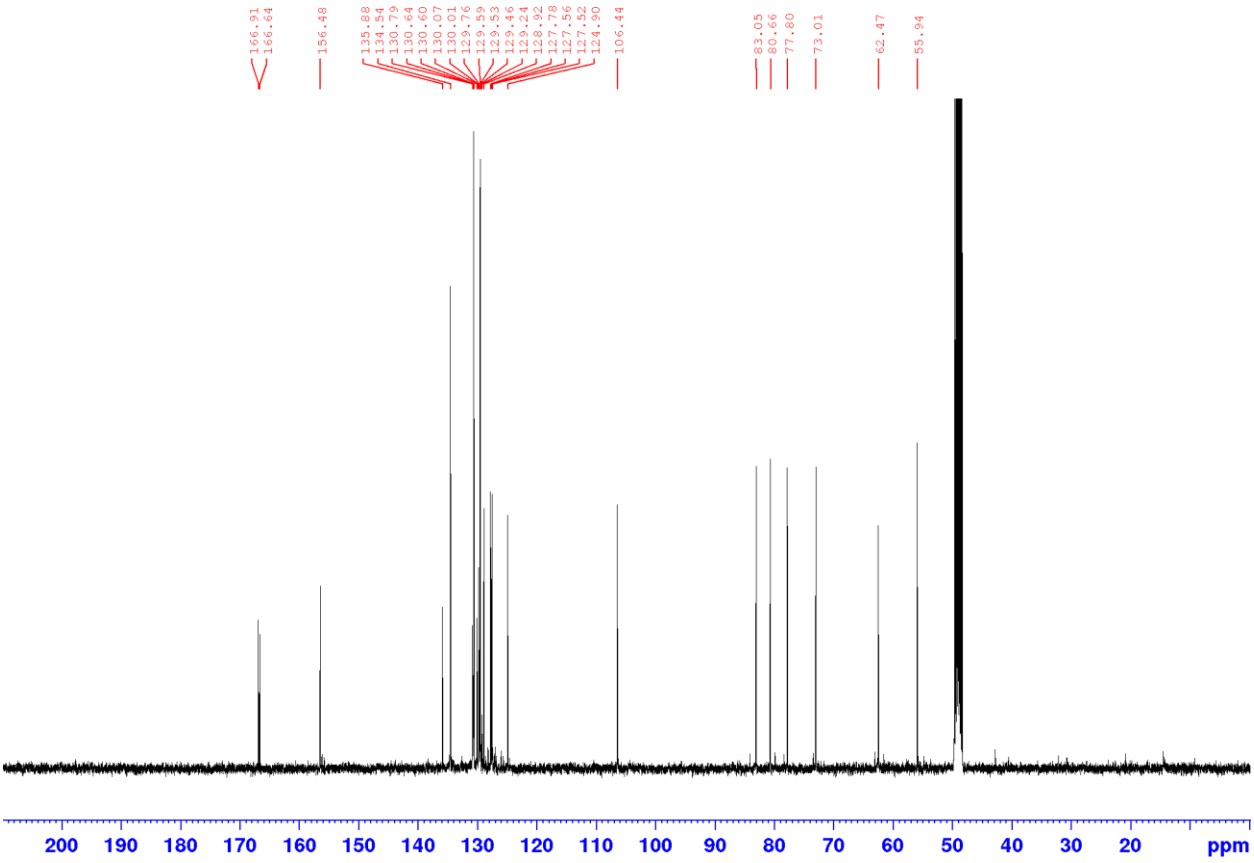


Figure S80. ^{13}C NMR of compound 10a.

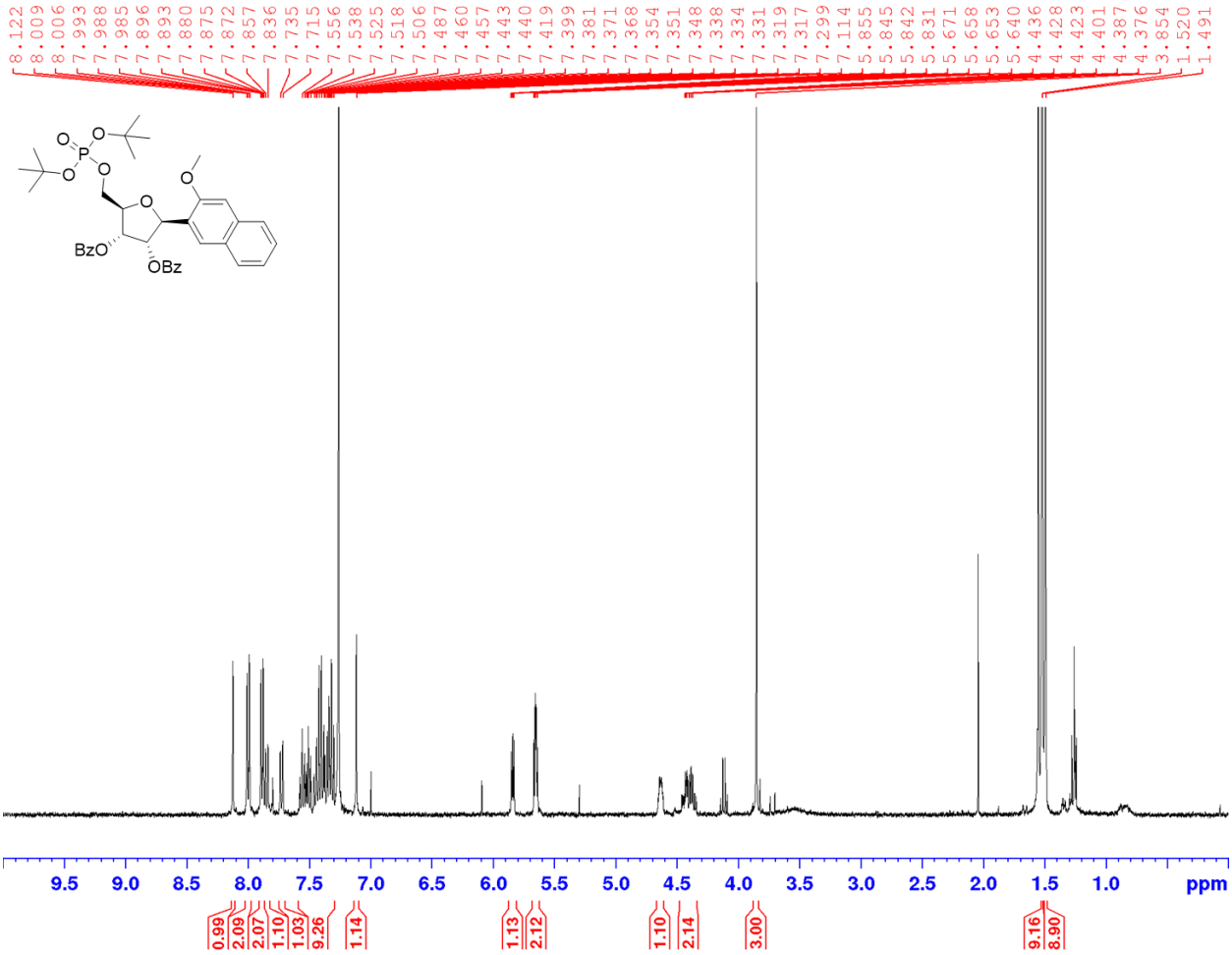


Figure S8149. ^{1}H NMR of compound 11a.

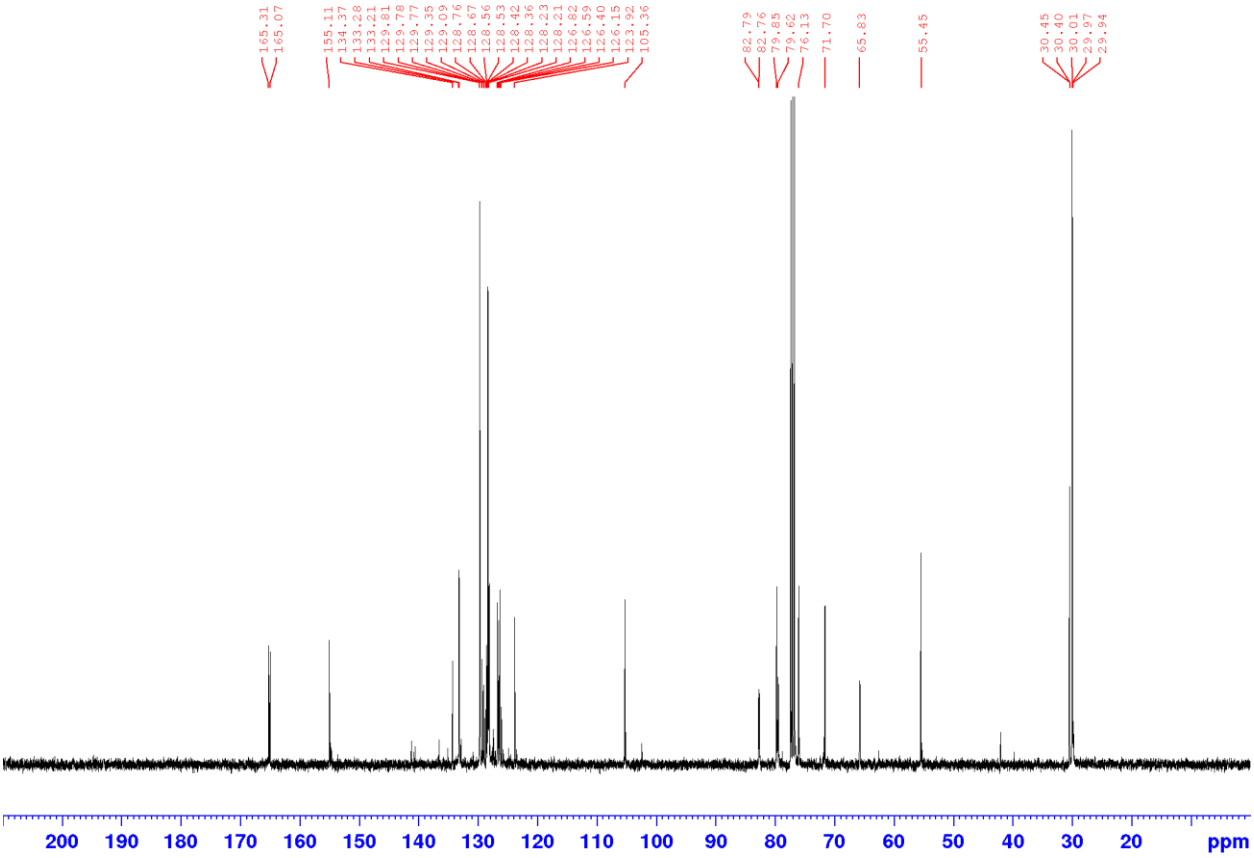


Figure S82. ^{13}C NMR of compound 11a.

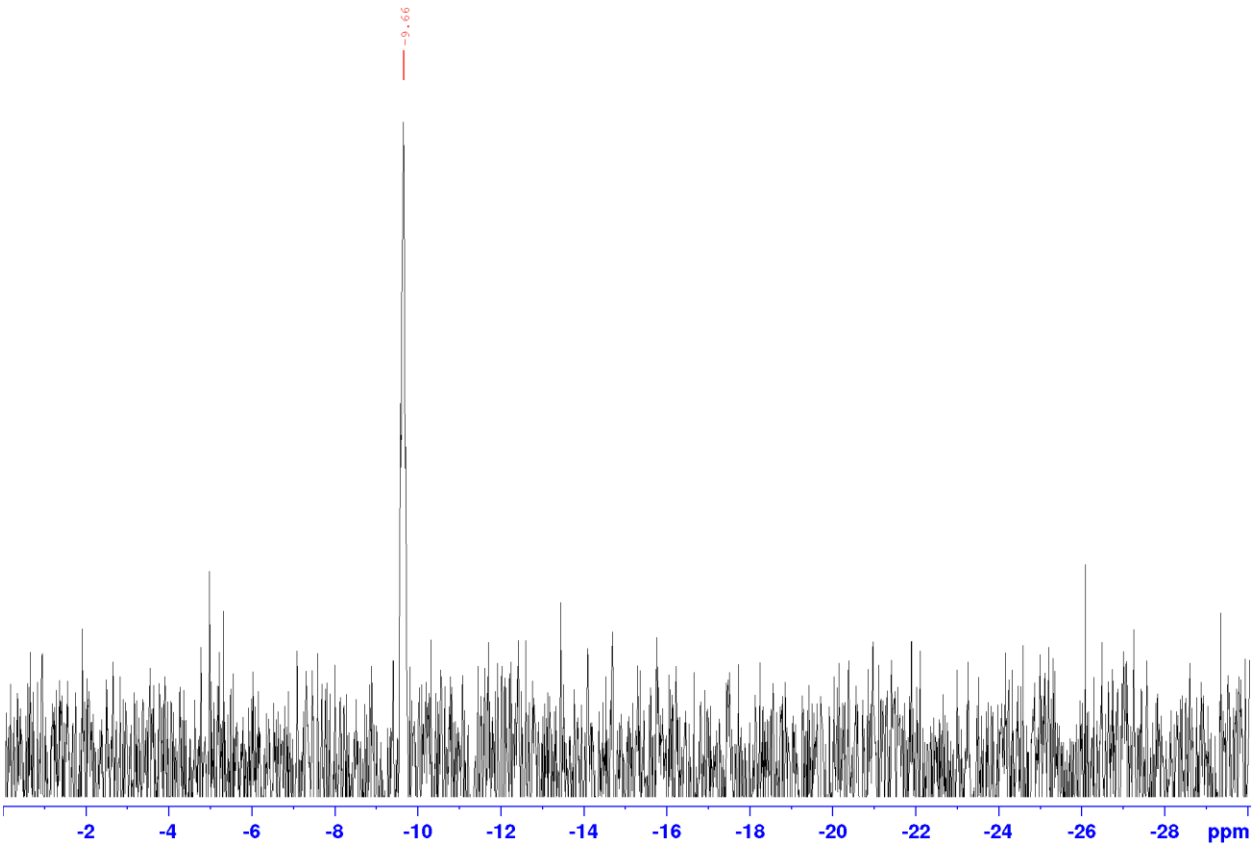


Figure S83. ^{31}P Proton decoupled NMR of compound 11a.

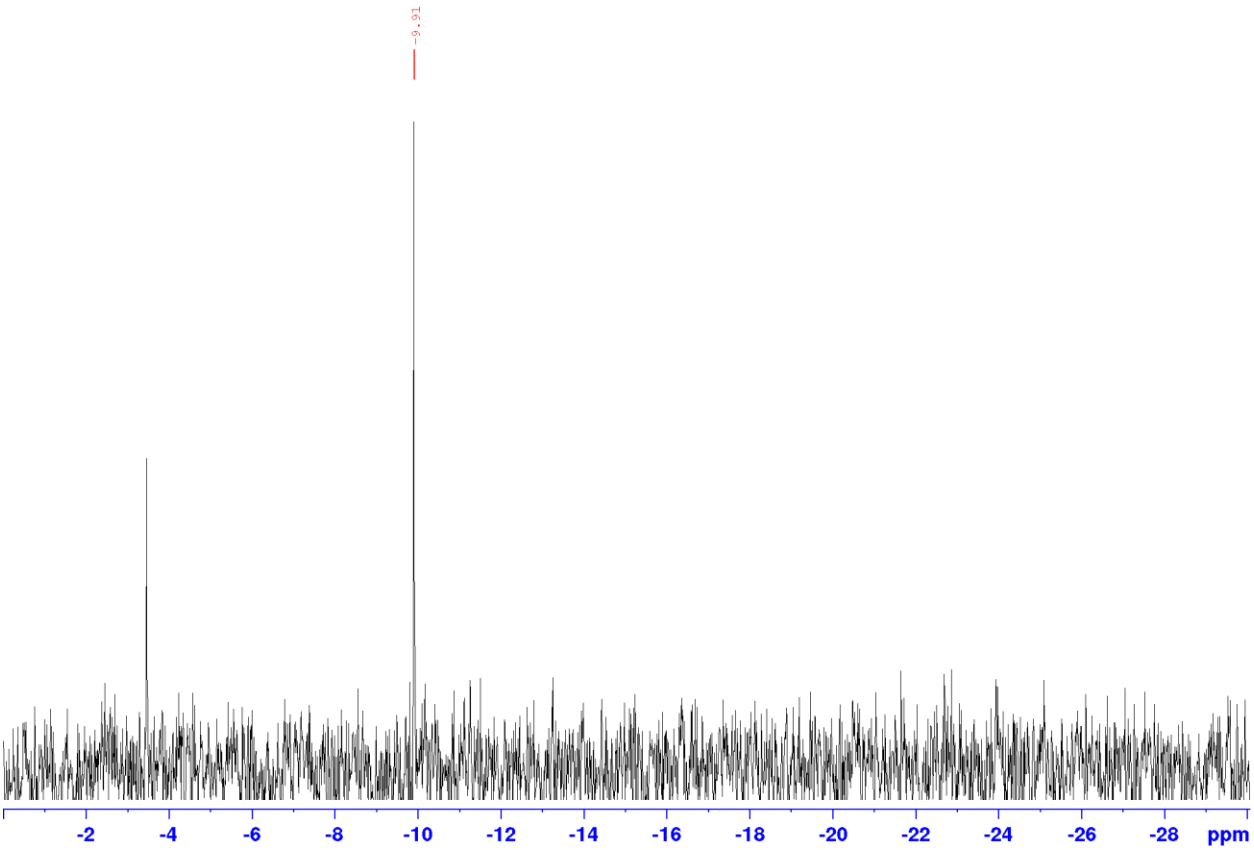


Figure S84. ^{31P} NMR no proton decoupled of compound 11a.

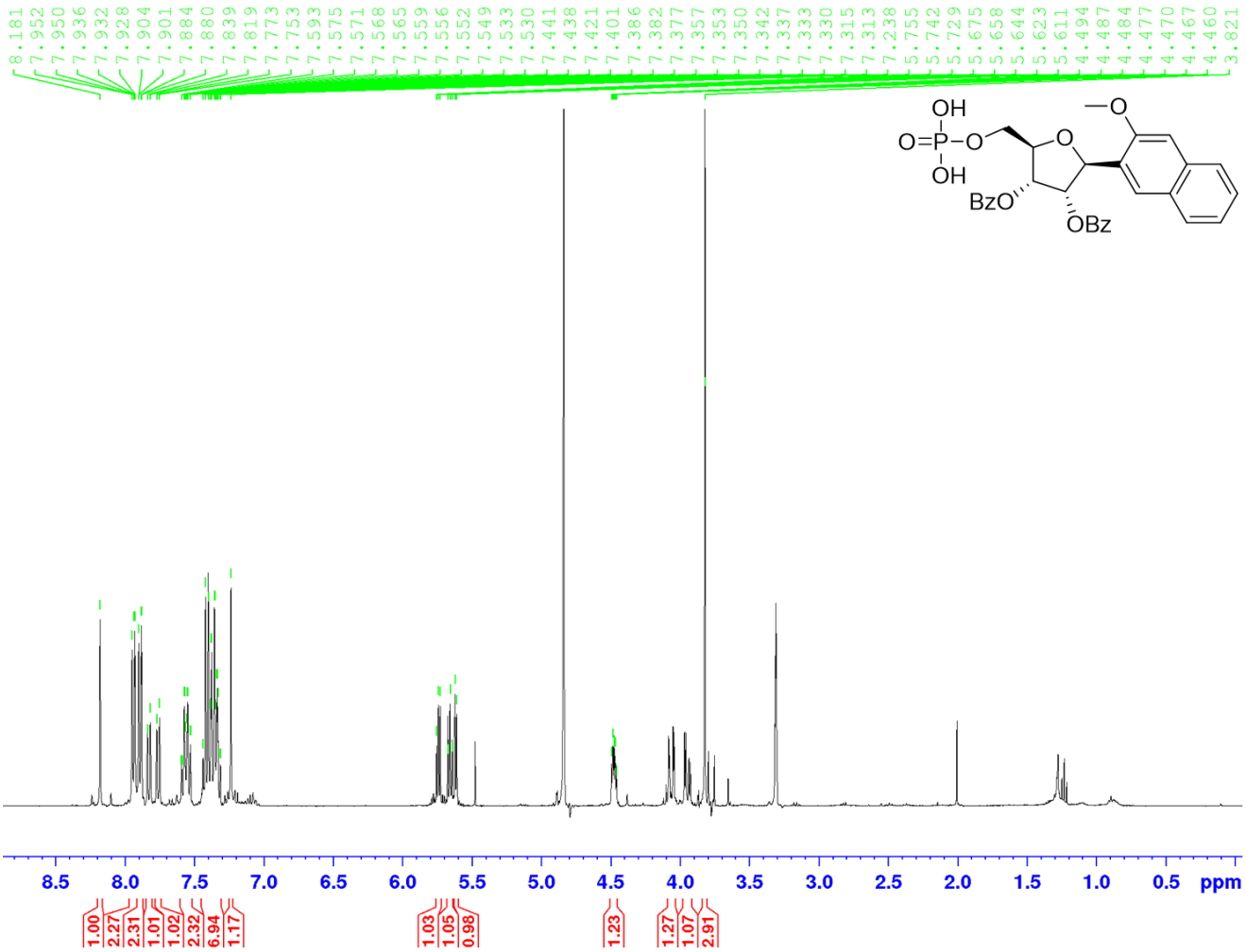


Figure S8550. ^{1}H NMR of compound 12a.

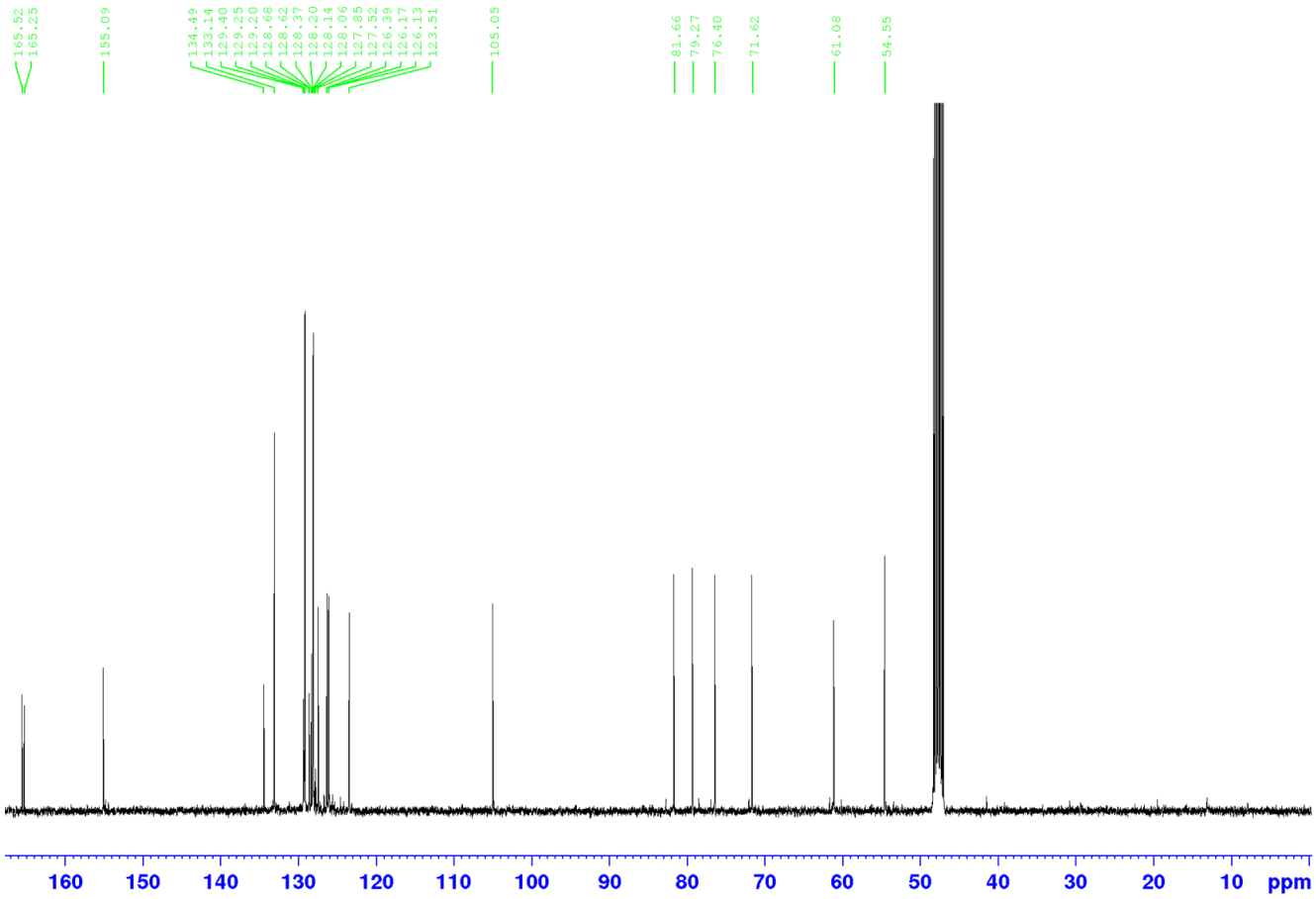


Figure S86. ^{13}C NMR of compound 12a.

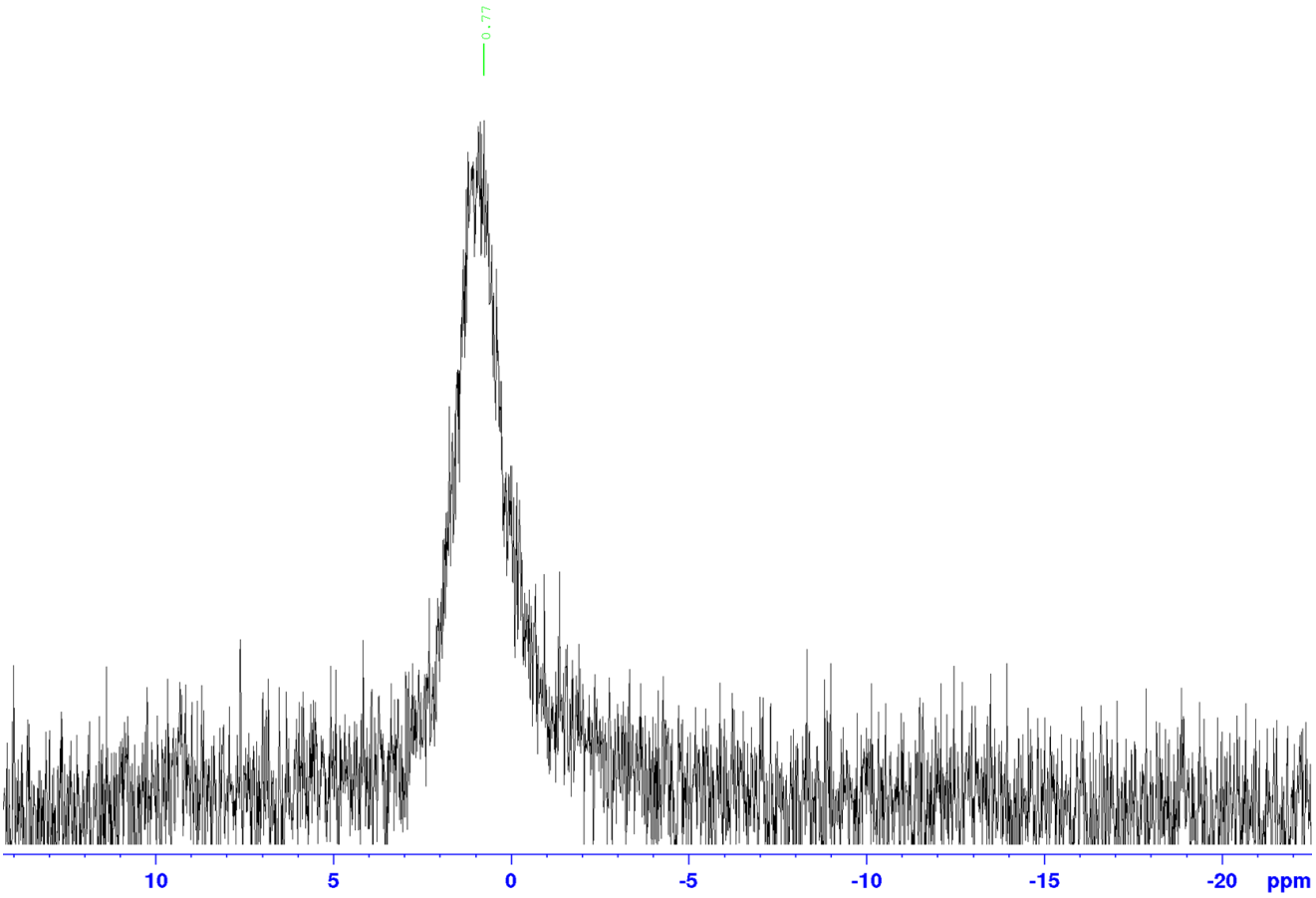


Figure S87. ^{31}P Proton decoupled NMR of compound 12a.

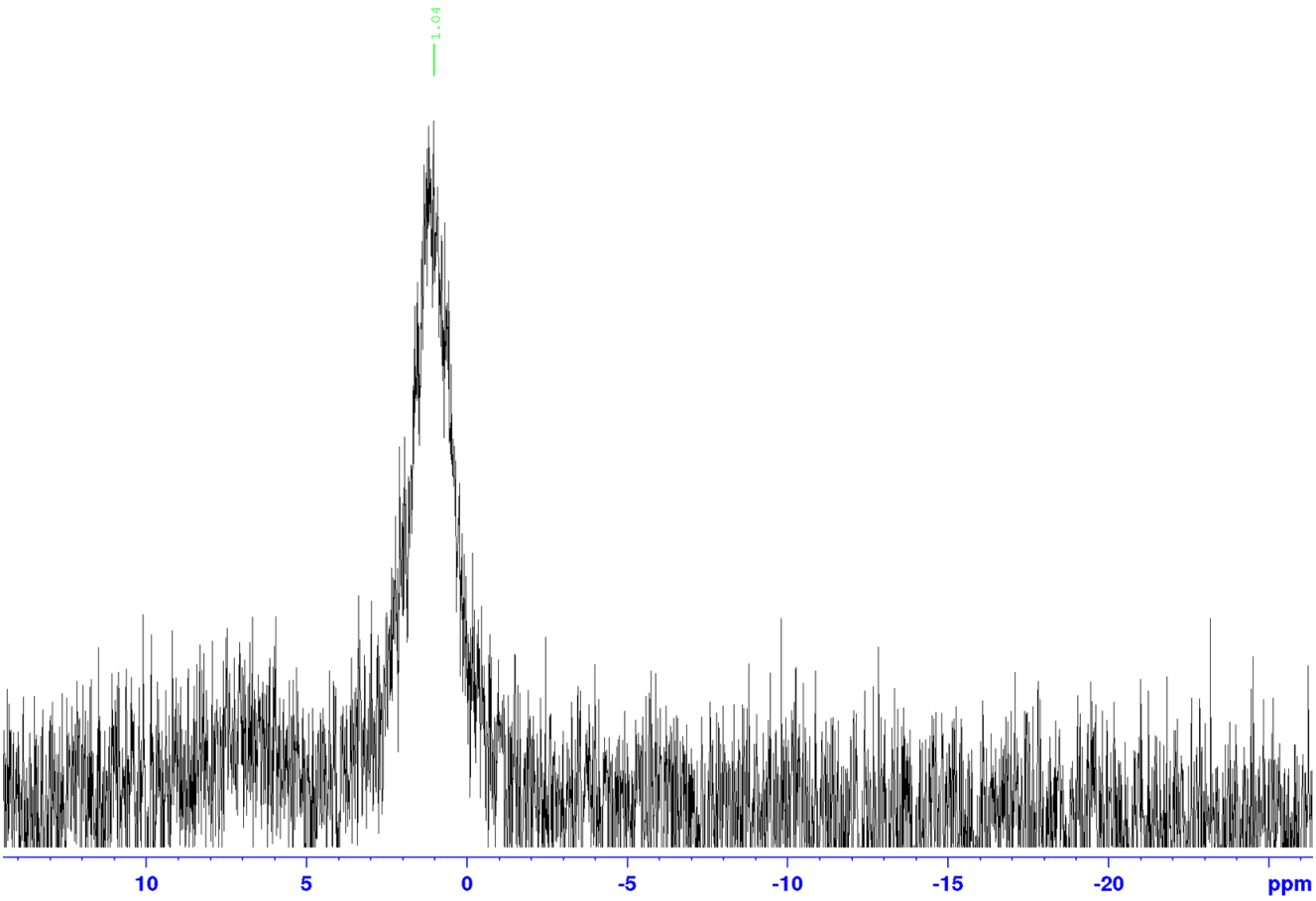


Figure S8851. ^{31}P no proton decoupled NMR of compound 12a.

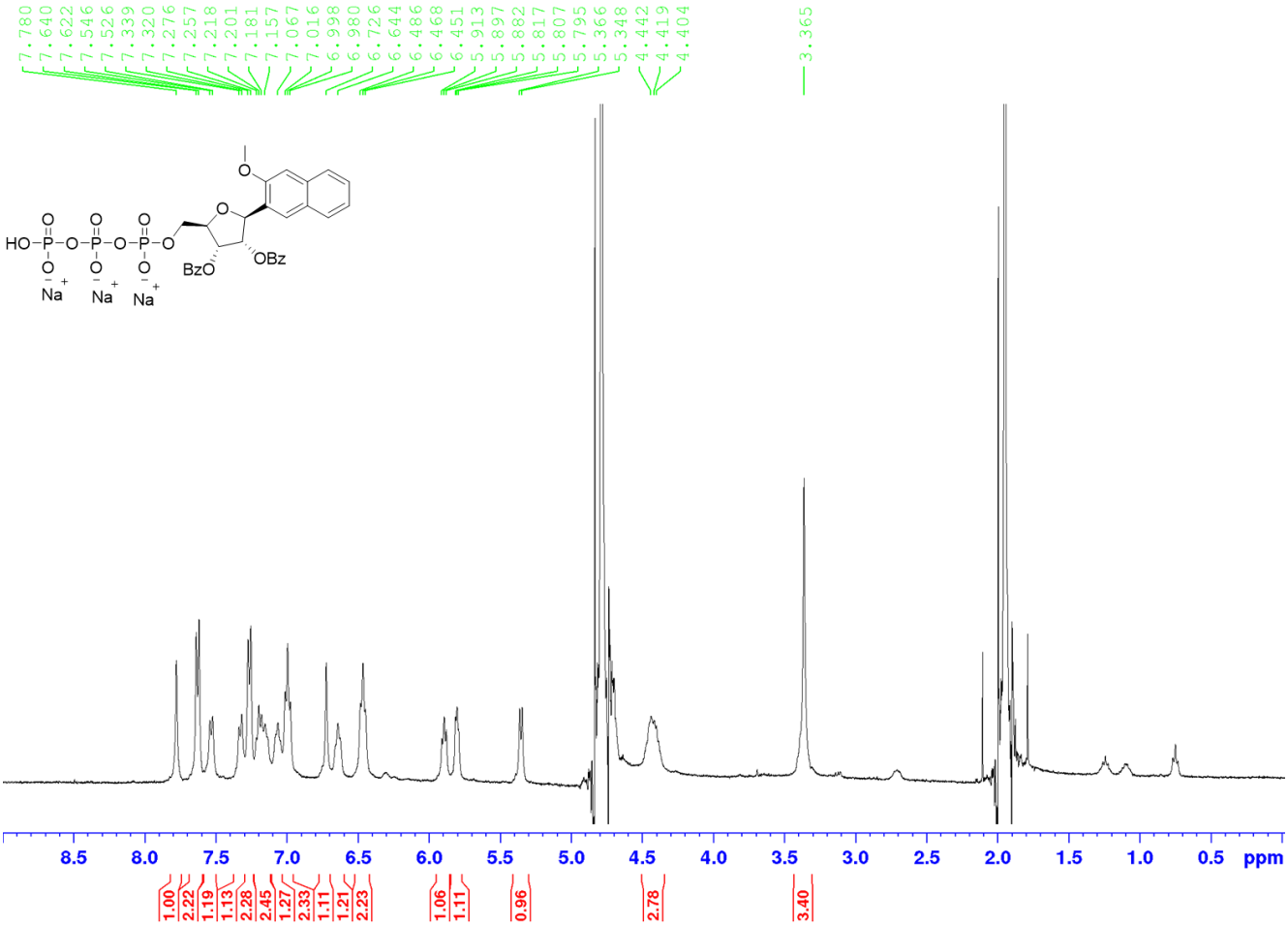


Figure S8952. ^{1}H NMR of compound 13a.

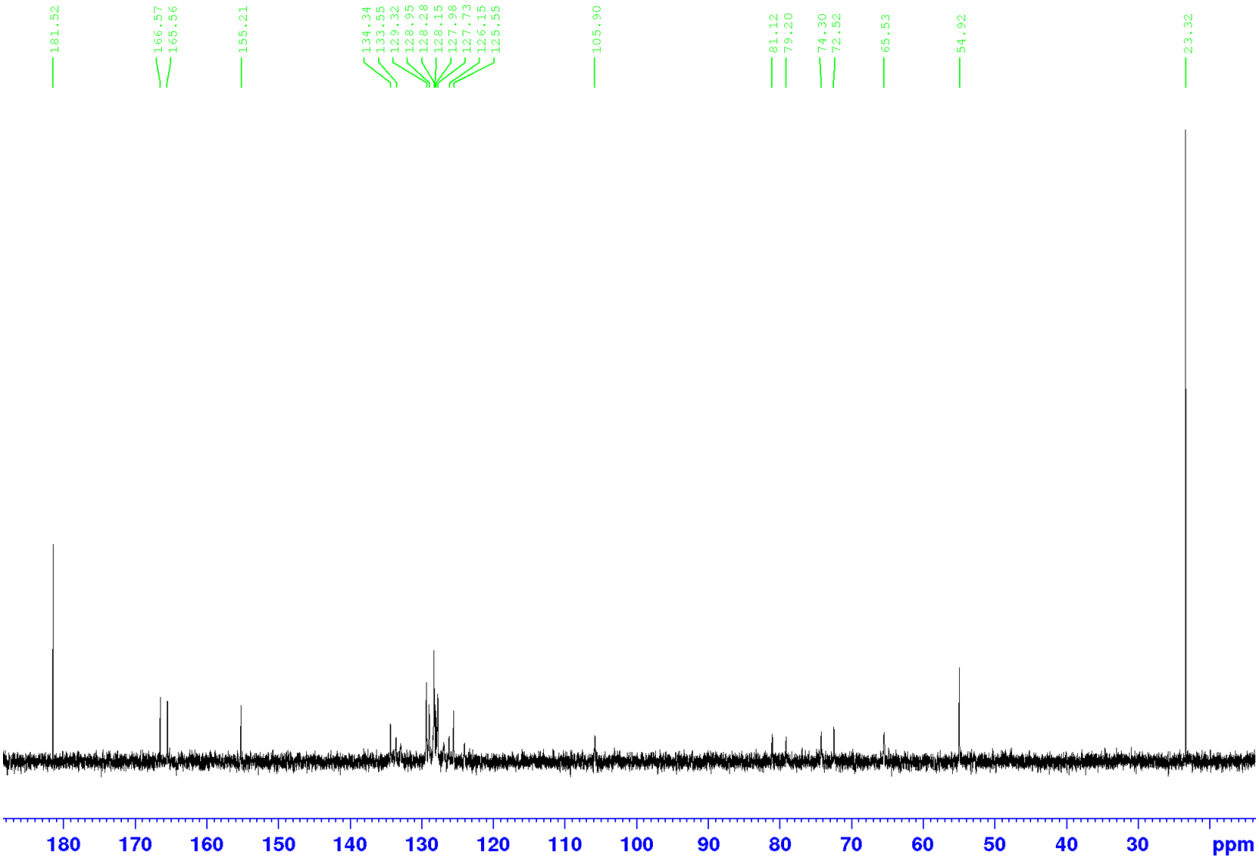


Figure S9053. ^{13}C NMR of compound 13a.

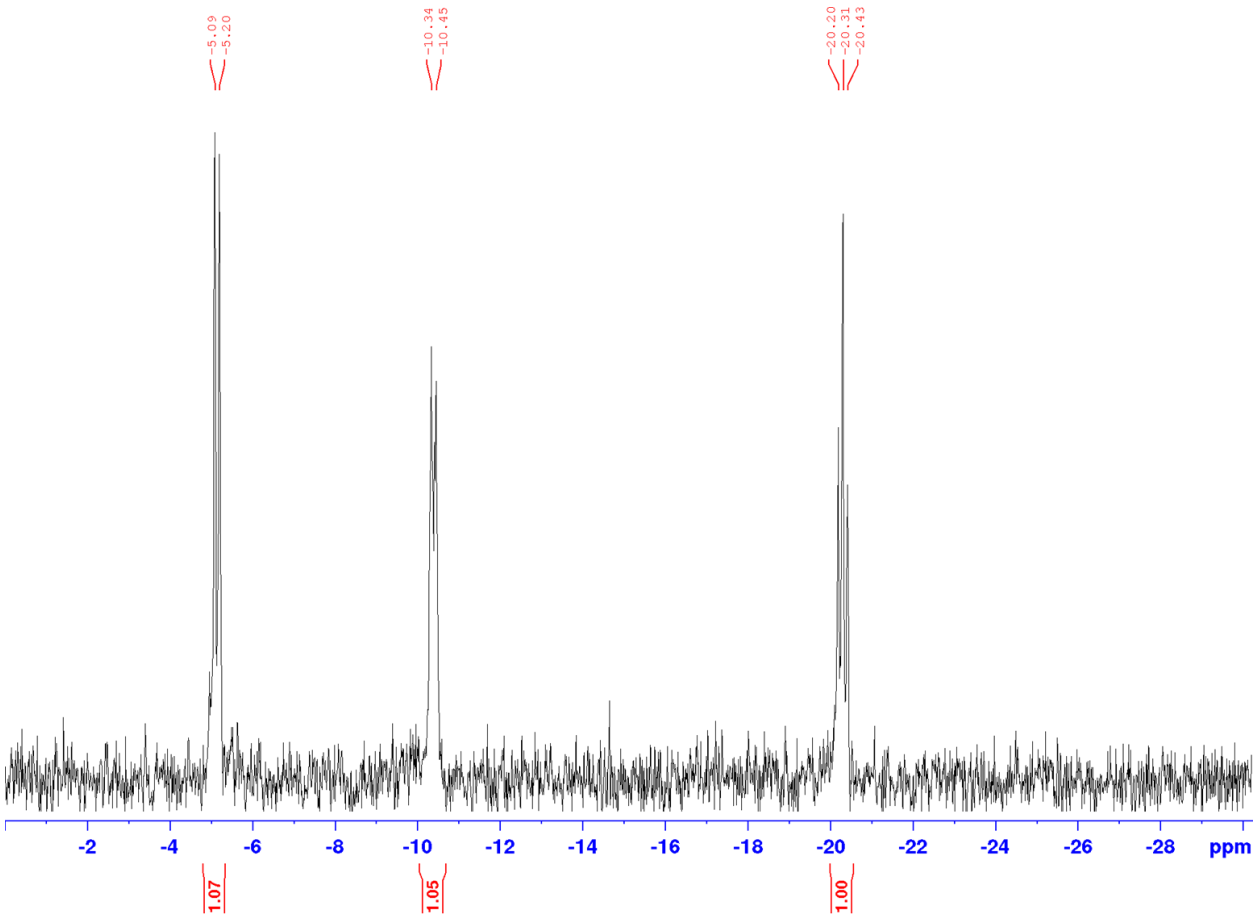


Figure S9154. ^{31}P Proton coupled NMR of compound 13a.

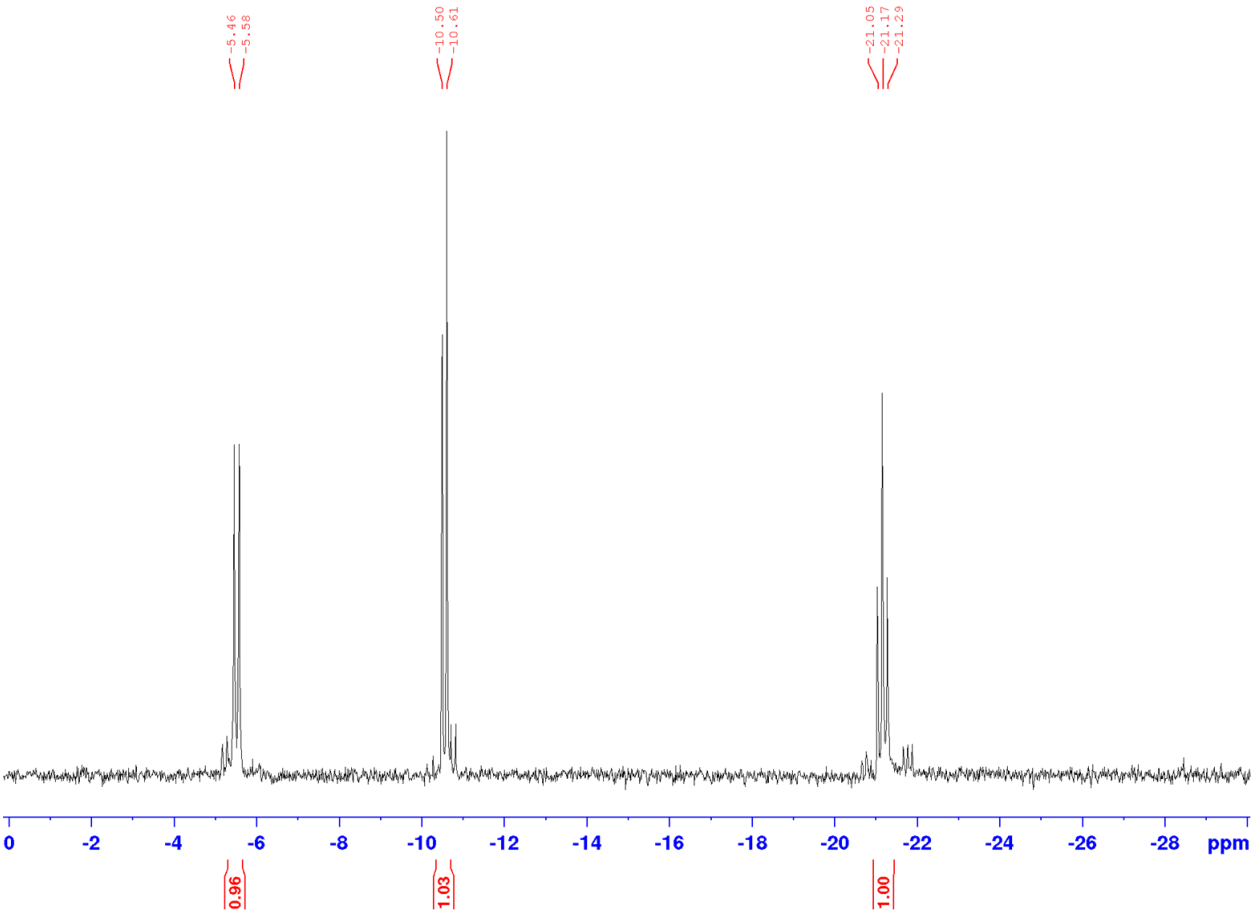


Figure S92. ^{31}P no proton coupled NMR of compound 13a.

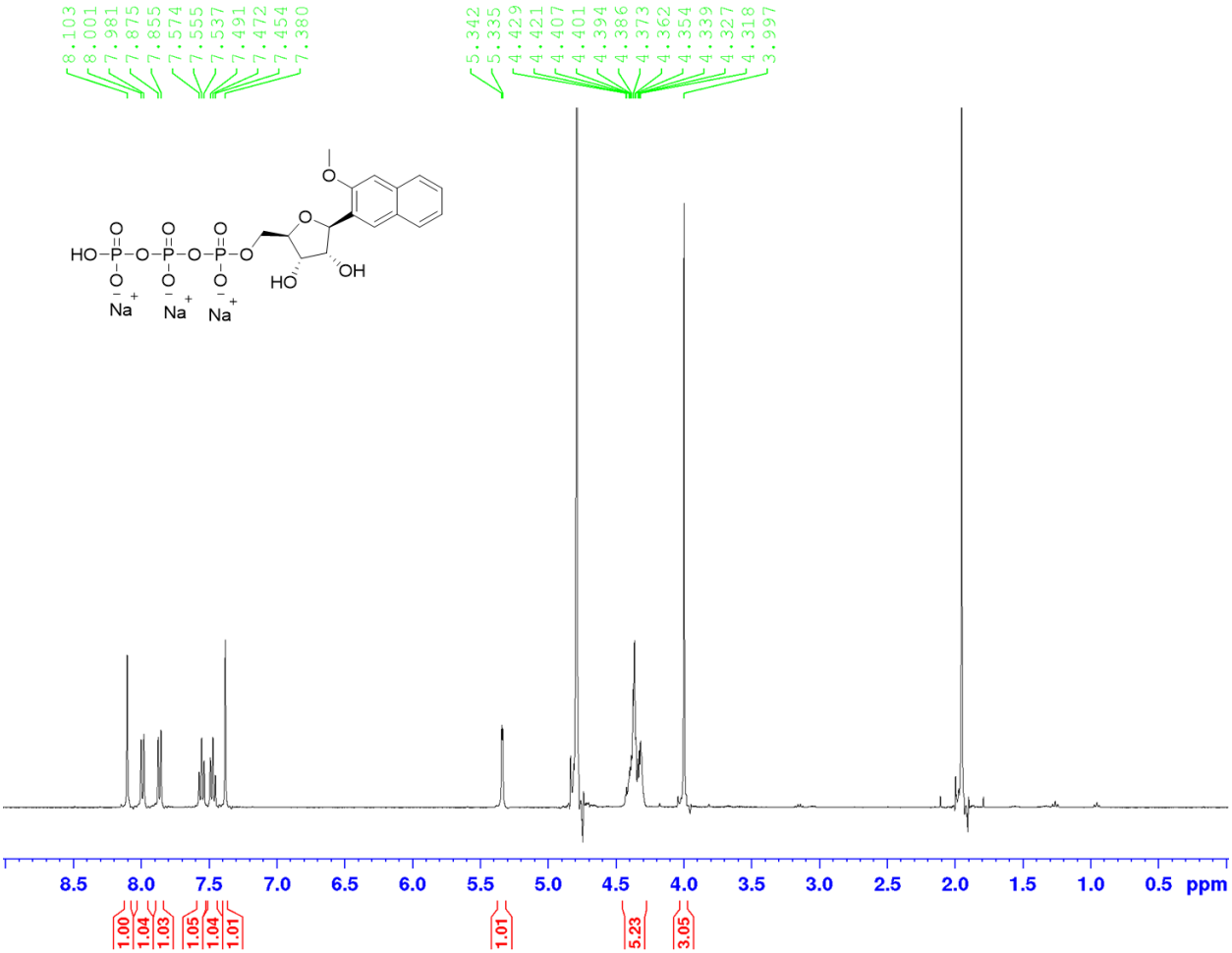


Figure S553. ^{1}H NMR of compound 14a.

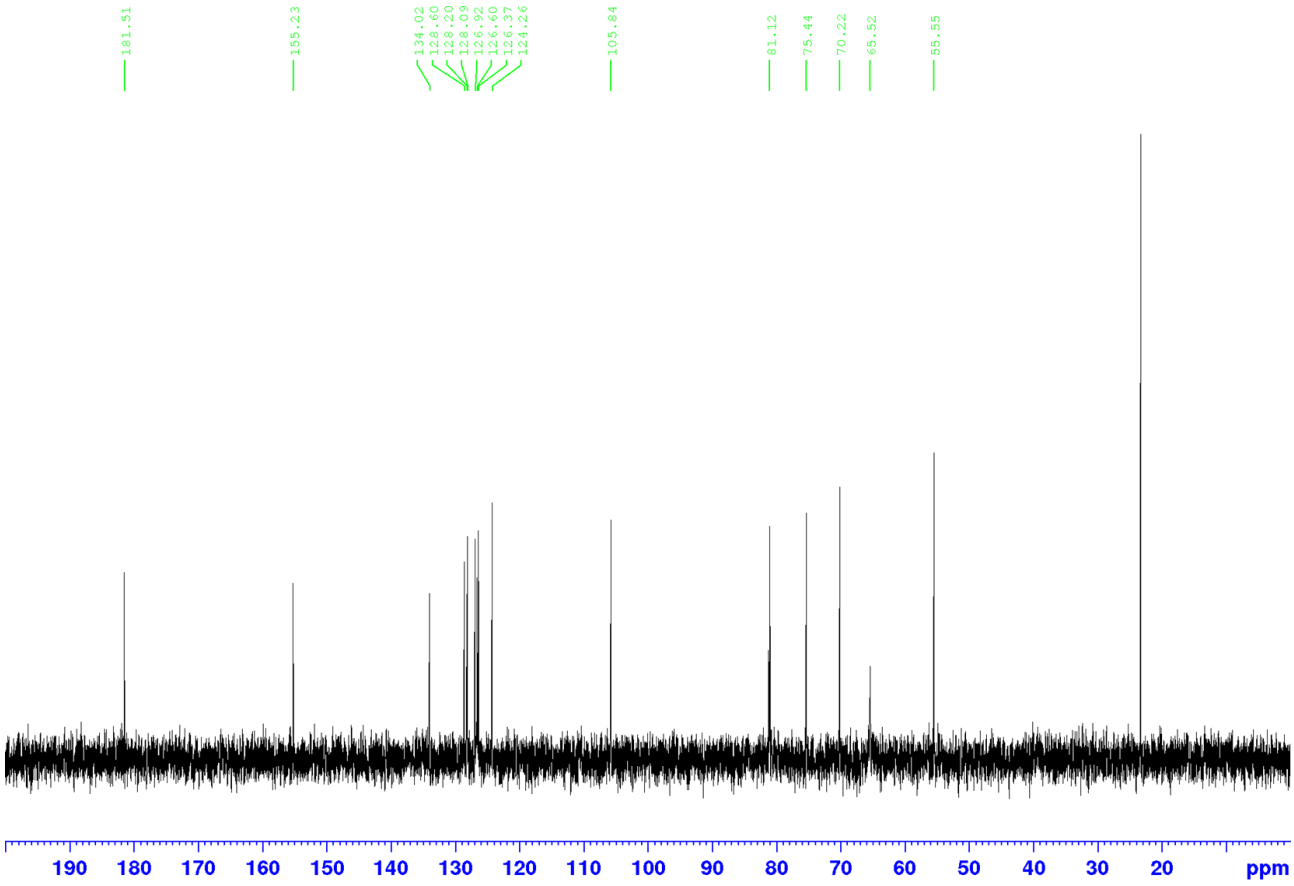


Figure S564. ^{13}C NMR of compound 14a.

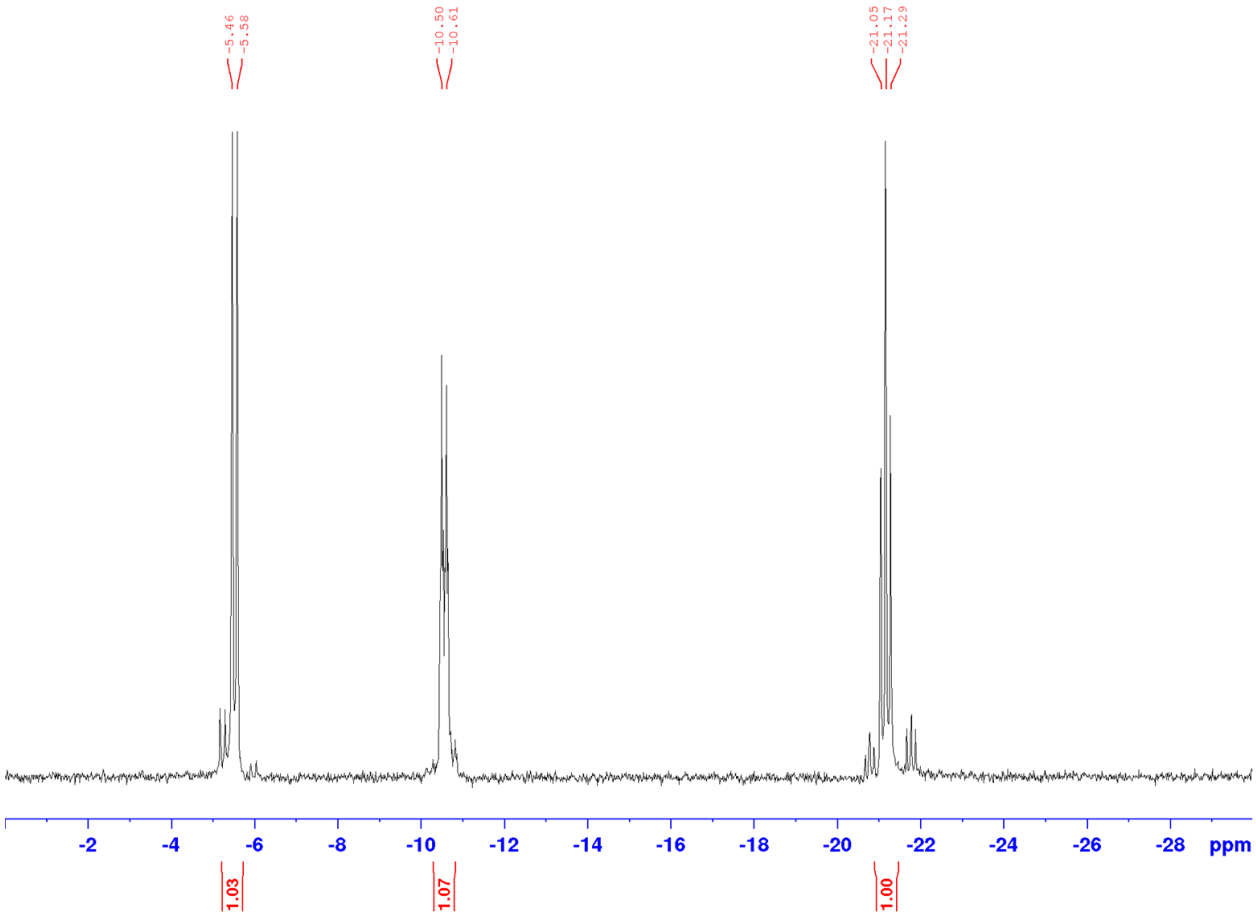


Figure S575. ^{31}P Proton coupled NMR of compound 14a.

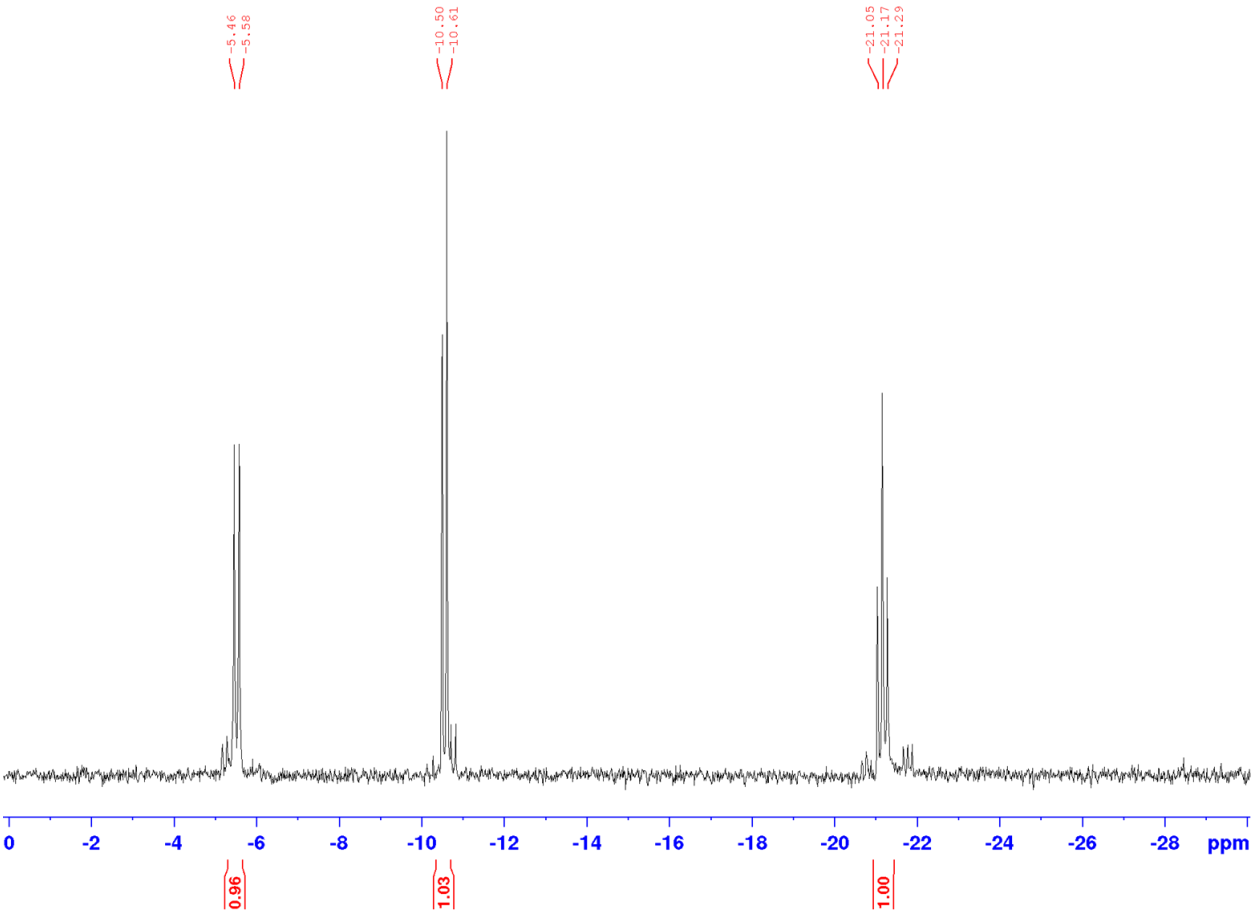


Figure S586. ^{31}P no proton coupled NMR of compound 14a.

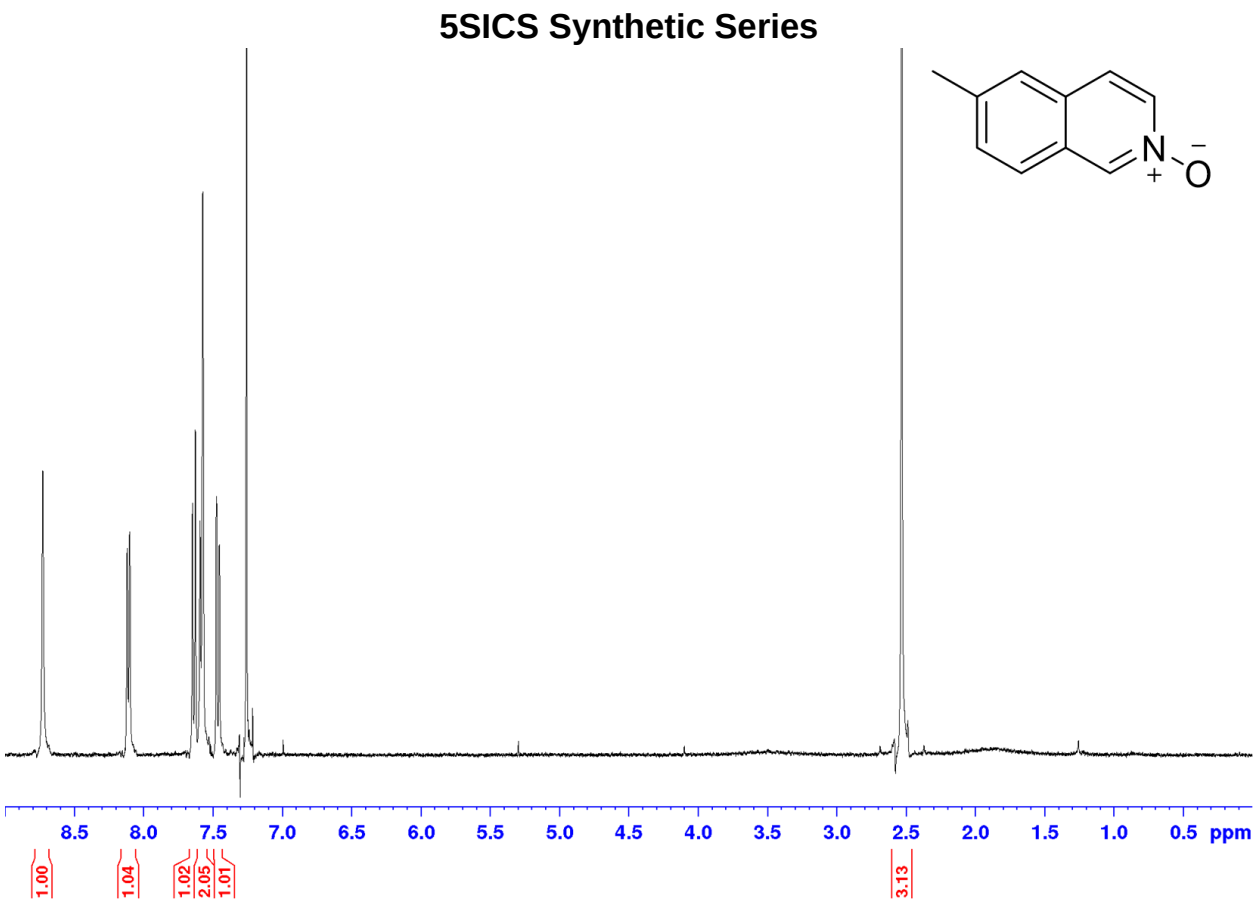


*Figure S9759. [1]H NMR of compound **16**.*

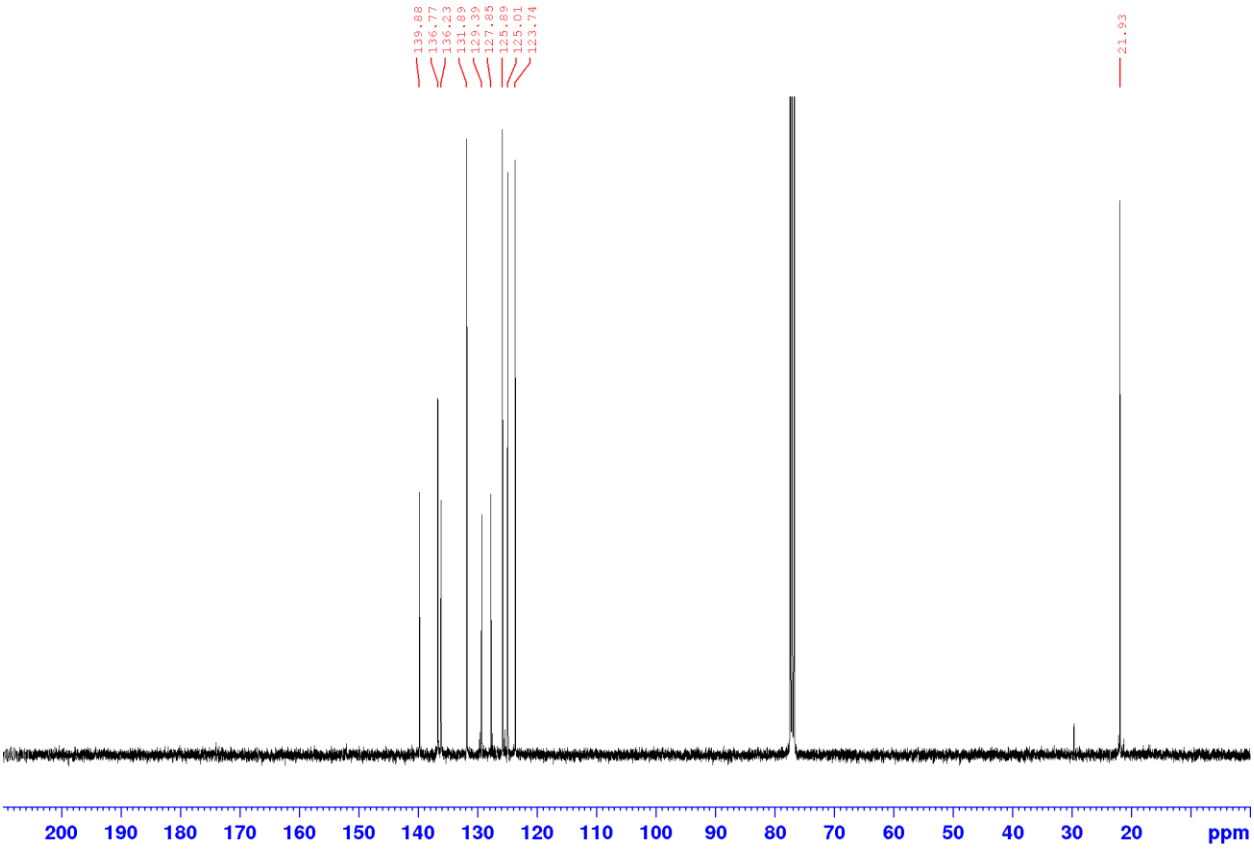


*Figure S9860. ^{13}C NMR of compound **16**.*

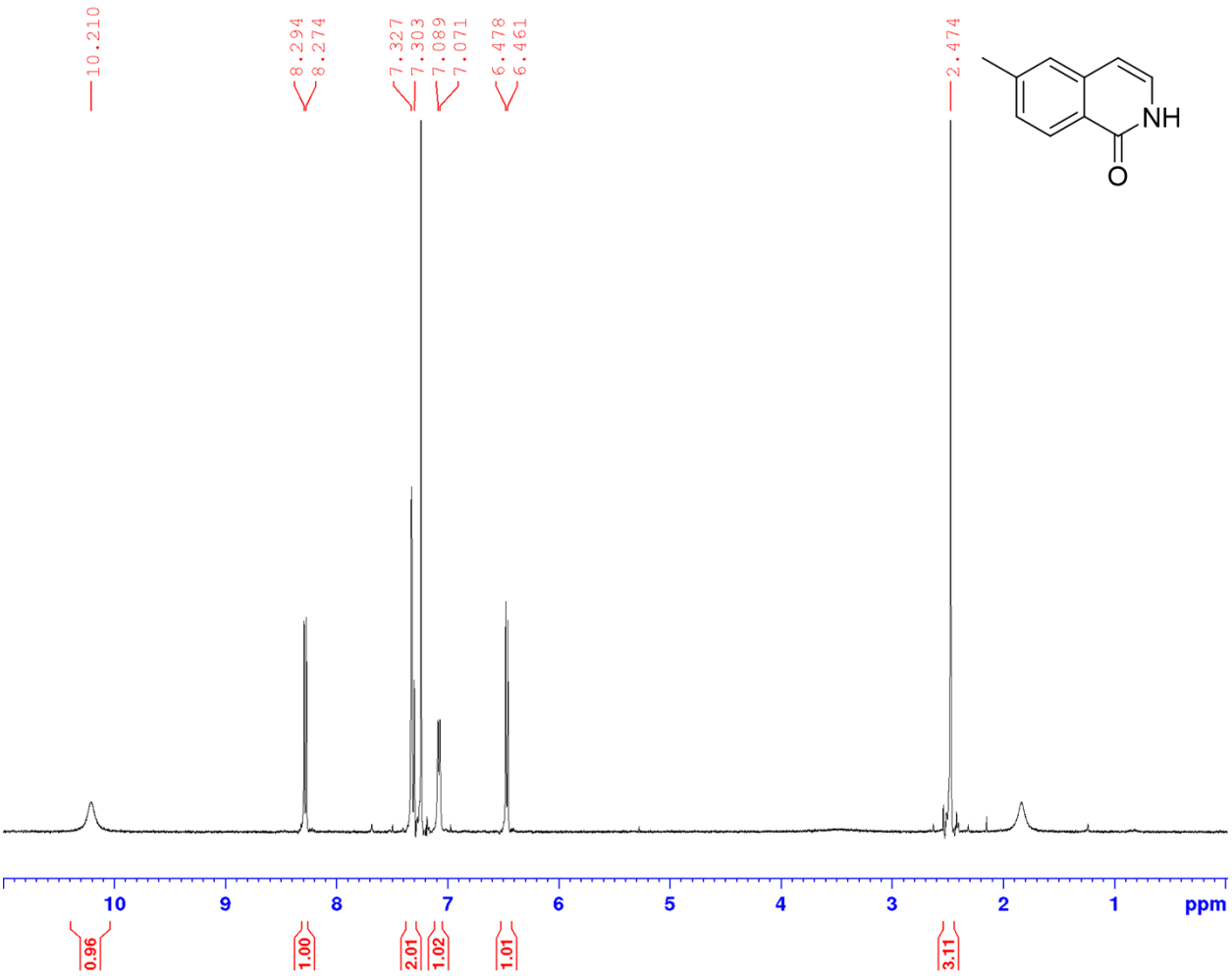


*Figure S99. ^{1}H NMR of compound **17**.*

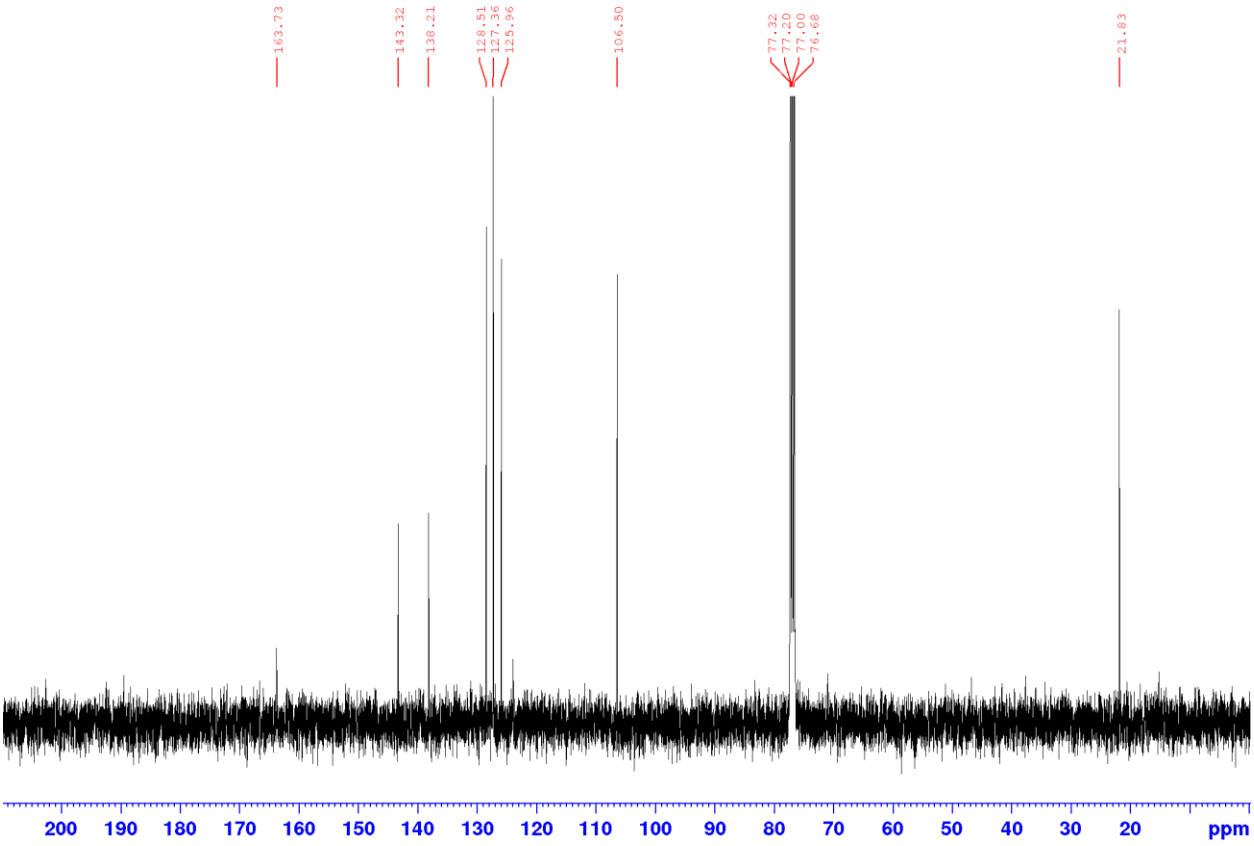


*Figure S100. ^{13}C NMR of compound **17**.*

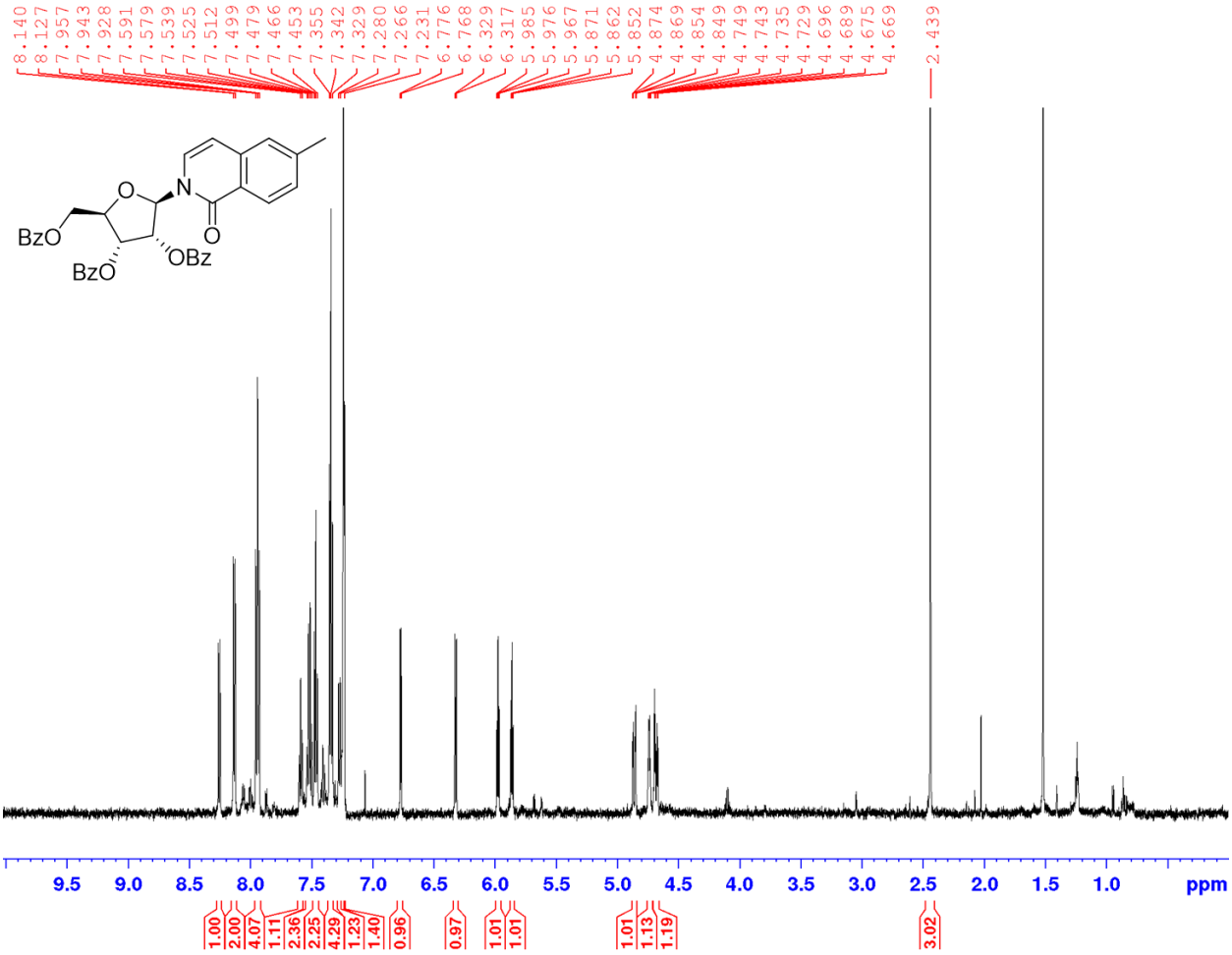


*Figure S611. ^{1}H NMR of compound **18**.*

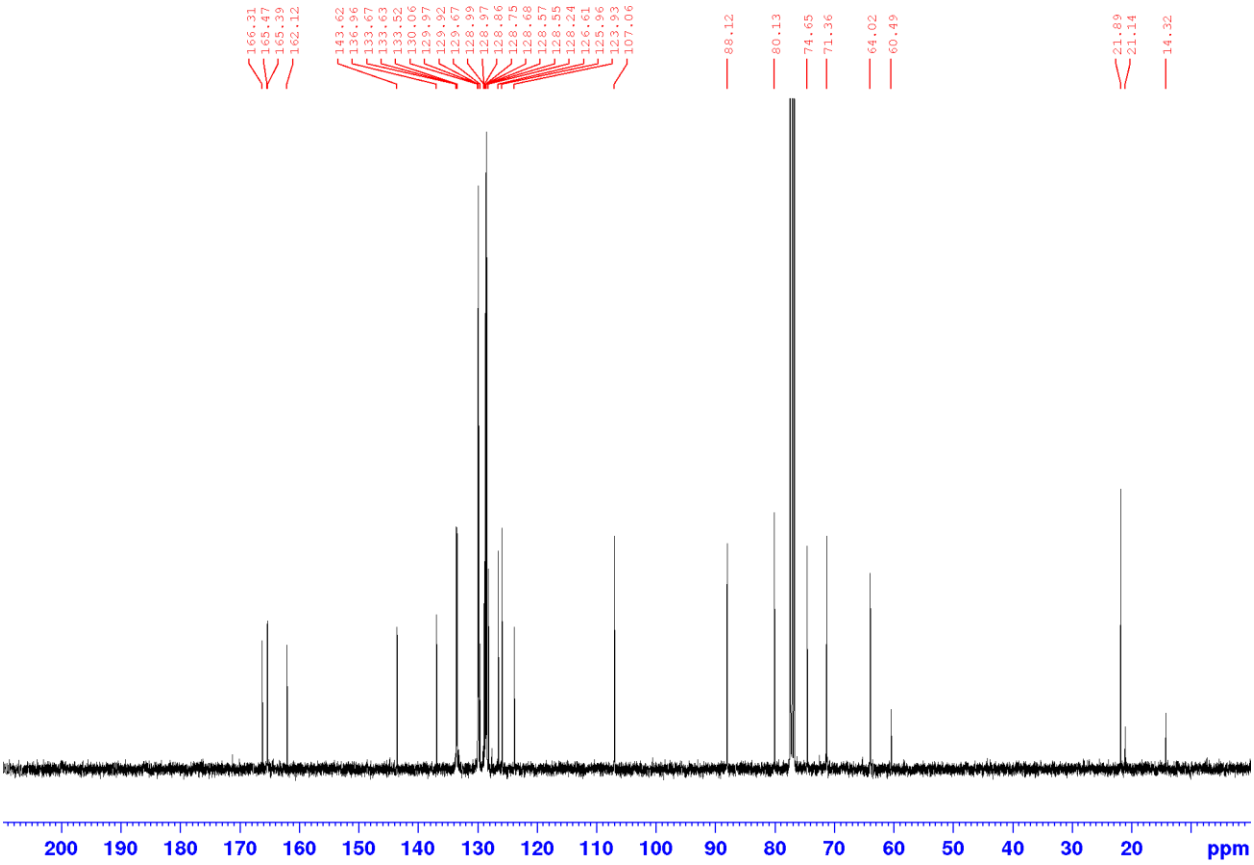


*Figure S622. ^{13}C NMR of compound **18**.*

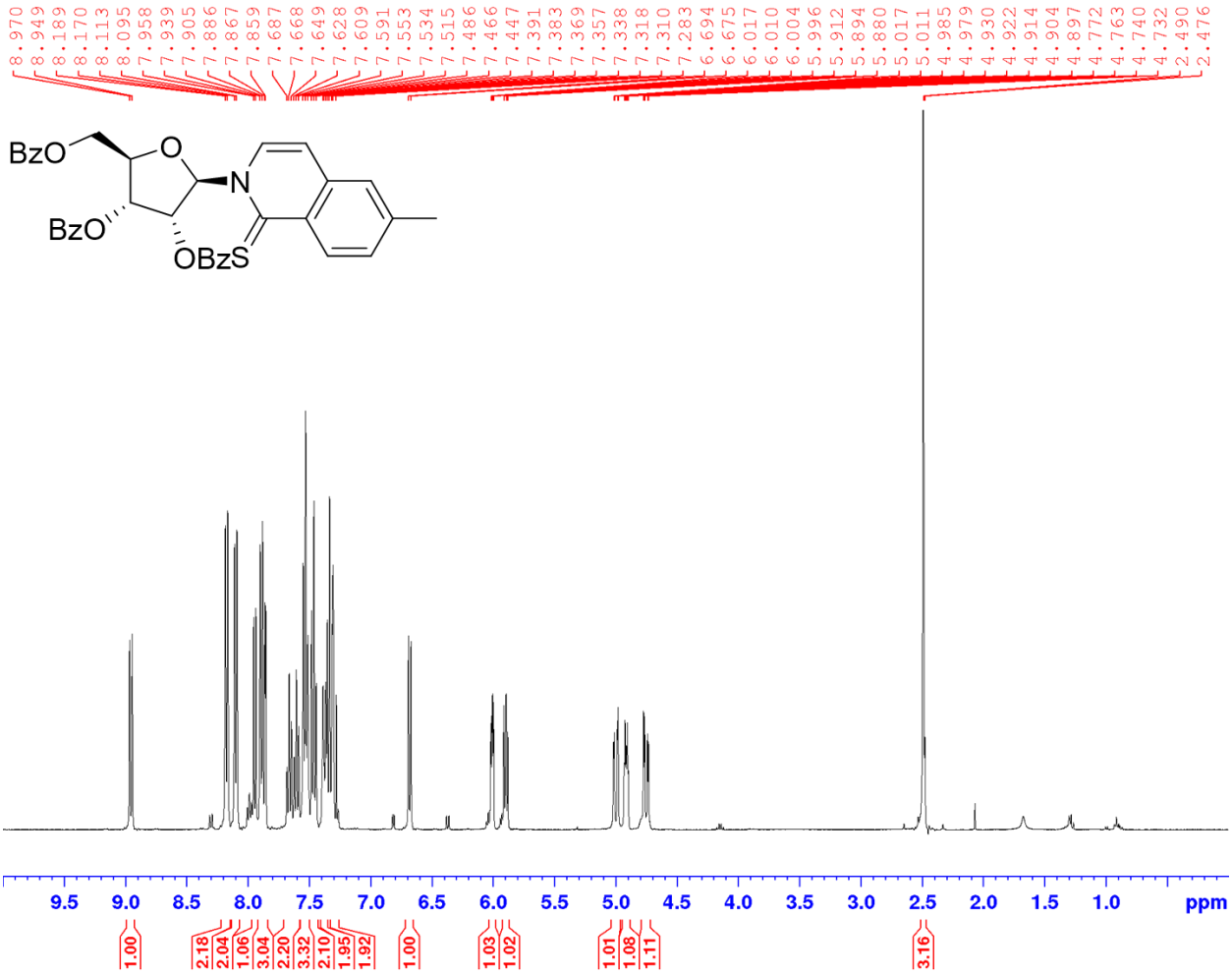


*Figure S633. ^{1}H NMR of compound **19**.*

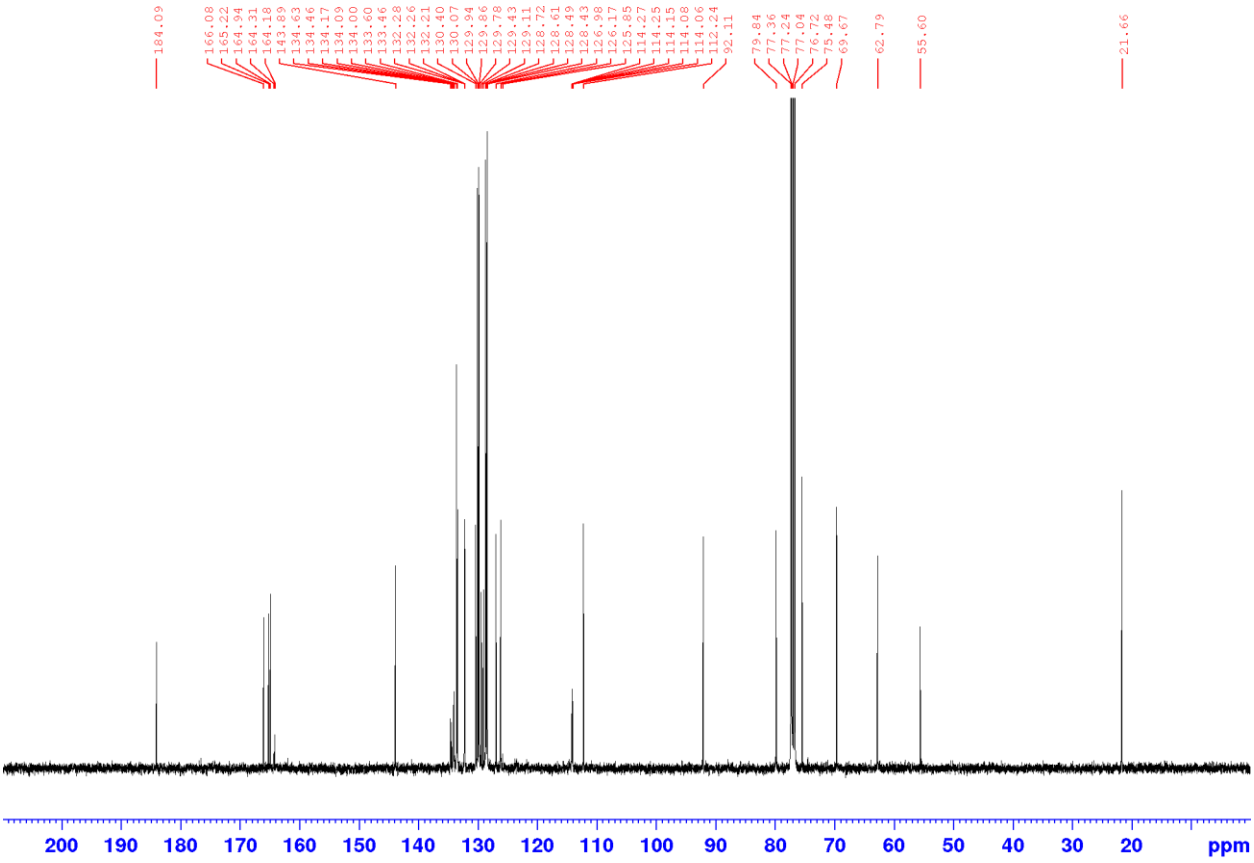


*Figure S644. ^{13}C NMR of compound **19**.*

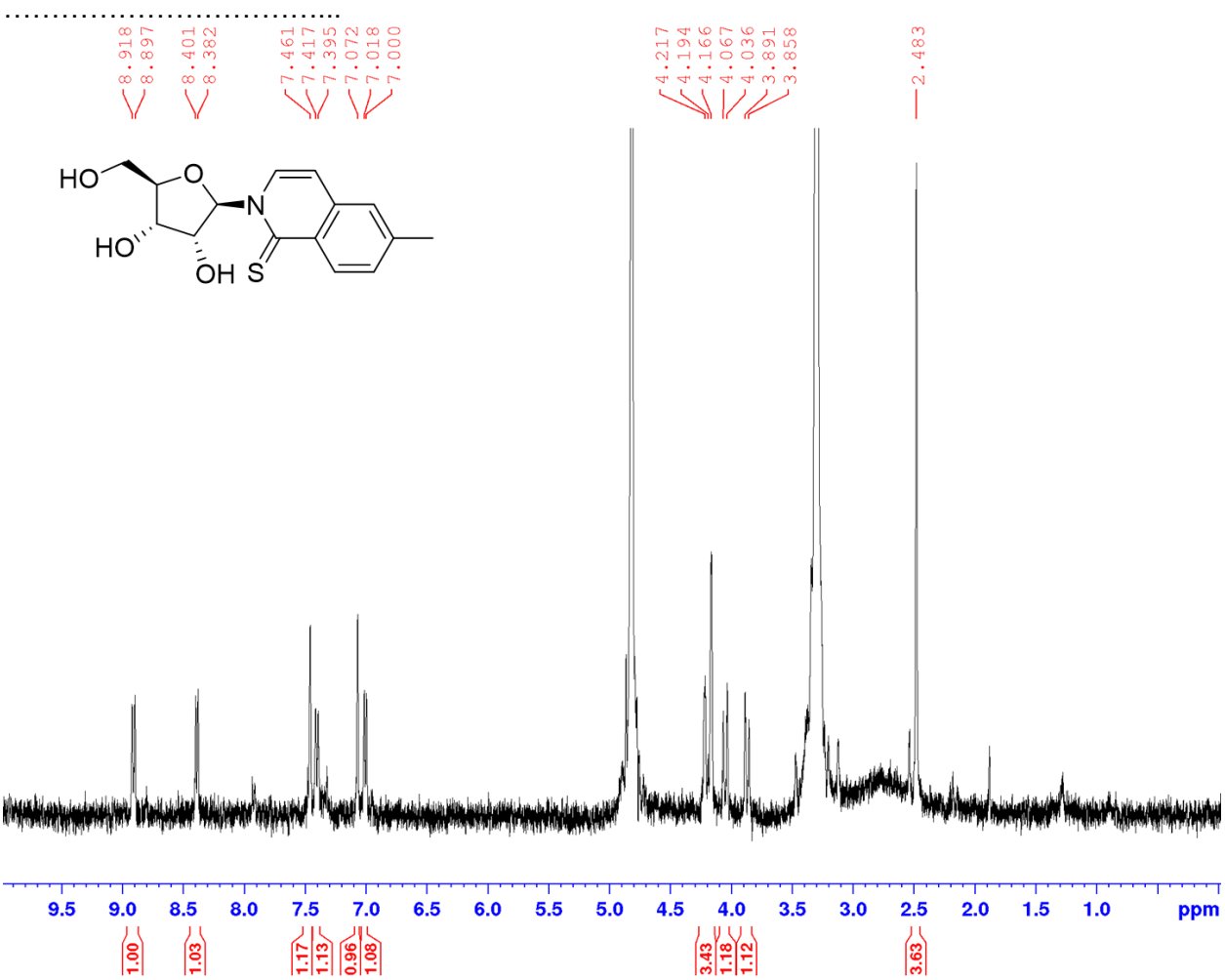


*Figure S655. ^{1}H NMR of compound **20.***

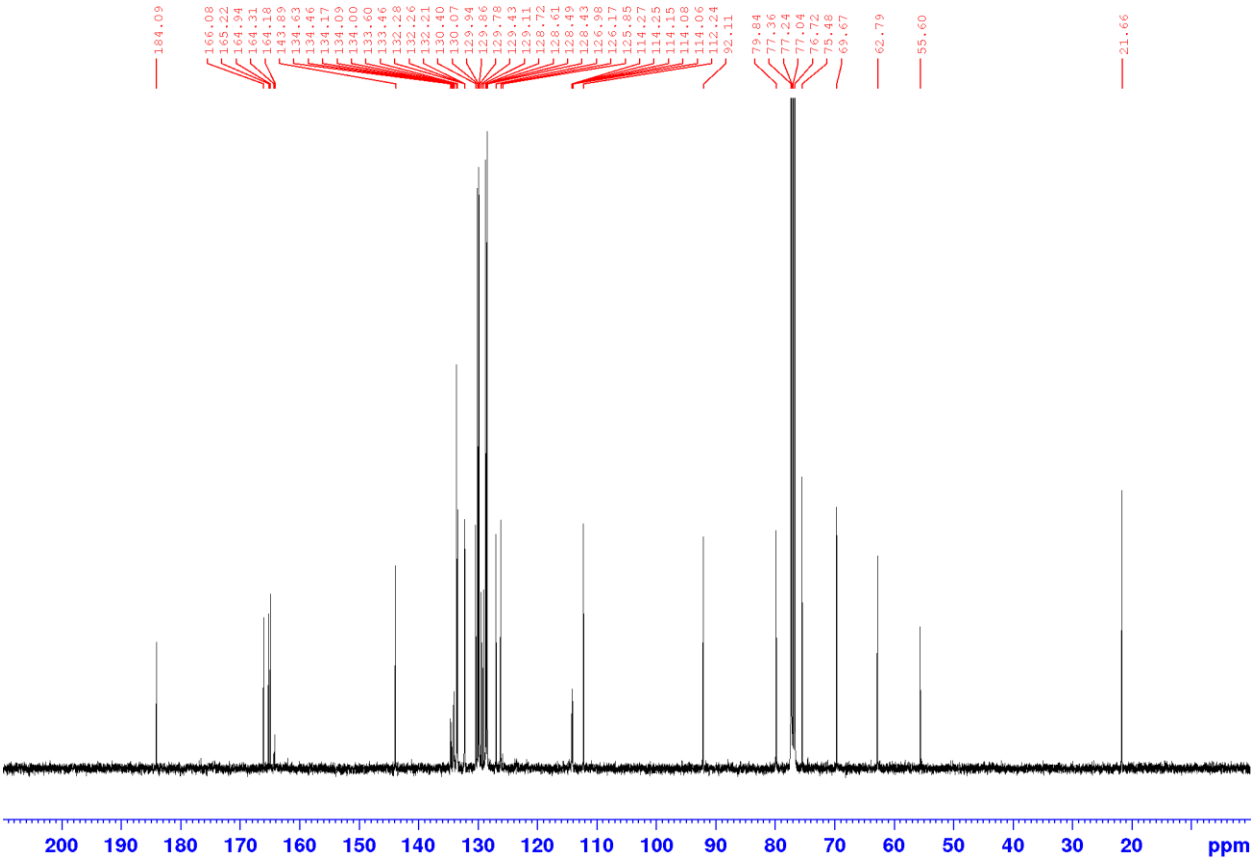


*Figure S666. ^{13}C NMR of compound **20**.*

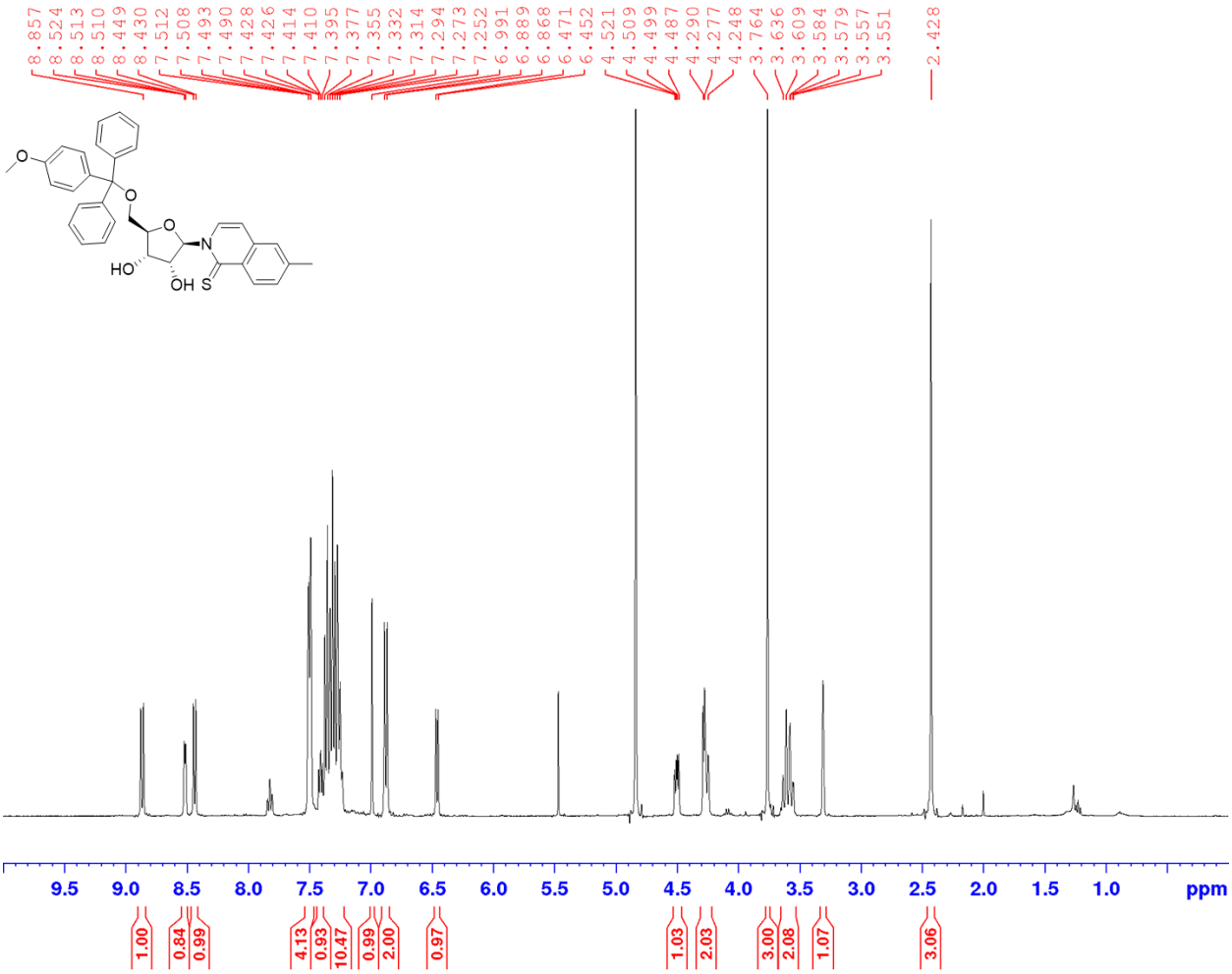


*Figure S6707. ^{1}H NMR of compound **8b**.*

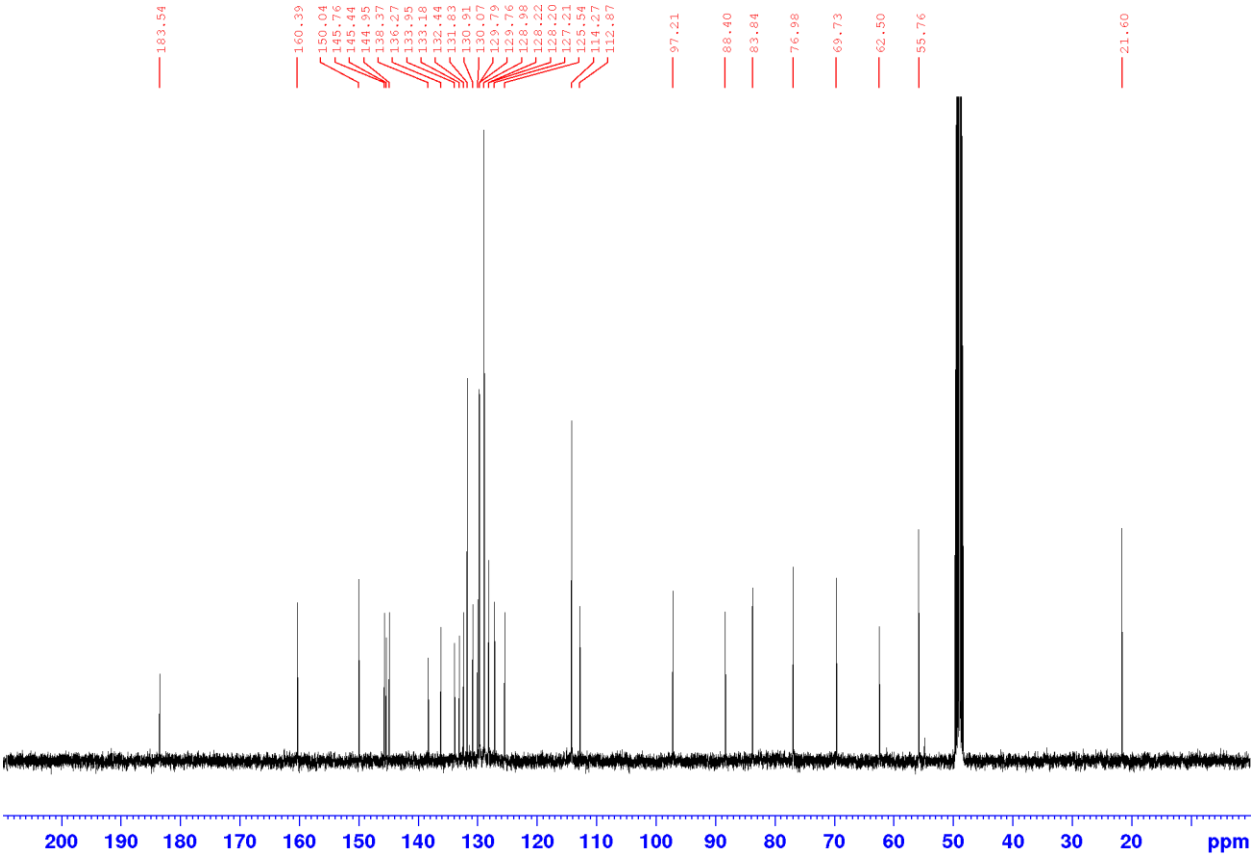


*Figure S6808. ^{13}C NMR of compound **8b**.*

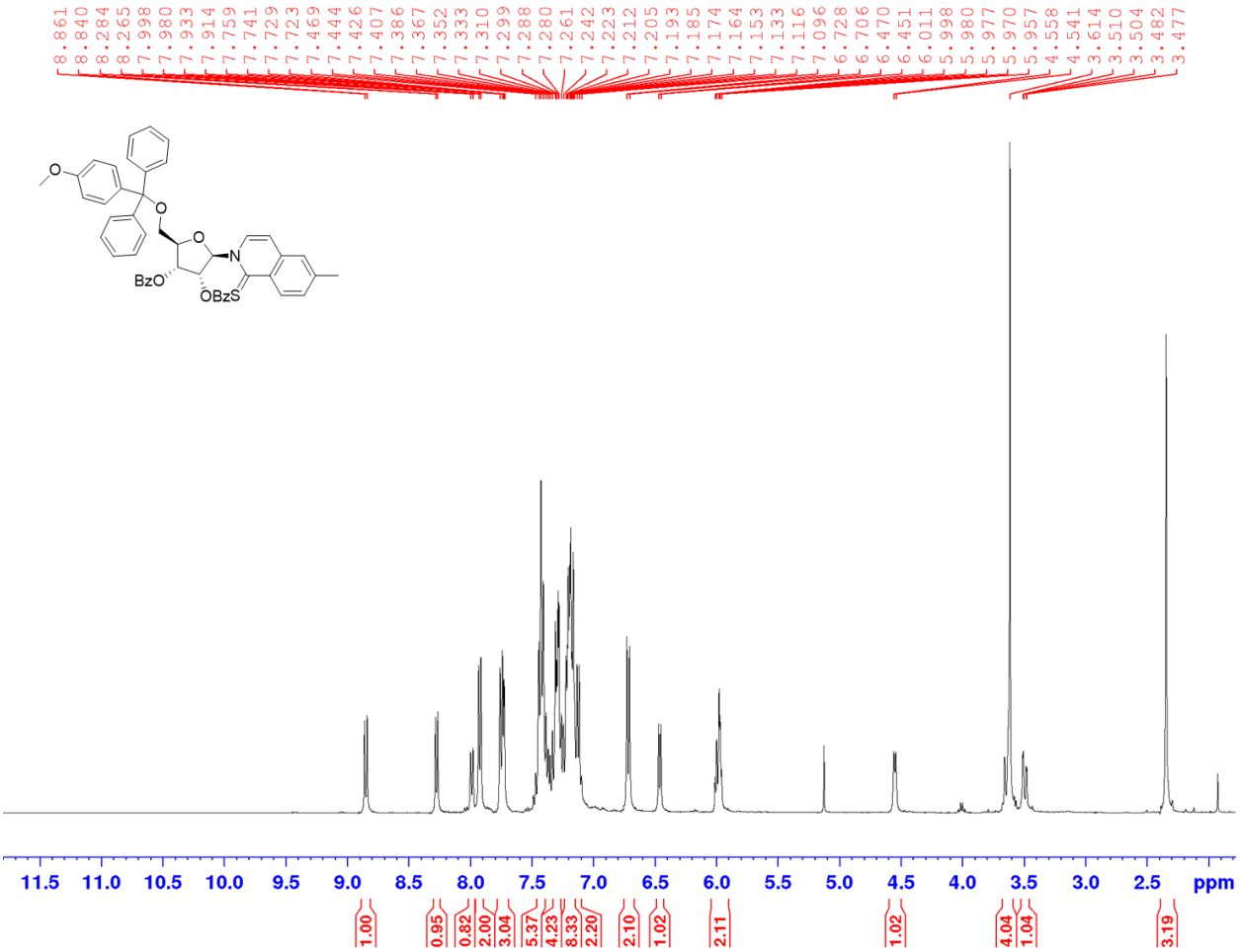


*Figure S6909. ^{1}H NMR of compound **9b**.*

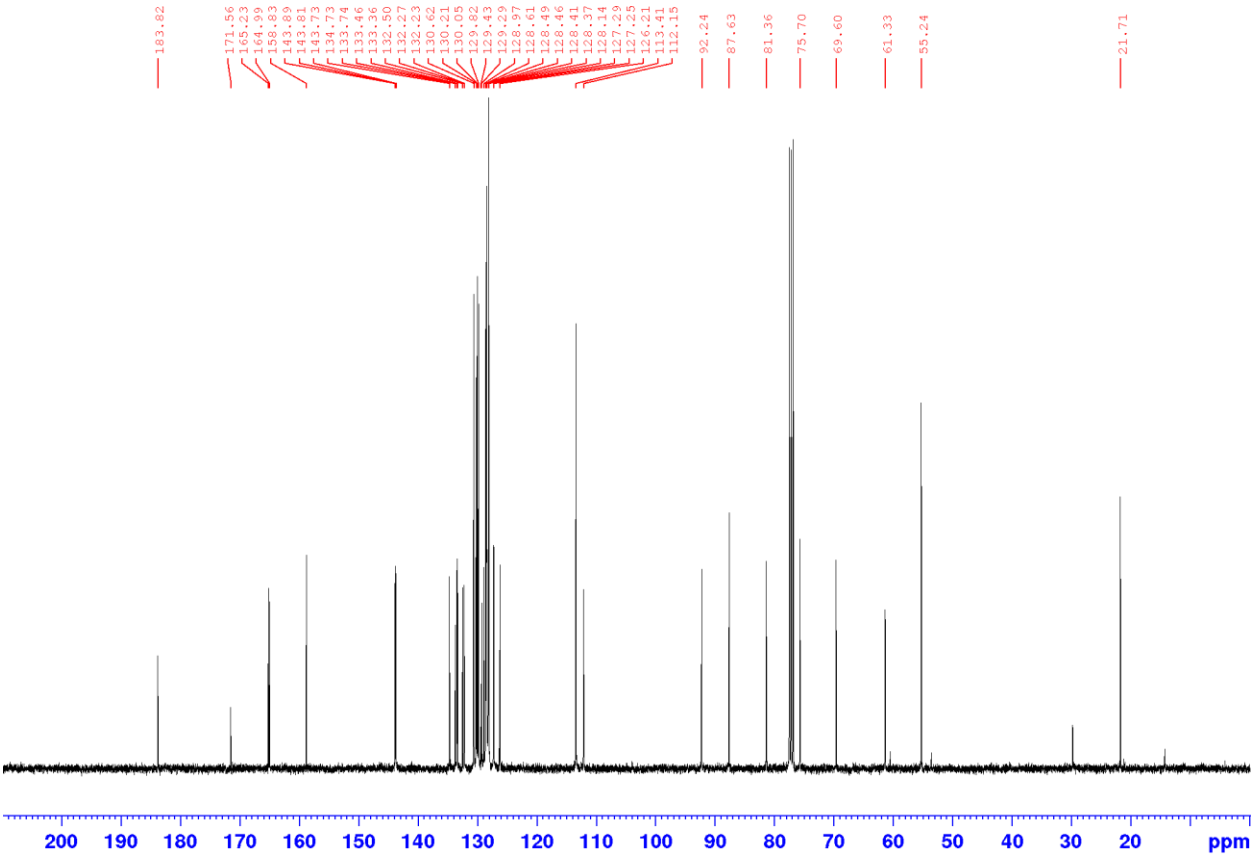


*Figure S110. ^{13}C NMR of compound **9b**.*

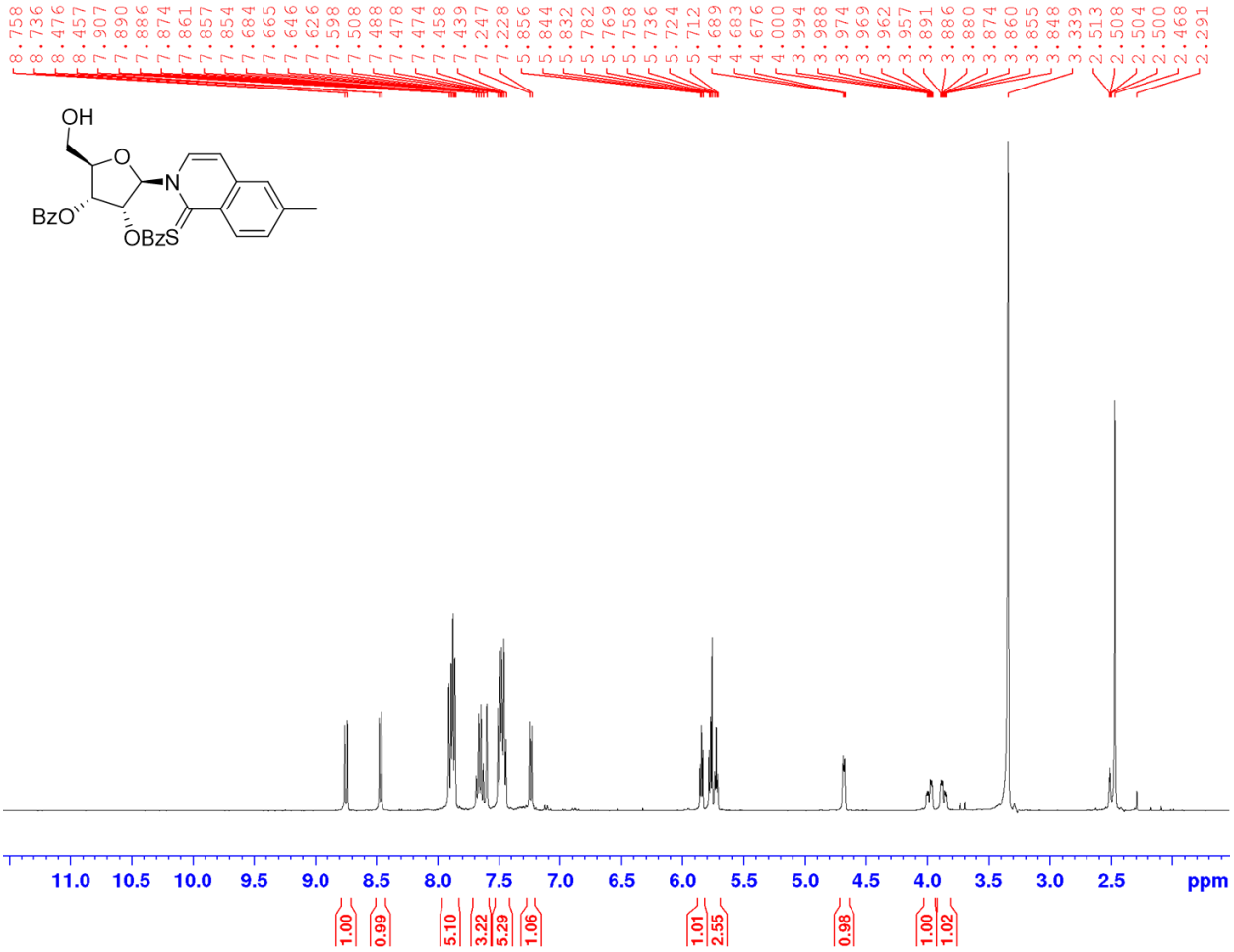


*Figure S701. ^{1}H NMR of compound **10b**.*

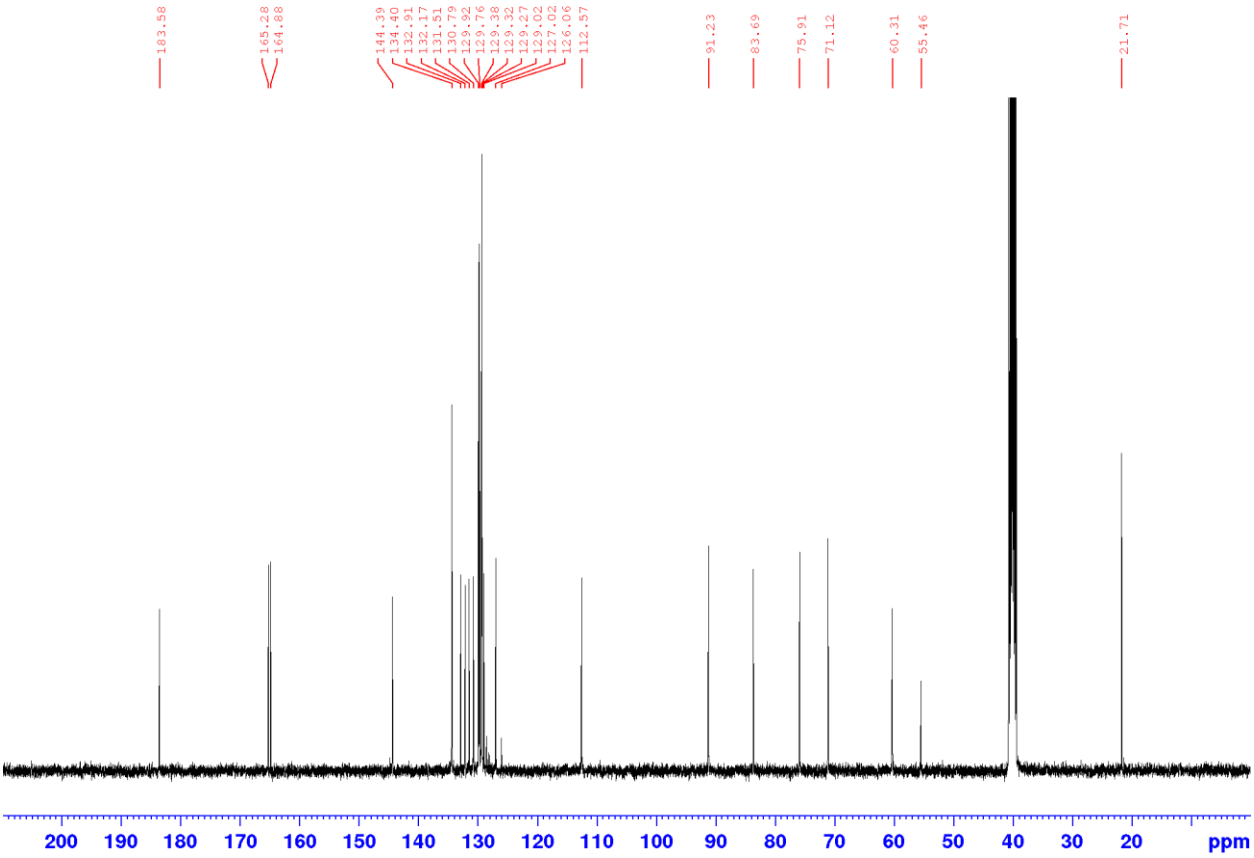


*Figure S712. ^{13}C NMR of compound **10b**.*

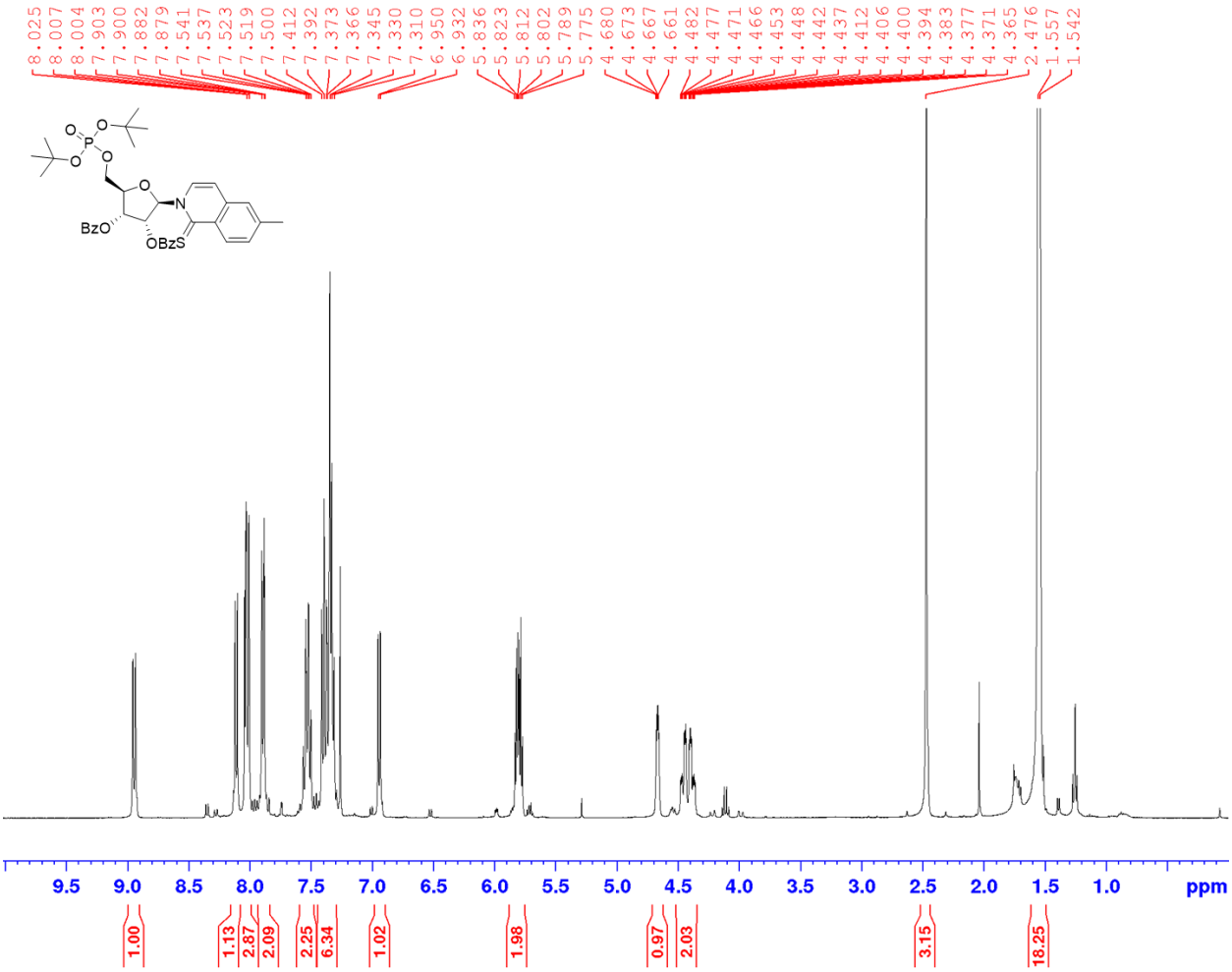


*Figure S723. ^{1}H NMR of compound **11b**.*

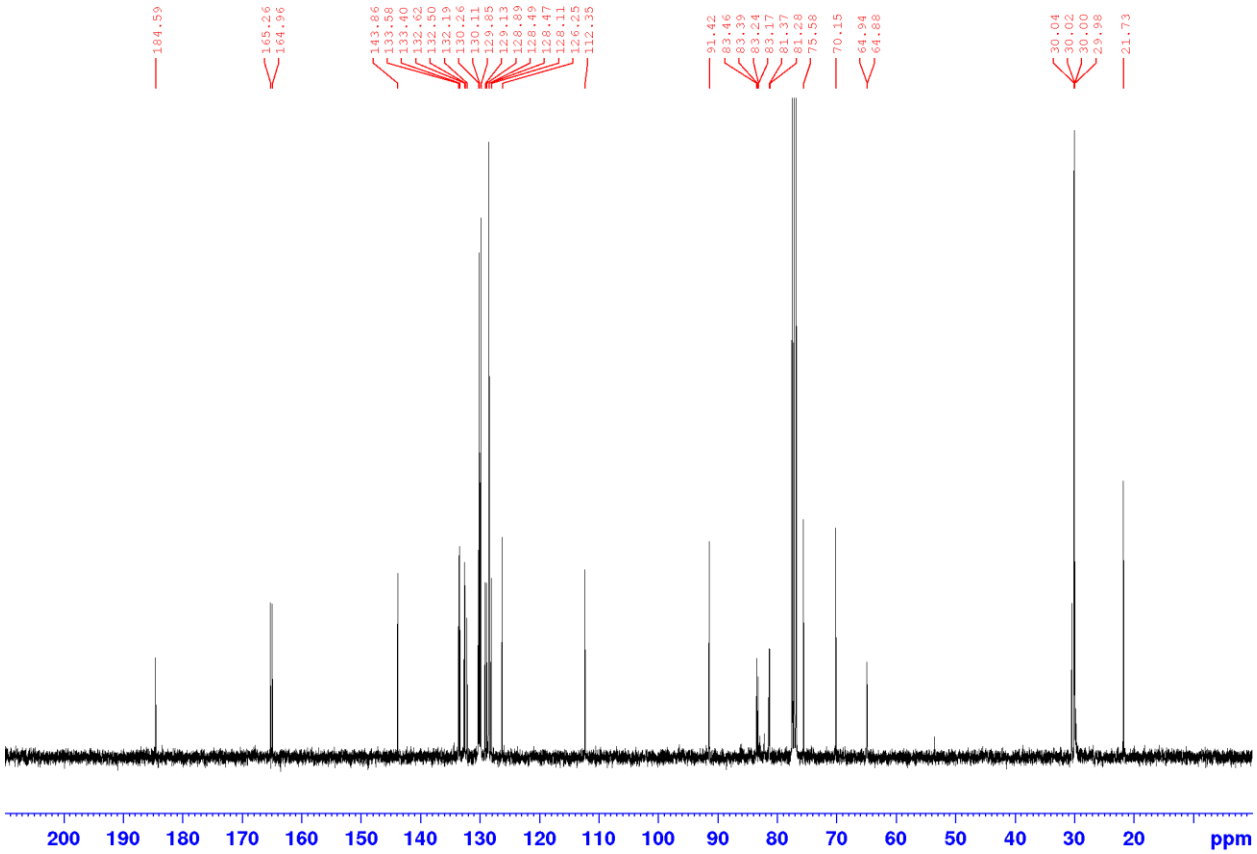


*Figure S734. ^{13}C NMR of compound **11b**.*

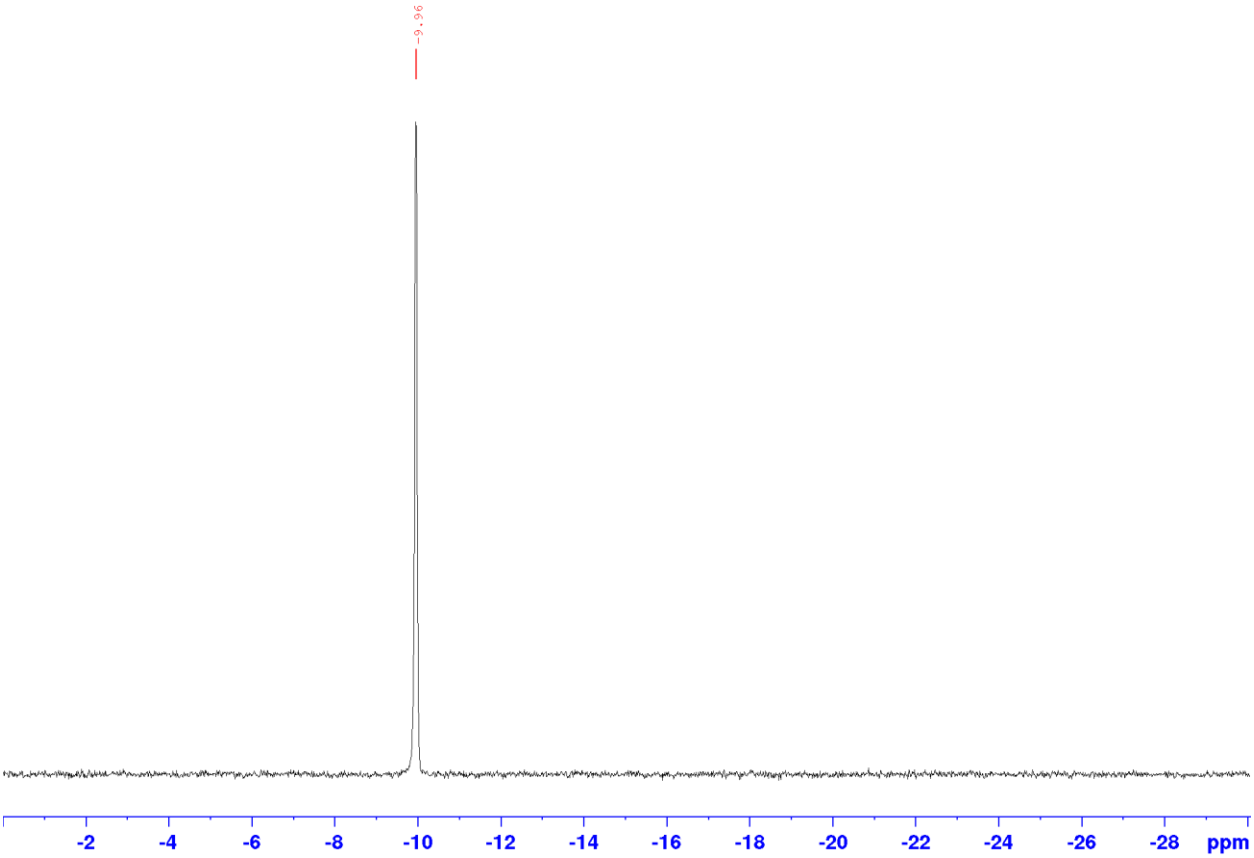


*Figure S745. ^{31}P Proton decoupled NMR of compound **11b**.*

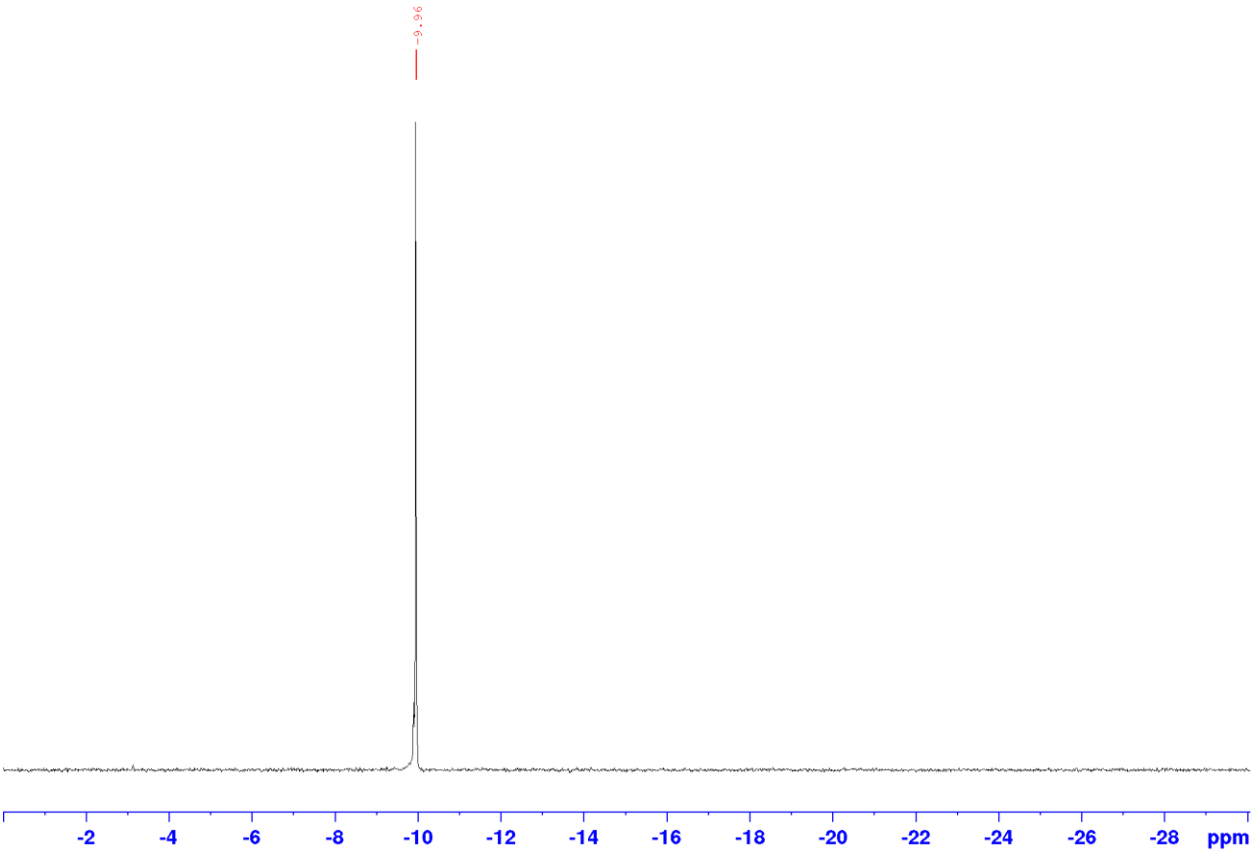


*Figure S756. ^{31}P no proton coupled NMR of compound **11b**.*

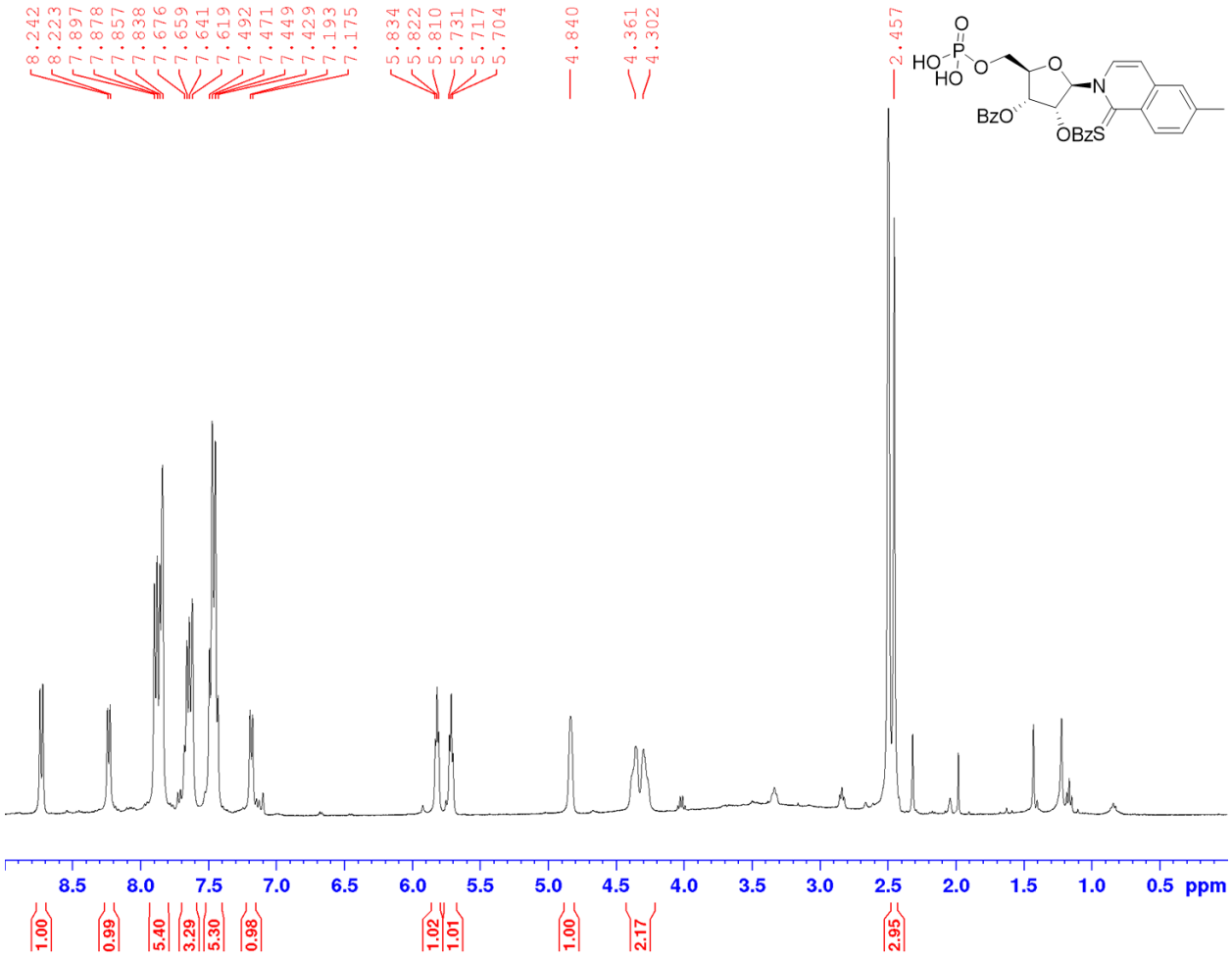


*Figure S7617. ^{1}H NMR of compound **12b**.*

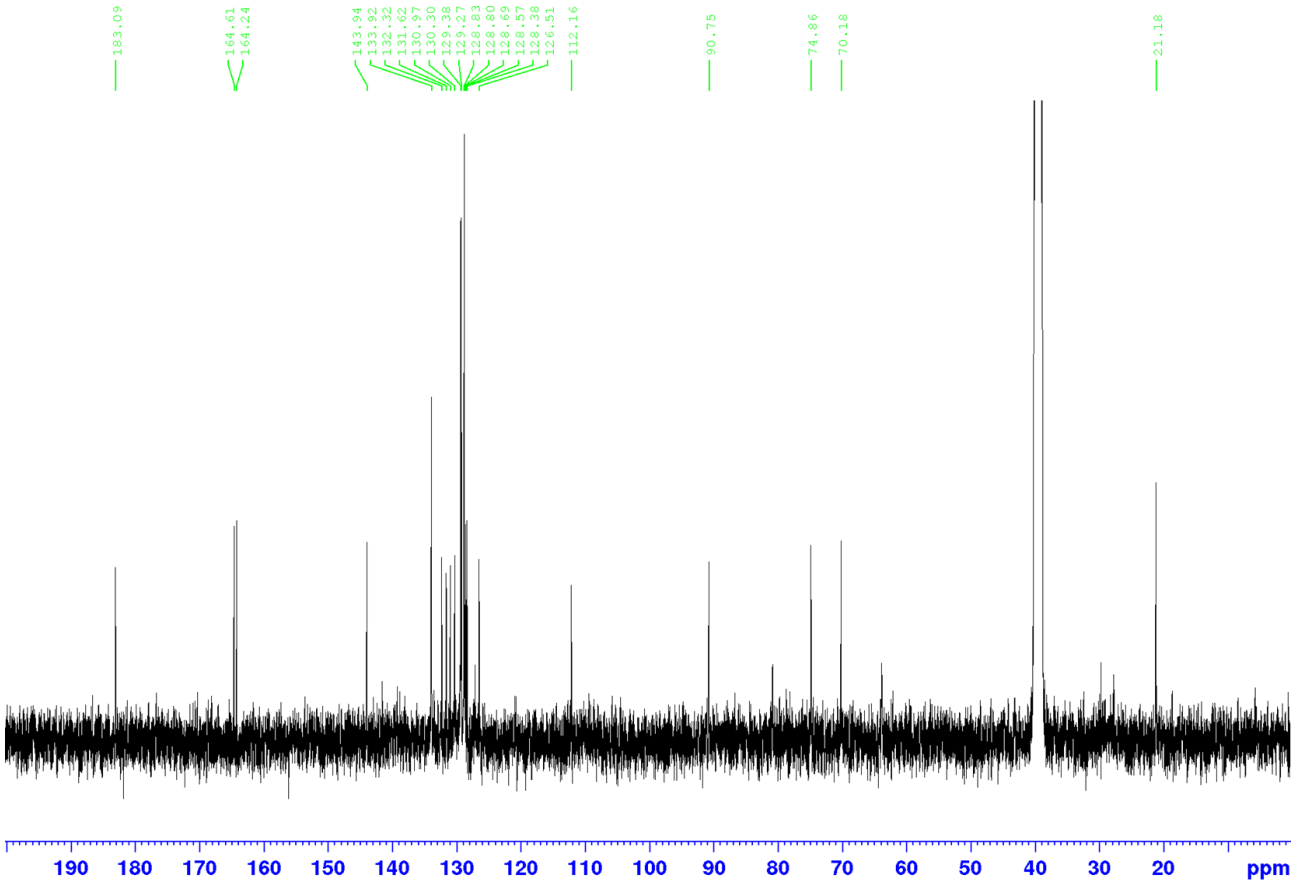


*Figure S7718. ^{13}C NMR of compound **12b**.*

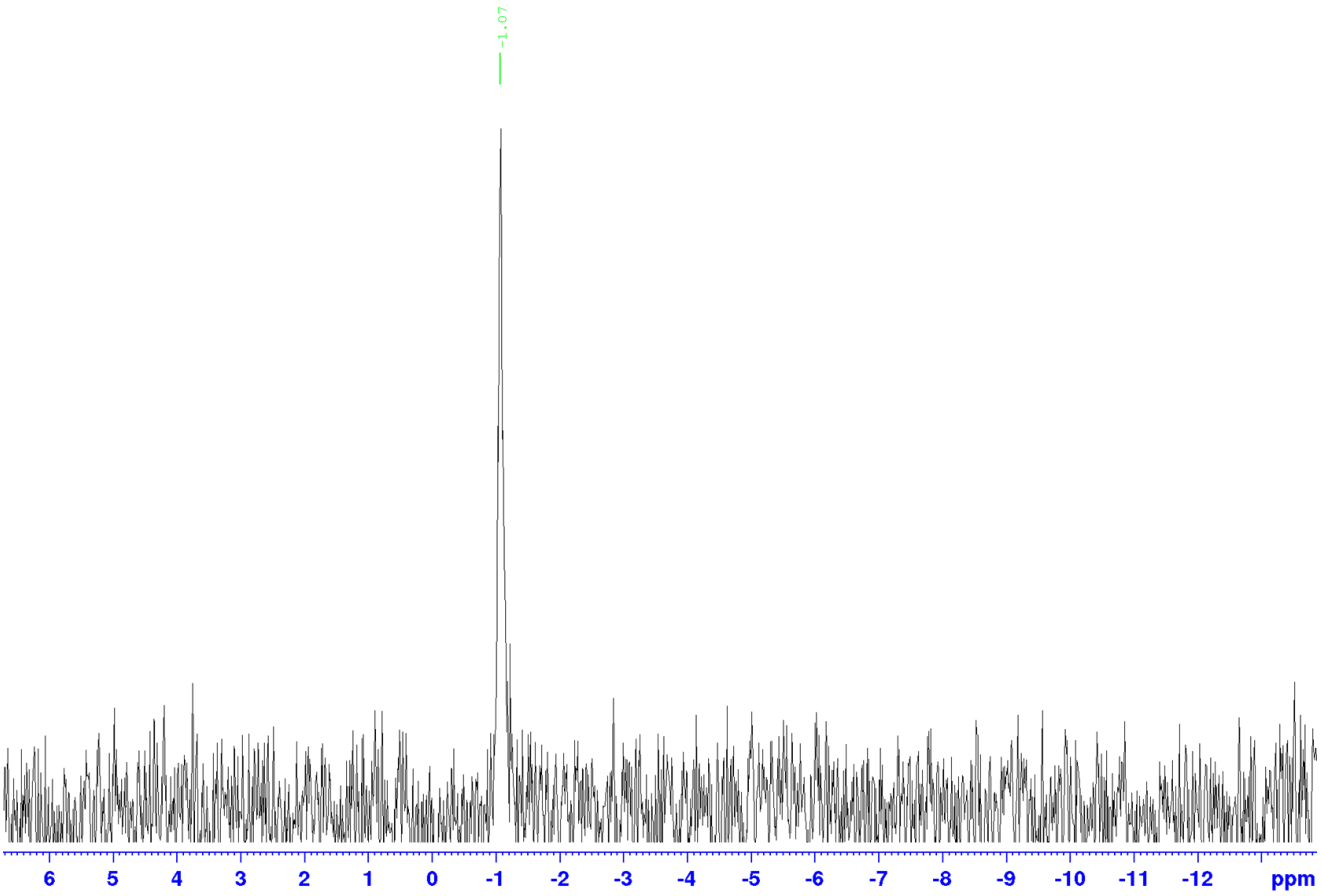


*Figure S7819. ^{31}P Proton decoupled NMR of compound **12b**.*

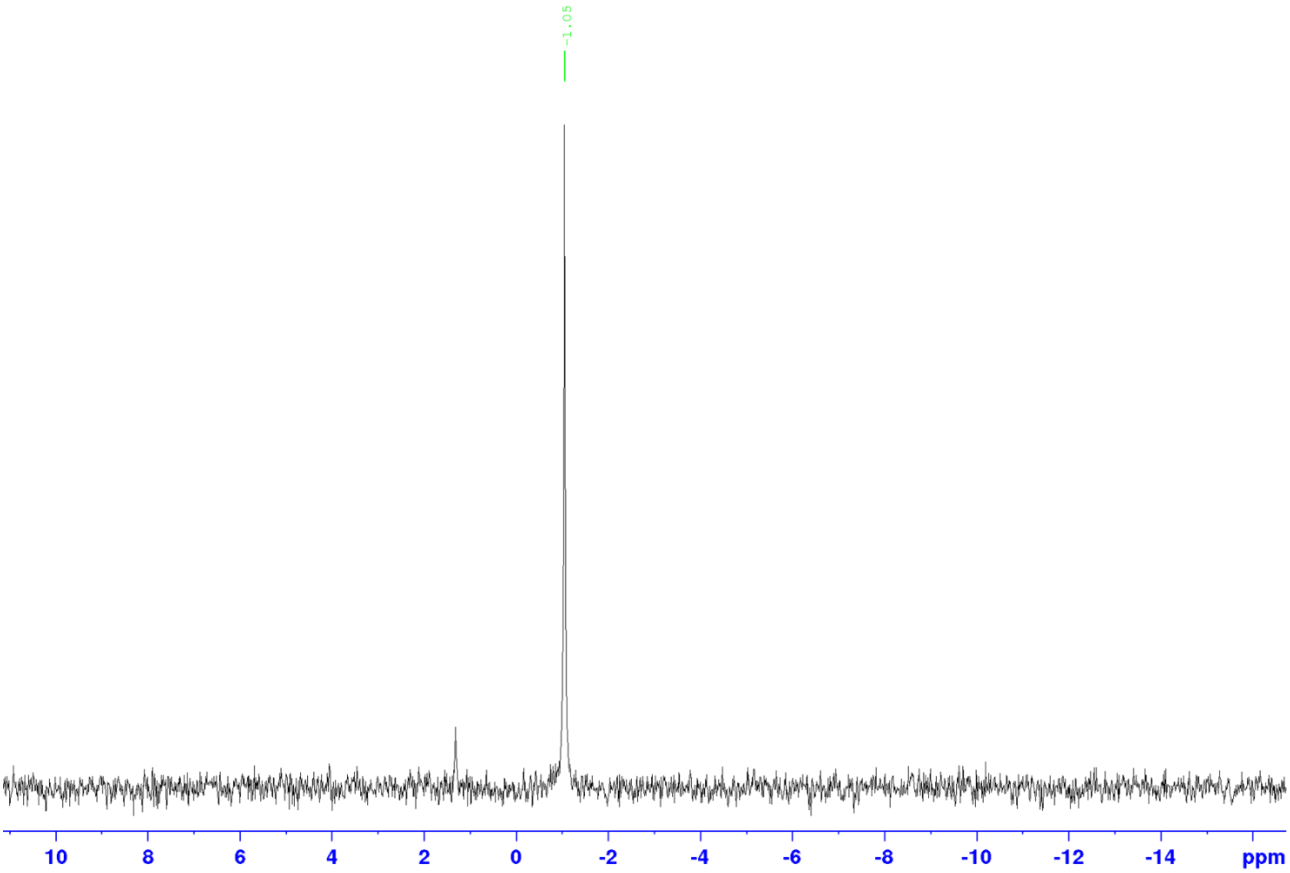


*Figure S7920. ^{31}P no proton decoupled NMR of compound **12b**.*

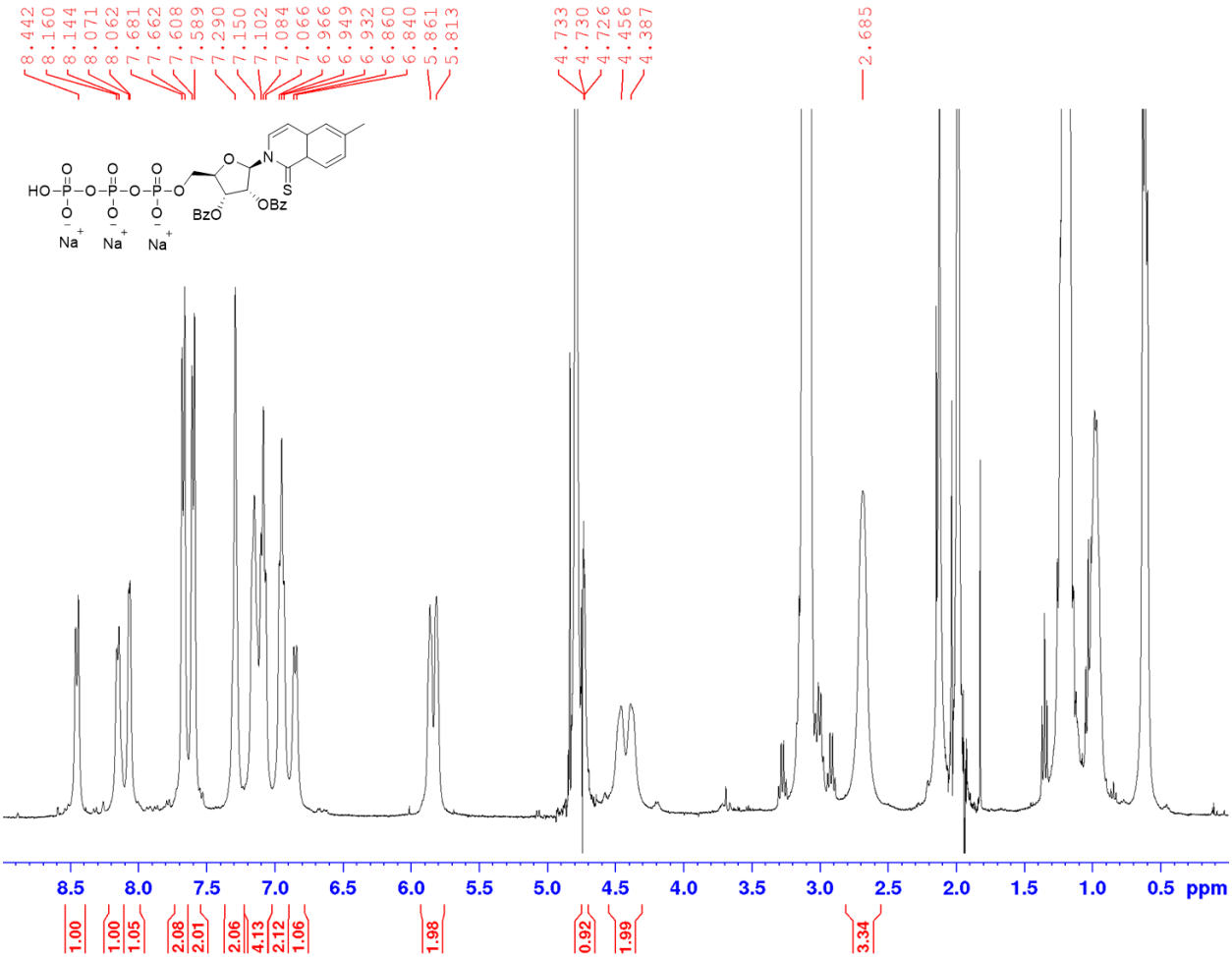


*Figure S8021. ^{1}H NMR of compound **13b**.*

Carbon nmr not available due to solubility

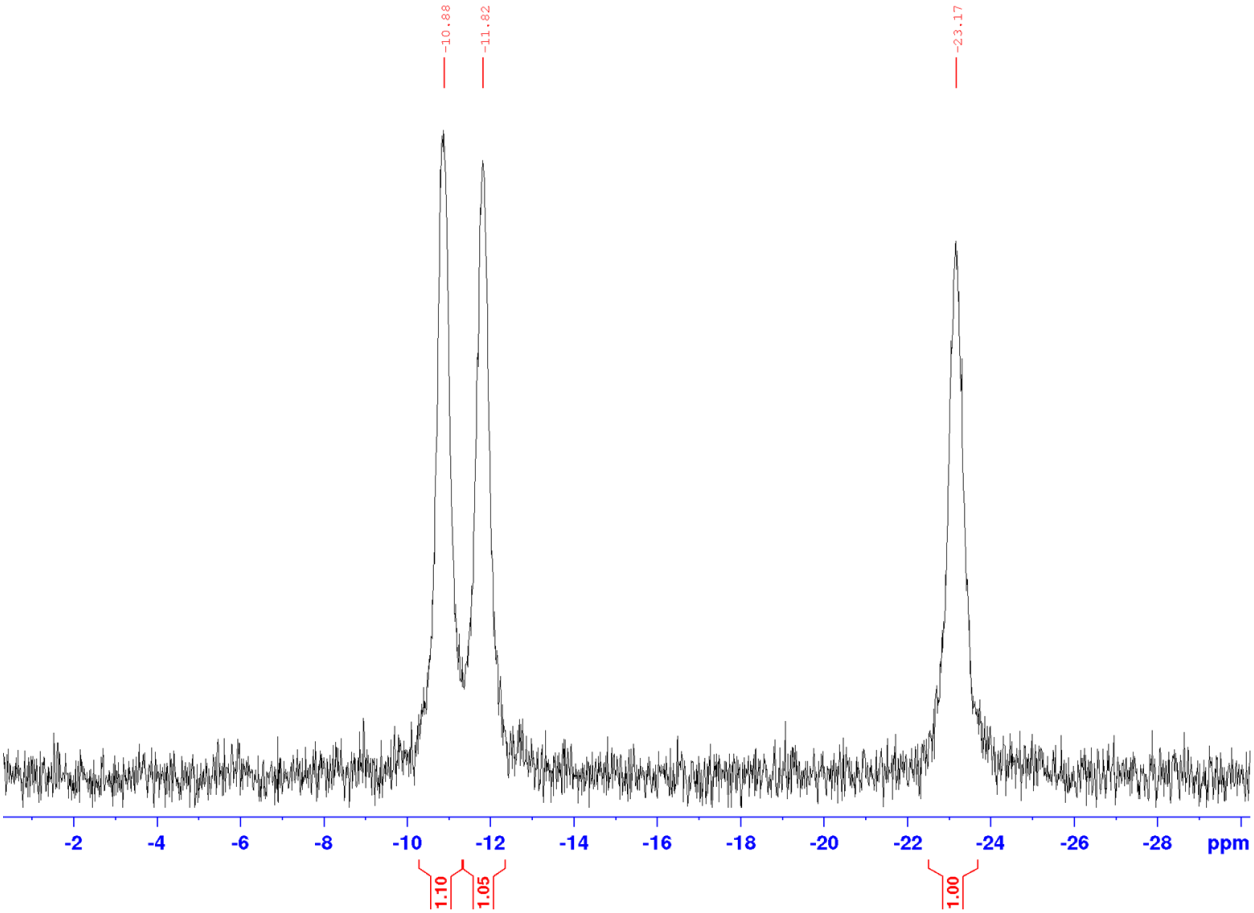


*Figure S812. ^{31}P Proton decoupled NMR of compound **13b**.*

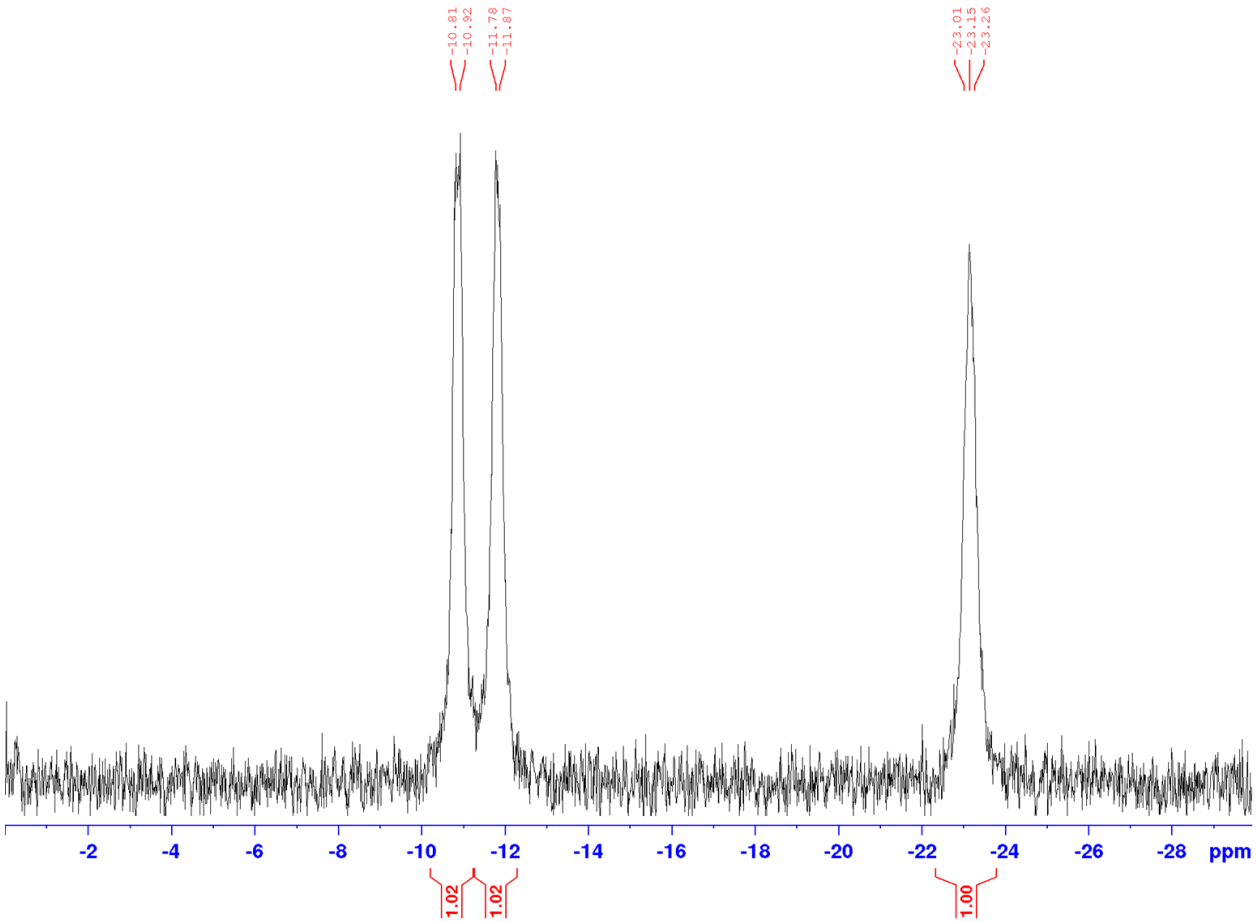


*Figure S823. ^{31}P no proton decoupled NMR of compound **13b.***

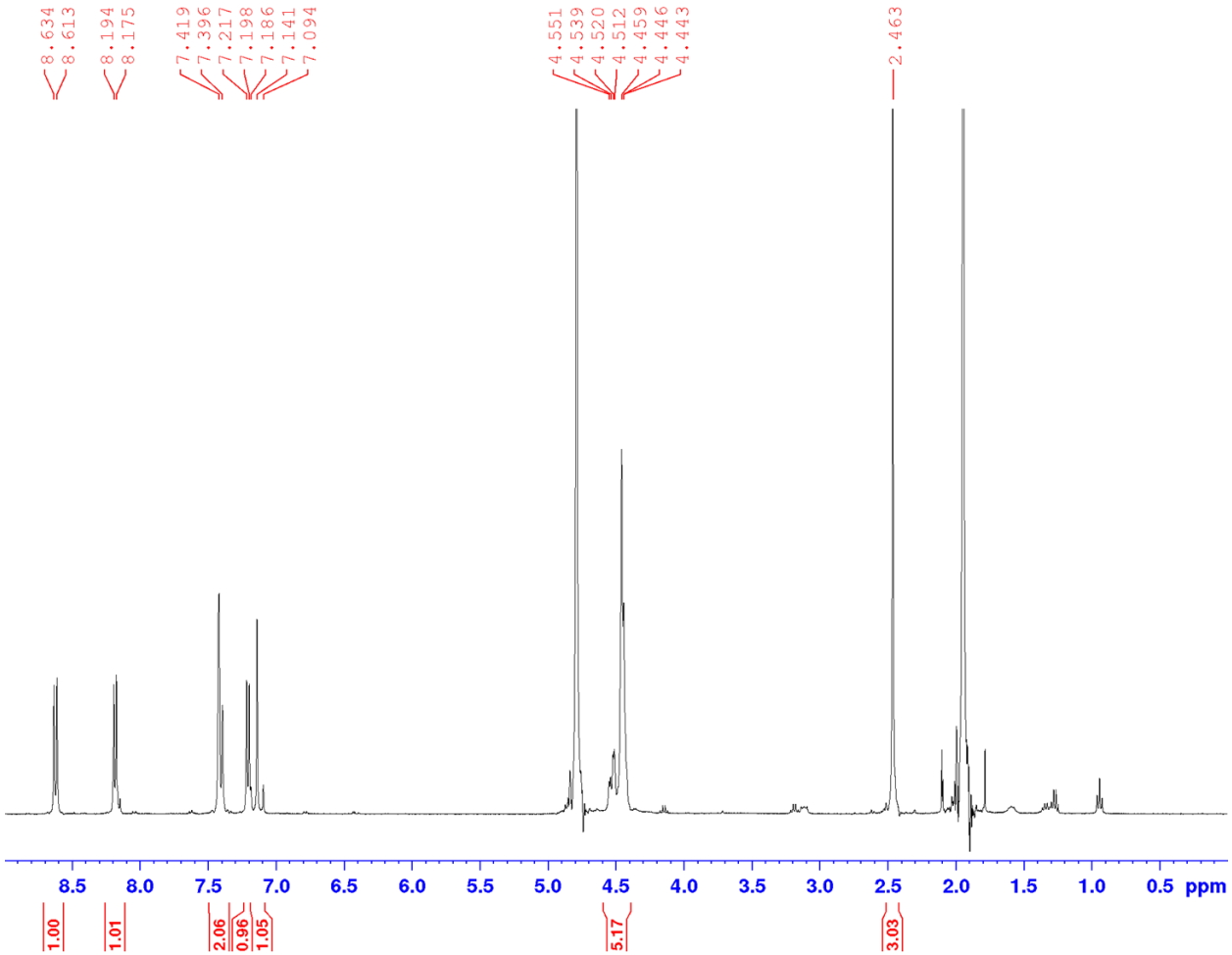


*Figure S834. ^{1}H NMR of compound **14b.***

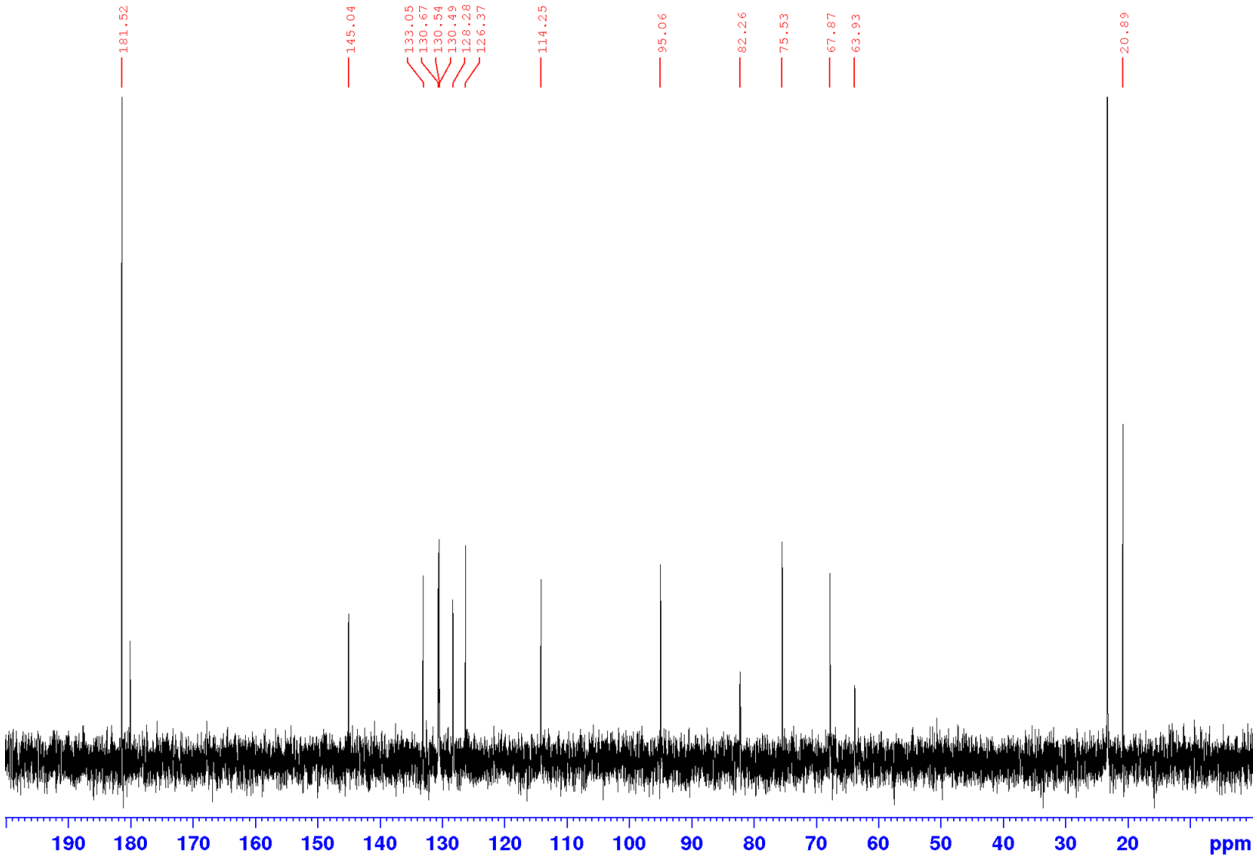


Figure S845. ^{13}C NMR of compound 14b.

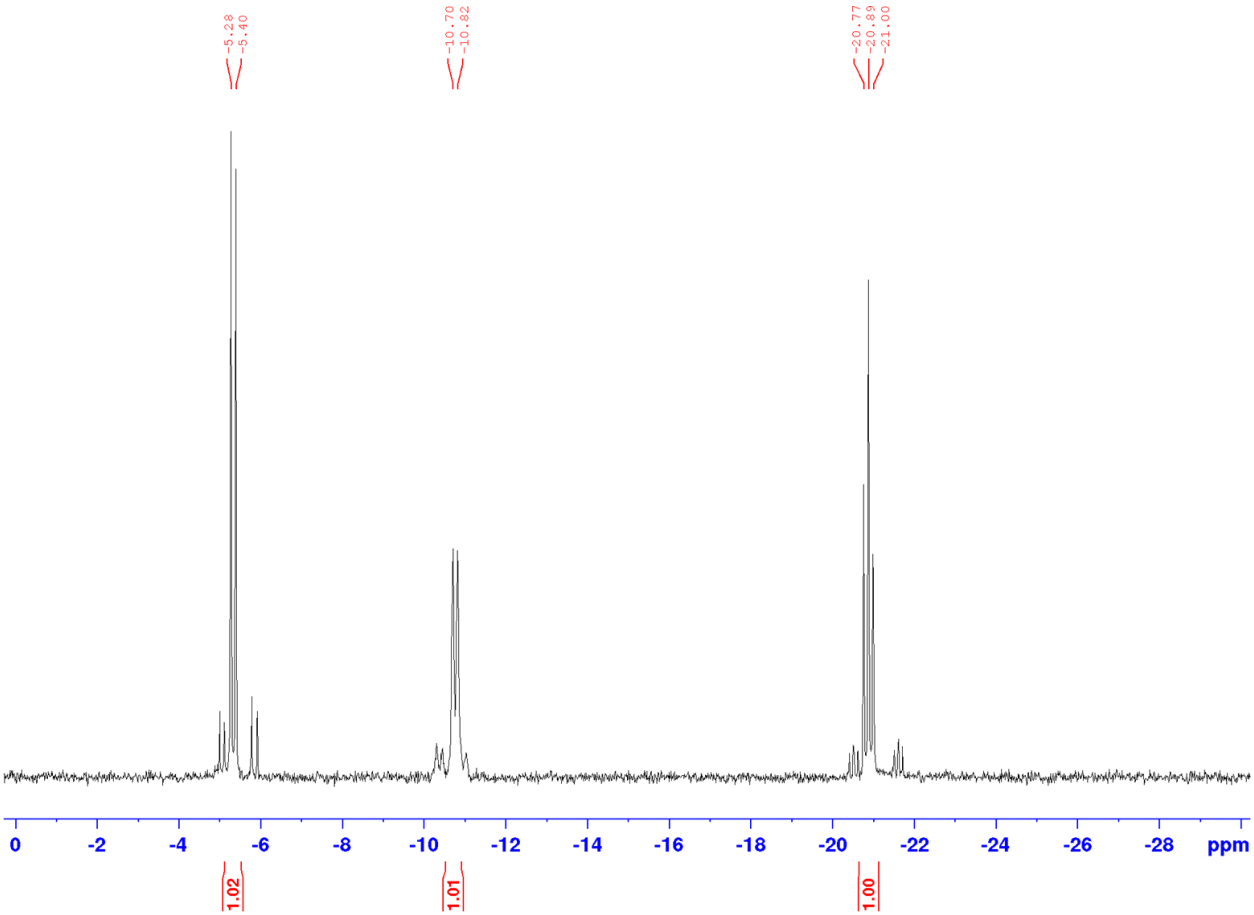


*Figure S856. ^{31}P Proton decoupled NMR of compound **14b.***

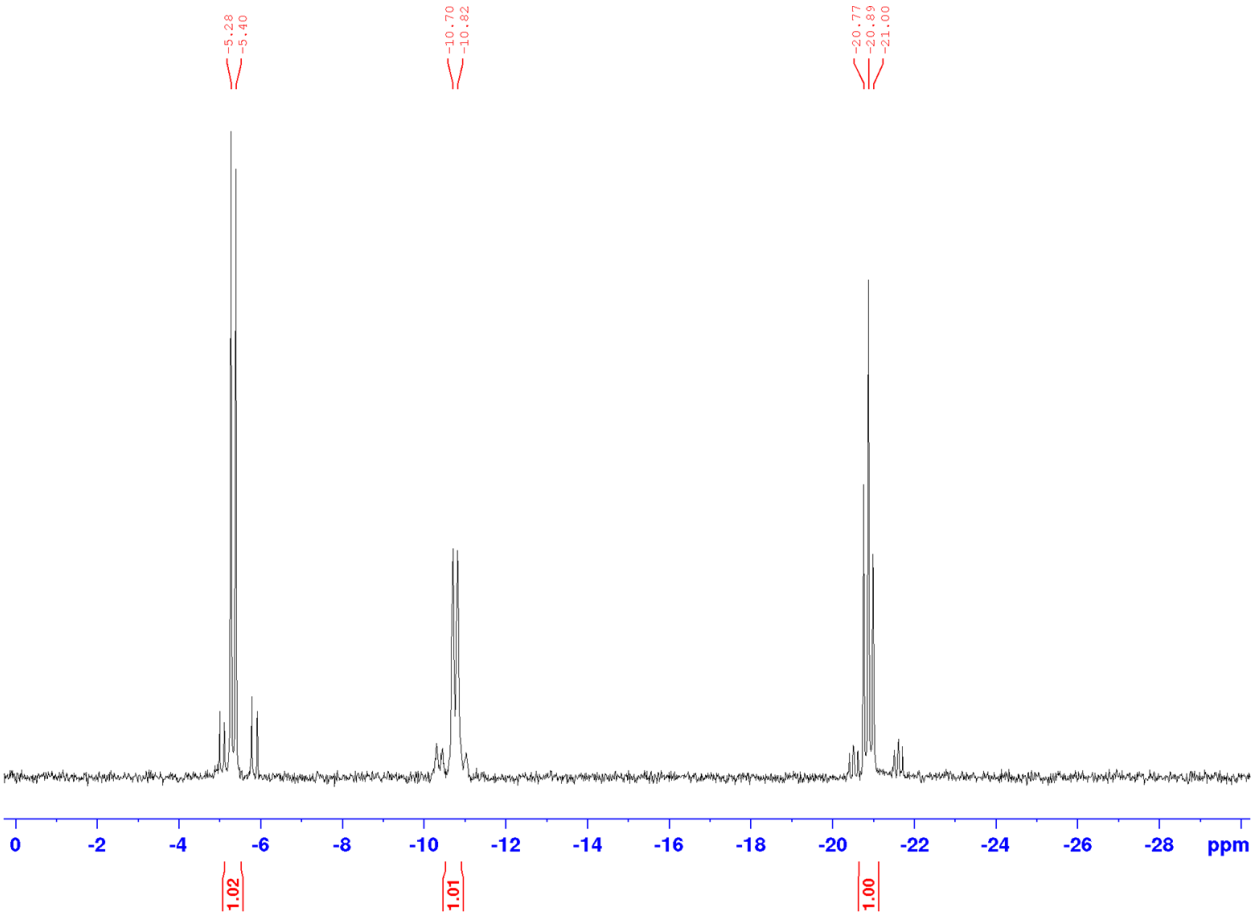


*Figure S8627. ^{31}P no proton decoupled NMR of compound **14b.***

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