SUPPLEMENTARY INFORMATION

Propargyl Uridine-Based Synthesis of Glycosylated RNA Analogues

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Section S1: General information

Section S2: Synthesis details

Section S3: RNA sequences used in the study

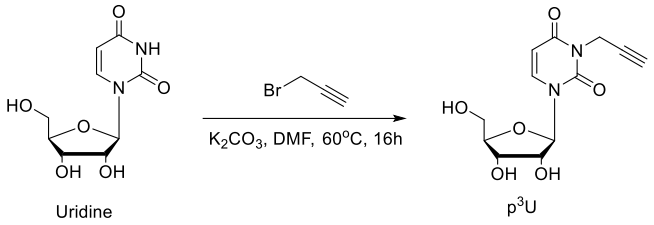
Section S4: Mass spectra

Section S5: NMR spectra

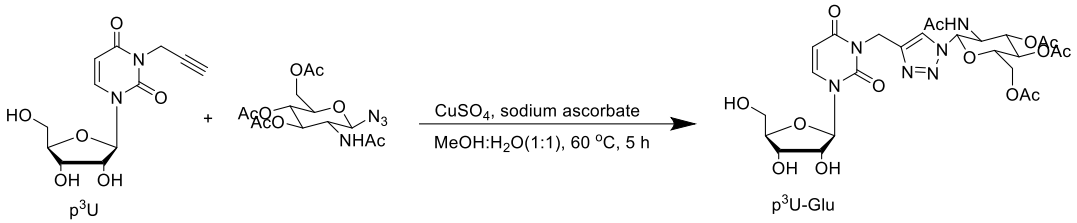
Section S1: General information

All reagents were obtained from commercial suppliers or synthesized by us. The ^{1}H NMR ,^{13}C NMR and ^{31}P NMR spectra were collected by Bruker 300 MHz or 400 MHz or 500 MHz spectrophotometer. Chemical shifts δ are given in ppm relative to the residual proton signals of the deuterated solvent DMSO-d_6 or $CDCl_3$ for ^{1}H ,^{13}C and ^{31}P NMR. Multiplicities are reported as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m). HRMS were obtained using electrospray ionization (ESI) mass spectrometer. HPLC data was collected with Waters ACQUITY H-Class equipped with an Ultimate AQ-C18 5 μm analysis column (250 × 4.6 mm) for nucleotide or nucleoside. RNA oligoes were synthesized by Suzhou Biosyntech Co., Ltd (Suzhou, China).

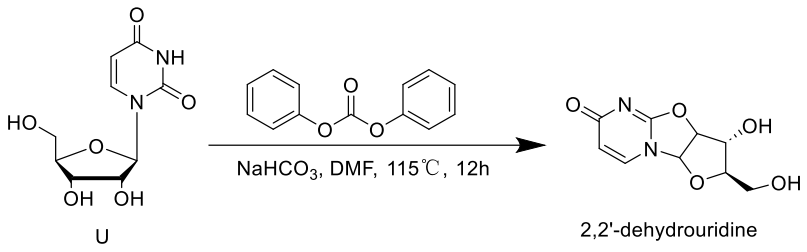
Section S2: Synthesis details



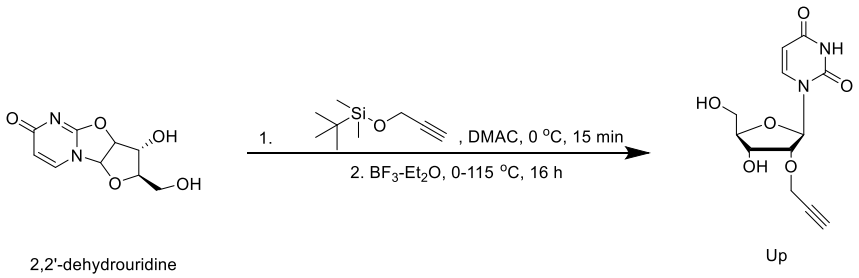
Uridine (730 mg, 2.90 mmol) was dissolved in DMF (30 mL). 3-Bromopropyne (310 μL) and K_2CO_3 (450 mg) were added successively. The reaction mixture was stirred at 60 °C for 16 h, then concentrated under reduced pressure. The crude product was purified by column chromatography (dichloromethane/methanol = 20:1) to afford N^3-propargyl uridine (p^3U) as a white solid (639 mg, 78% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.01-7.98 (d, J = 15.0 Hz, 1H), 5.84-5.82 (m, 2H), 5.40-5.39 (d, J = 5.0 Hz, 1H), 5.11-5.07 (m, 2H), 4.07-4.04 (m, 1H), 3.99-3.96 (q, J = 5.0 Hz, 1H), 3.88-3.86 (m, 1H), 3.66-3.54 (m, 2H), 3.10-3.09 (m, 1H).[1]



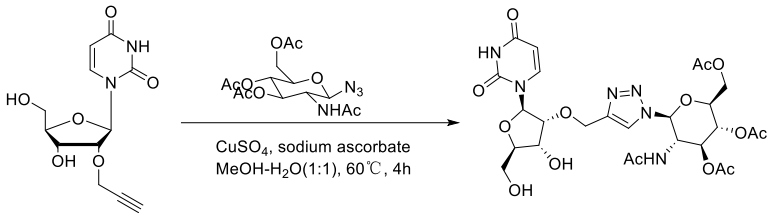
N^3-Propargyl uridine (p^3U) (150 mg, 0.54 mmol) was dissolved in MeOH/H_2O (4 mL, V/V = 1/1). 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide (201 mg, 0.54 mmol), anhydrous $CuSO_4$ (81.4 mg, 0.51 mmol), and sodium ascorbate (101 mg, 0.51 mmol) were added. The reaction mixture was stirred at 60 °C for 5 h, filtered, and concentrated under reduced pressure to give the crude product. Purification by column chromatography (dichloromethane/methanol = 6:1) afforded a white solid (248 mg, 68% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.09 (s, 1H), 8.05-8.00 (m, 2H), 6.11-6.09 (d, J = 10.0 Hz, 1H), 5.82-5.80 (m, 2H), 5.40-5.32 (m, 2H), 5.12-5.06 (m, 4H), 4.99-4.96 (m, 1H), 4.57-4.51 (q, J = 10.0 Hz, 1H), 4.22-4.19 (m, 1H), 4.16-4.12 (dd, J = 12.5, 5.0 Hz, 1H), 4.06-4.05 (m, 2H), 3.98 (s, 1H), 3.87-3.85 (m, 1H), 3.67-3.64 (m, 1H), 3.58-3.54 (m, 1H), 2.003-1.995 (d, J = 4.0 Hz, 6H), 1.94 (s, 3H), 1.58 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.9, 169.5, 169.4, 169.2, 161.4, 150.5, 142.7, 139.4, 122.2, 100.8, 89.1, 84.7, 84.5, 73.7, 73.4, 72.3, 69.5, 68.0, 61.8, 60.5, 52.0, 35.6, 22.2, 20.4, 20.3, 20.2. HRMS-ESI: m/z calculated for $C_{26}H_{34}N_6O_{14}Na^+$ $[M+Na]^+$: 677.20250, found: 677.20252.



Uridine (200 mg, 0.82 mmol) was dissolved in DMF (900 μL). Diphenyl carbonate (186 mg, 0.9 mmol) and sodium bicarbonate (6.4 mg, 0.02 mmol) were added, and the reaction mixture was stirred at 115 °C for 12 h. The reaction was cooled to room temperature, evaporated to dryness and dissolved in methanol. A large amount of white solid precipitated out, which was filtered to afford 2,2'-*O*-anhydrouridine as a white solid (58 mg, 31% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.85-7.82 (d, J = 7.5 Hz, 1H), 6.31-6.29 (d, J = 5.7 Hz, 1H), 5.88-5.83 (m, 2H), 5.20-5.18 (d, J = 5.7 Hz, 1H), 4.99-4.96 (t, J = 5.1 Hz, 1H), 4.38 (m, 1H), 4.08-4.07 (m, 1H), 3.28-3.15 (m, 2H).[2]

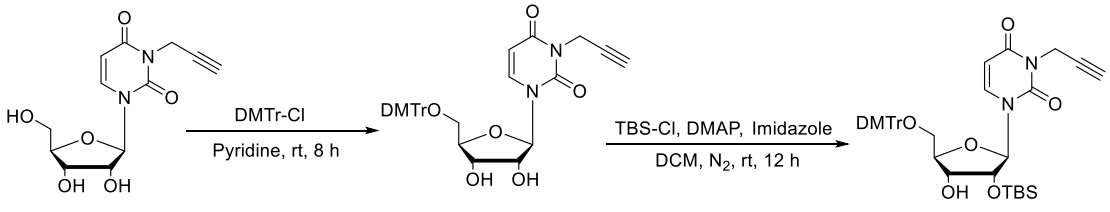


2,2'-O-Anhydrouridine (226 mg, 1 mmol) was dissolved in DMAC, and the air was replaced with nitrogen. Under a nitrogen atmosphere, *tert*-butyldimethyl(2-propynyloxy)silane (1.01 μL) was added. Boron trifluoride diethyl etherate (309 μL) was added dropwise at 0 °C, and stirring was continued at 0 °C for 15 min. The reaction mixture was heated to 115 °C and stirred for 16 h. The mixture was filtered and concentrated under reduced pressure to give the crude product. Purification by column chromatography (dichloromethane/methanol = 10:1) afforded 2'-O-propargyl uridine (Up) as a white solid (85 mg, 30% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.3 (s, 1H), 7.93-7.91 (d, J = 8.0 Hz, 1H), 5.88-5.87 (d, J = 5.5 Hz, 1H), 5.66-5.64 (d, J = 8.0 Hz, 1H), 5.23-5.22 (m, 1H), 5.16-5.14 (m, 1H), 4.32-4.21 (m, 2H), 4.15-4.13 (m, 1H), 4.10-4.06 (m, 1H), 3.88-3.86 (m, 1H), 3.65-3.54 (m, 2H), 3.41-3.40 (t, J = 2.5 Hz, 1H), 3.07-3.03 (q, J = 7.5 Hz, 2H). HRMS-ESI: m/z calculated for $C_{12}H_{14}N_2O_6Na^+$ [M+Na]$^+$: 305.07441, found: 305.07364.[3]

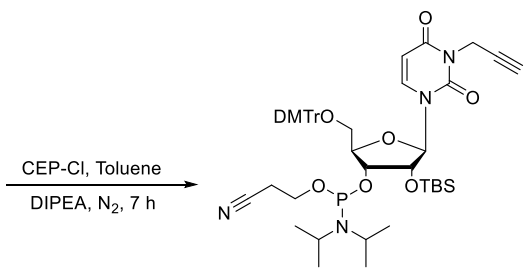


Up Up-Glu

2'-O-Propargyl uridine (Up) (85 mg, 0.3 mmol) was dissolved in MeOH/H_2O (4 mL, V/V = 1/1). 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide (112 mg, 0.3 mmol), anhydrous $CuSO_4$ (52 mg, 0.3 mmol), and sodium ascorbate (60 mg, 0.3 mmol) were added. The reaction mixture was stirred at 60 °C for 4 h, filtered, and concentrated under reduced pressure to give the crude product. Purification by column chromatography (dichloromethane/methanol = 10:1) afforded a white solid (93 mg, 46% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.34 (br s, 1H), 8.28 (s, 1H), 8.12-8.10 (d, J = 9.2 Hz, 1H), 7.91-7.88 (d, J = 8.4 Hz, 1H), 6.12-6.09 (d, J = 10.0 Hz, 1H), 5.90-5.89 (d, J = 4.4 Hz, 1H), 5.63-5.61 (d, J = 8.4 Hz, 1H), 5.38-5.33 (t, J = 10.0 Hz, 1H), 5.11-5.06 (t, J = 9.6 Hz, 1H), 4.70 (s, 2H), 4.60-4.52 (q, J = 9.6 Hz, 1H), 4.33-4.22 (m, 1H), 4.18-4.14 (m, 2H), 4.07-4.03 (m, 2H), 3.89-3.86 (m, 1H), 3.67-3.64 (m, 1H), 3.58-3.54 (m, 1H), 3.17 (s, 2H), 2.000-1.998 (s+s, 6H), 1.95 (s, 3H), 1.59 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.5, 170.1, 169.8, 163.5, 150.7, 144.3, 140.7, 123.3, 102.1, 86.7, 85.13, 85.06, 81.0, 73.8, 72.6, 68.5, 68.4, 63.0, 62.0, 60.6, 52.5, 48.8, 22.5, 20.7, 20.6, 20.5. HRMS-ESI: *m/z* calculated for $C_{26}H_{33}N_6O_{14}^-$ [M-H]$^-$: 653.20602, found: 653.20782.



p^{3}U 1 2



3

Synthesis of **1:** N^3-Propargyl uridine (p^3U) (2.6 g, 9.2 mmol) was dissolved in dry pyridine 10 mL). 4,4'-Dimethoxytrityl chloride (6.3 g, 18.4 mmol) was added, and the reaction was carried out under nitrogen protection at room temperature for 8 h. The reaction mixture was concentrated directly, dissolved in CH_2Cl_2 (200 mL), and washed successively with 5% $NaHCO_3$ solution (200 mL) and saturated NaCl solution (200 mL). The organic phase was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated. Purification by column chromatography (dichloromethane/ethyl acetate = 30:1) afforded the product as a white foam (1.37 g, 25% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.84-7.81 (d, J = 8.4 Hz, 1H), 7.39-7.24 (m, 9H), 6.92-6.89 (d, J = 8.7 Hz, 4H), 5.794-5.785 (d, J = 2.7 Hz, 1H), 5.58-5.57 (d, J = 4.5 Hz, 1H), 5.47-5.44 (d, J = 8.1 Hz, 1H), 5.21-5.19 (d, J = 5.4 Hz, 1H), 4.52-4.51 (d, J = 2.1 Hz, 2H), 4.10-3.99 (m, 4H), 3.74 (s, 6H), 3.28-3.21 (m, 2H), 3.14-3.13 (m, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.6, 158.8, 151.1, 144.4, 138.6, 135.4, 135.2, 130.2, 128.2, 128.1, 127.2, 113.4, 101.6, 91.4, 87.2, 84.5, 78.1, 76.1, 70.9, 70.8, 62.6, 55.4, 30.0. HRMS-ESI: m/z calculated for $C_{33}H_{32}N_2O_8Na^+$ $[M+Na]^+$: 607.20510 , found: 607.20509.

Synthesis of **2**: Compound **1** (1.37 g, 2.34 mmol) was dissolved in dry dichloromethane (10 mL). *Tert*-Butyldimethylsilyl chloride (530 mg, 3.5 mmol), imidazole (797 mg, 11.7 mmol), and 4-dimethylaminopyridine (29 mg, 0.23 mmol) were added. The reaction was stirred at room temperature under nitrogen atmosphere for 12 h. Purification by column chromatography (dichloromethane/ethyl acetate = 100/1-80/1) afforded a white foam (527 mg, 32% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94-7.92 (d, J = 8.0 Hz, 1H), 7.20 (m), 6.85-6.83 (d, J = 8.4 Hz, 4H), 6.01-6.00 (m, 1H), 5.39-5.37 (d, J = 8.0 Hz, 1H), 4.69 (m, 2H), 4.35 (m, 2H), 4.15-4.09 (m, 3H), 3.80 (s, 6H), 3.53-3.46 (m, 2H), 0.92 (s, 9H), 0.17-0.15 (s+s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.0, 158.89, 158.86, 150.3, 144.4, 138.6, 135.3, 135.1, 130.3, 130.2, 128.23, 128.18, 127.4, 113.47, 113.46, 101.9, 89.4, 87.4, 83.8, 78.4, 76.6, 70.8, 70.7, 62.6, 55.4, 30.3, 25.8, 18.2, -4.5, -5.1. HRMS-ESI: m/z calculated for $C_{39}H_{46}N_2O_8SiNa^+$ $[M+Na]^+$: 721.29156, found: 721.29160.

Synthesis of **3:** Compound **2** (86 mg, 0.12 mmol) was dissolved in dry toluene (4 mL). Under nitrogen atmosphere, N,N-diisopropylethylamine (105 μL, 0.6 mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (110 μL, 0.5 mmol) were added. The mixture was stirred at room temperature for 7 h. Purification by column chromatography (petroleum ether/ethyl acetate = 2:1) afforded the product as a white foam as a mixture of diastereoisomers with some remaining **2** (50 mg, 46% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16-8.13 (m, 1H), 7.38-7.24 (m), 6.86-6.83 (m, 4H), 6.14 (m, 1H), 5.39-5.35 (m, 1H), 5.30-5.31 (m, 2H), 4.69 (m, 2H), 4.32 (m, 2H), 4.12 (m, 1H), 3.81-3.80 (m, 6H), 3.66 (m, 2H), 3.33-3.32 (m, 1H), 2.59-2.58 (m, 2H), 2.17-2.16 (m, 1H), 1.26 (m), 0.80 (m, 9H), 0.09-0.01 (m). ^{13}C NMR (126 MHz, $CDCl_3$) δ

161.9, 161.8, 150.5, 150.4, 144.4, 144.3, 138.7, 138.6, 135.4, 135.3, 135.2, 135.0, 130.4, 130.2, 128.4, 128.1, 127.4, 117.8, 117.4, 113.4, 101.9, 101.8, 89.3, 88.8, 87.5, 87.3, 83.36, 83.33, 83.2, 78.21, 78.18, 76.2, 76.1, 75.50, 75.46, 72.51, 72.46, 72.4, 72.3, 70.7, 63.0, 62.5, 58.9, 58.8, 57.9, 57.7, 43.5, 43.4, 43.1, 43.0, 24.9, 24.8, 24.7, 20.7, 20.6, 20.4, 20.3, 18.2, -4.60, -4.64. ^{31}P NMR (162 MHz, DMSO-d_6) δ 149.12, 148.54. HRMS-ESI: m/z calculated for $C_{48}H_{63}N_4O_9PSiNa^+$ $[M+Na]^+$: 921.39940, found: 921.39832.

Section S3: RNA sequences used in the study

The RNA oligos were synthesized following the standard protocol with an RNA solid-phase synthesizer (Suzhou Biosyntech Co., Ltd (Suzhou, China)).

Table S1. RNA sequences

Name	**Sequences**
p^3U-RNA	**5'-CAUGXUGCA-3' (X=p^3U)**
Up-RNA	**5'-CAUGYUGCA-3' (Y=Up)**

RNA reaction

p^3U -RNA

The modified RNA strand (5 OD) was dissolved in DEPC-treated water, and its concentration was determined to be 2123.6 ng/μL using a NanoDrop spectrophotometer. Stock solutions were prepared as follows: $CuSO_4$, NaACS and THPTA (0.5 mmol each) were dissolved in 1 mL of DEPC-treated water; 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide (0.5 mmol) was dissolved in 1 mL of DMSO; MopS and NaCl (0.5 mmol each) were dissolved in 1 mL of DEPC-treated water. To 2 μL of the RNA solution were added 2 μL of $CuSO_4$ solution, 2 μL of THPTA solution, 10 μL of MopS solution, 10 μL of NaCl solution, followed by 2 μL of NaACS solution and 2 μL of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide solution. Subsequently, 24 μL of DEPC-treated water and 48 μL of DMSO were added to afford a total reaction volume of 100 μL. The reaction mixture was vortexed and incubated at 37 °C for 12 h. After the reaction, ethanol precipitation was carried out. The product was analyzed by HPLC.

Up-RNA

The modified RNA strand (2 OD) was dissolved in DEPC-treated water, and its concentration was determined to be 1727.2 ng/μL using a NanoDrop spectrophotometer. Stock solutions were prepared as follows: $CuSO_4$, NaACS, and THPTA solutions (0.5 mmol each) were dissolved in

1 mL of DEPC-treated water; 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide (0.5 mmol) was dissolved in 1 mL of DMSO; Mops and NaCl (0.5 mmol each) were dissolved in 1 mL of DEPC-treated water. To 2 μL of the RNA solution were added 2 μL of $CuSO_4$ solution, 2 μL of THPTA solution, 10 μL of Mops solution, 10 μL of NaCl solution, followed by 2 μL of NaACS solution and 2 μL of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide solution. Then 24 μL of DEPC-treated water and 48 μL of DMSO were added to give a total reaction volume of 100 μL. The reaction mixture was vortexed and incubated at 37 °C for 12 h. After completion of the reaction, ethanol precipitation was performed. The product was analyzed by HPLC.

Ethanol precipitation

To a centrifuge tube was added 360 μL of anhydrous ethanol. The tube was stored at -80 °C in a refrigerator for 5 h, then centrifuged at 15000 rpm and 4 °C for 30 min. The supernatant was discarded, and the pellet was washed twice with 500 μL of 75% ethanol, followed by centrifugation at 15000 rpm for 5 min each time. The pellet was air dried at room temperature for 5 min, then dissolved in 30 μL of DEPC treated water. The RNA concentration was determined using a NanoDrop spectrophotometer.

HPLC analysis

The HPLC analysis of the reaction mixture was performed using a Waters acquity H-Class system equipped with an Ultimate AQ-C18 analytical column (5 μm, 250 × 4.6 mm). The mobile phase consisted of TEAA buffer (triethylammonium acetate, 100 mM, pH 7.2) and acetonitrile. The gradient elution program was as follows: a reduction from 93% TEAA buffer to 85% for 15 minutes, followed by a gradual increase to 93% TEAA buffer over 16 to 20 minutes, maintained at this concentration for 5 minutes, with a total duration of 25 minutes. The detection wavelength was set at 260 nm. For analysis, the reaction mixture was diluted with 30 μL of DEPC-treated water in a capped HPLC bottle, and 15 μL of the diluted solution was injected.

Enzymatic digestion of the RNA strand

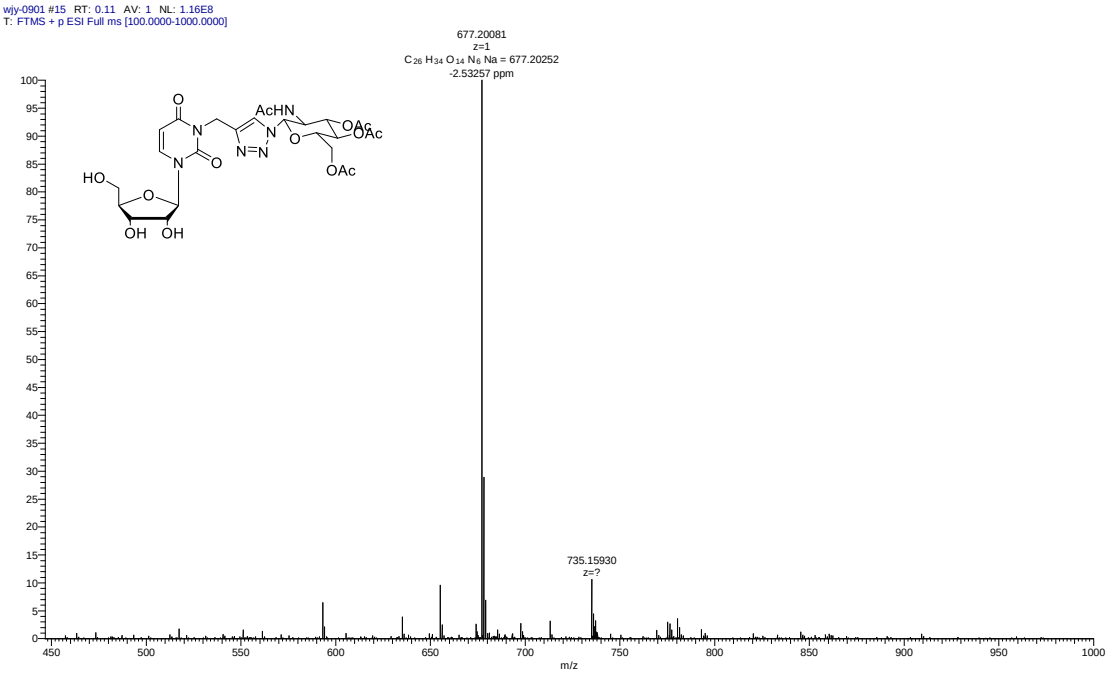
An aliquot of the product RNA strand solution was digested by adding 0.6 μL of CIP enzyme solution (1 U/μL, NEB M0290L), 0.6 μL of 0.3 M sodium acetate solution, and 0.6 μL of Nuclease P1 solution (0.1 U/μL, Sigma N8630-1VL). The mixture was vortexed and incubated at 37 °C overnight. The resulting mixture was analyzed by HPLC.

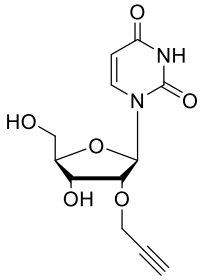
HPLC analysis of Enzymatic digestion

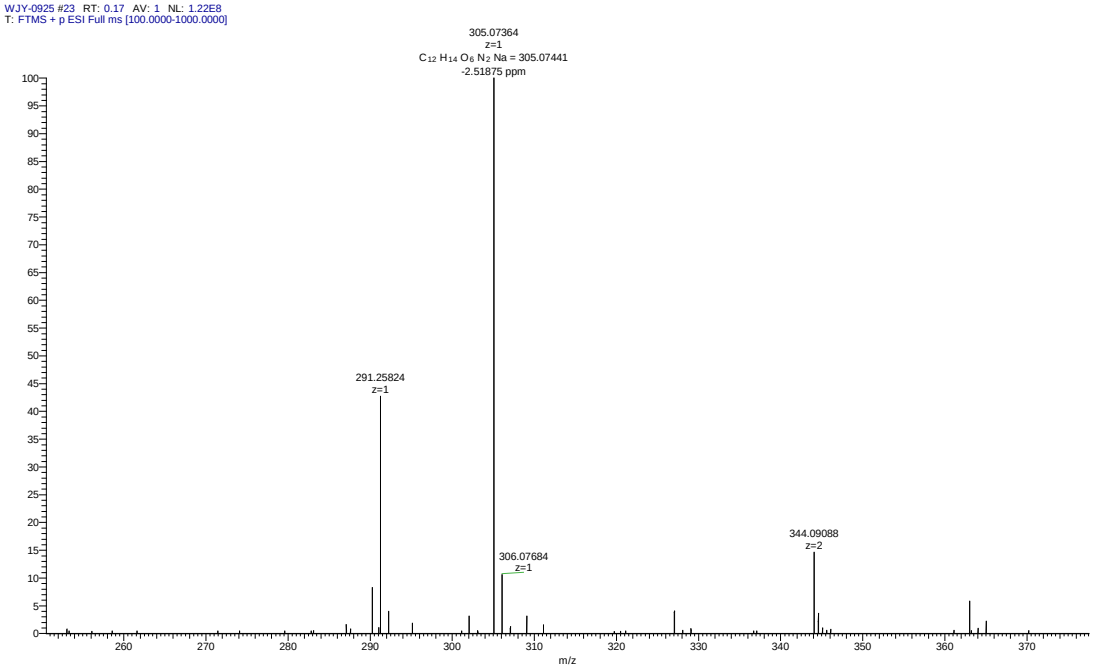
HPLC analysis of the reaction mixture was performed on a Waters ACQUITY H-Class system equipped with an Ultimate AQ-C18 analytical column (5 μm, 250 × 4.6 mm). The mobile phase

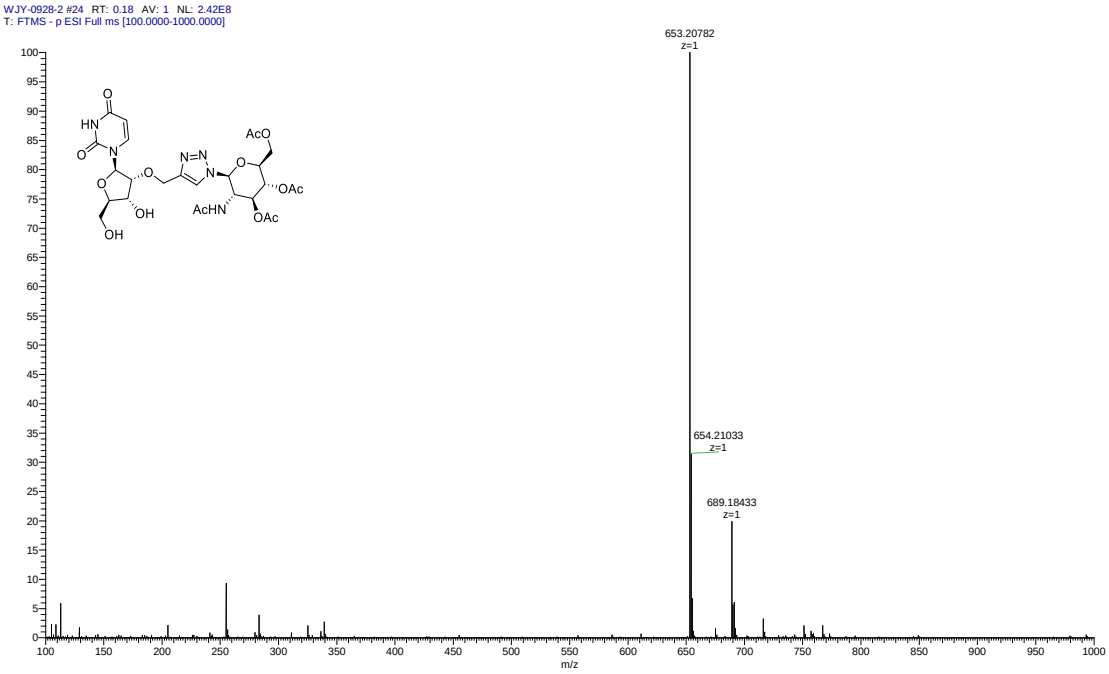
consisted of TEAA buffer (triethylammonium acetate, 100 mM, pH 7.2) and acetonitrile. The gradient elution program was set as follows: 95% TEAA buffer was maintained for the first 6 min, then linearly decreased to 0% over 9 min and held for 10 min. Subsequently, the proportion of TEAA buffer was gradually increased back to 95% from 31 to 35 min and maintained for 3 min. The total run time was 38 min. The detection wavelength was 260 nm. For analysis, the reaction mixture was diluted with 30 μL of DEPC-treated water in an HPLC vial with an insert, and 25 μL of the resulting solution was injected.

Section S4: Mass Spectra





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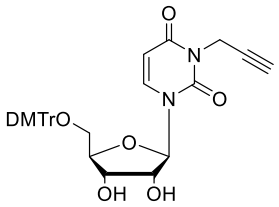
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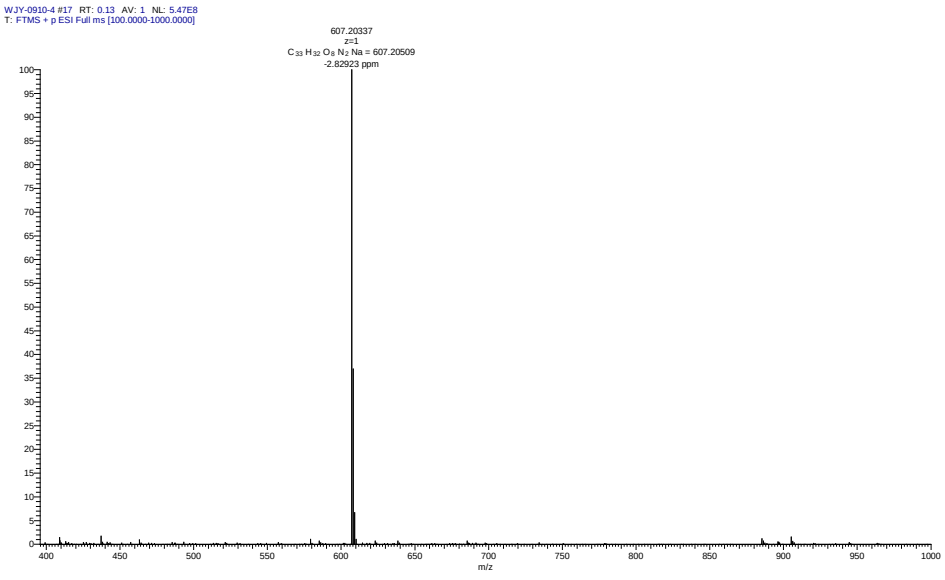
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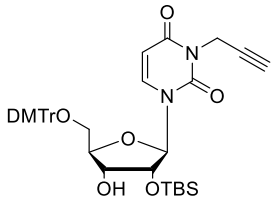
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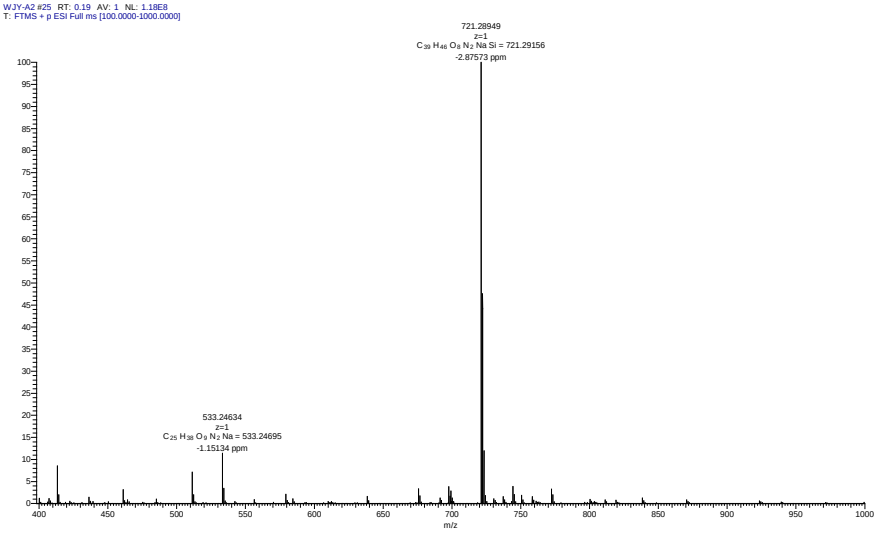
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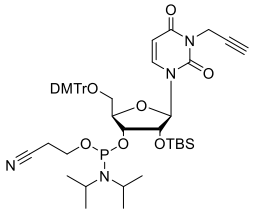
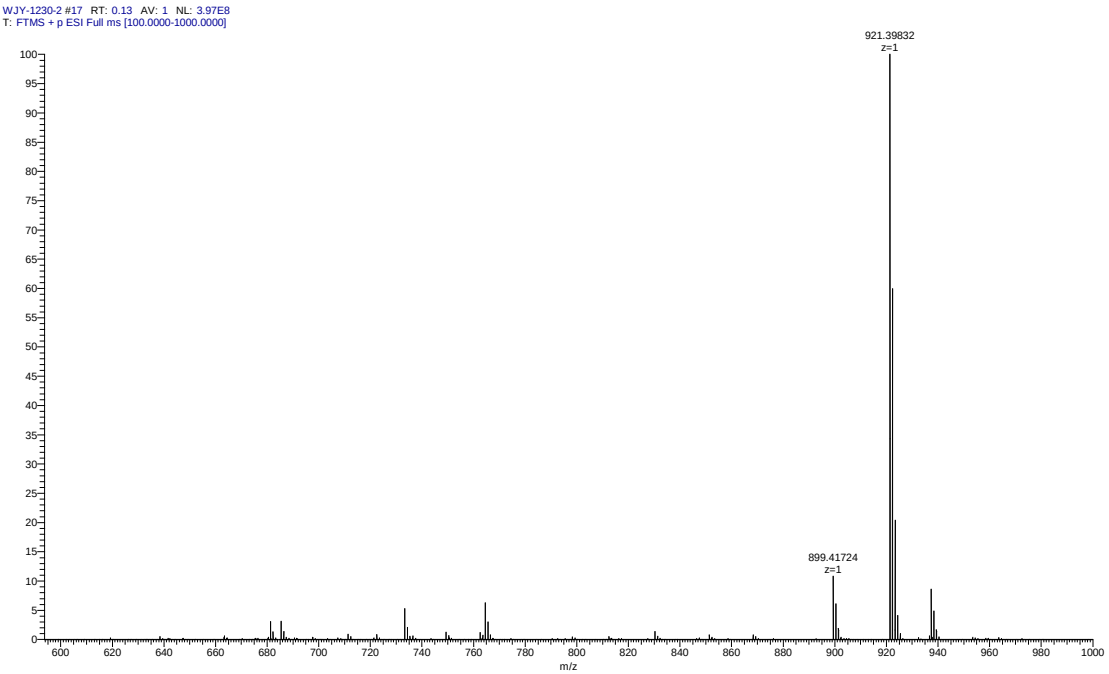
m/z


O
N
N
O
DMTrO
O
O
P
O
OTBS
N
N


WJY-1230-2 #17 RT: 0.13 AV: 1 NL: 3.97E8
T: FTMS + p ESI Full ms [100.0000-1000.0000]
921.39832
z=1
899.41724
z=1
m/z

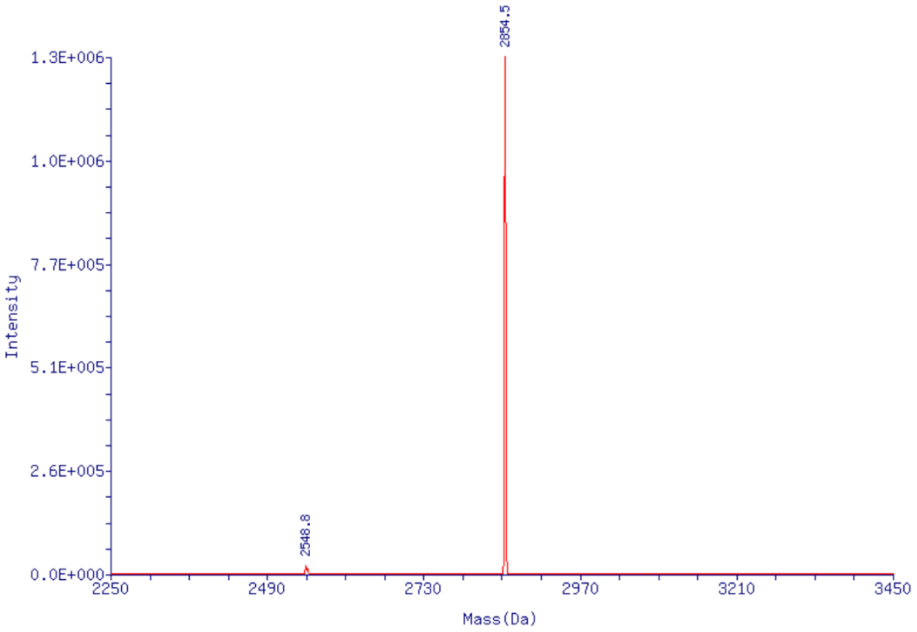


Figure S1: Mass spectrum of 5'-CAUG(p^3U)UGCA-3'

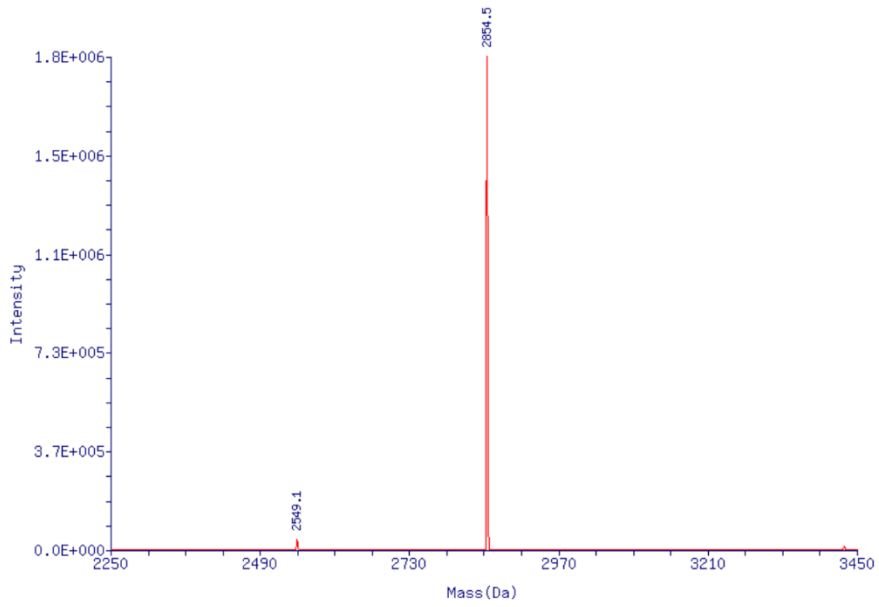


Figure S2: Mass spectrum of 5'-CAUGY(Up)UGCA-3'

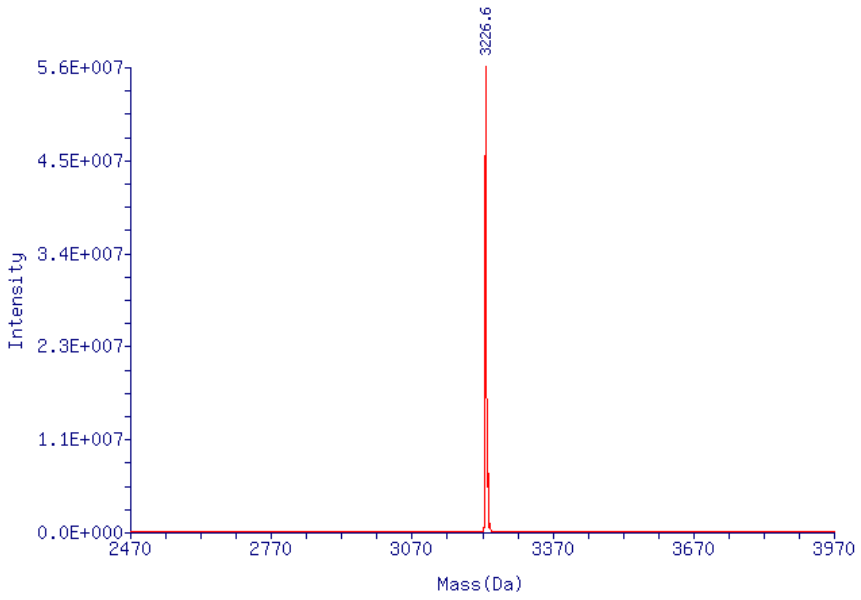


Figure S3: Mass spectrum of 5'-CAUG(p^3U-Glu)UGCA-3'

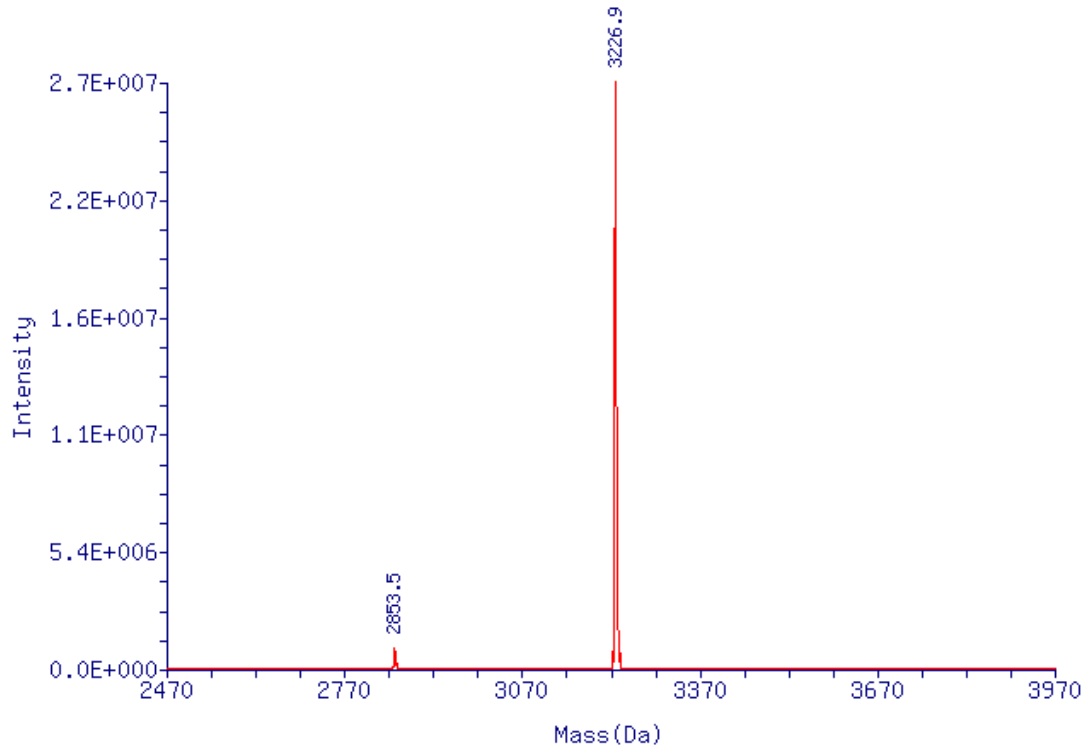
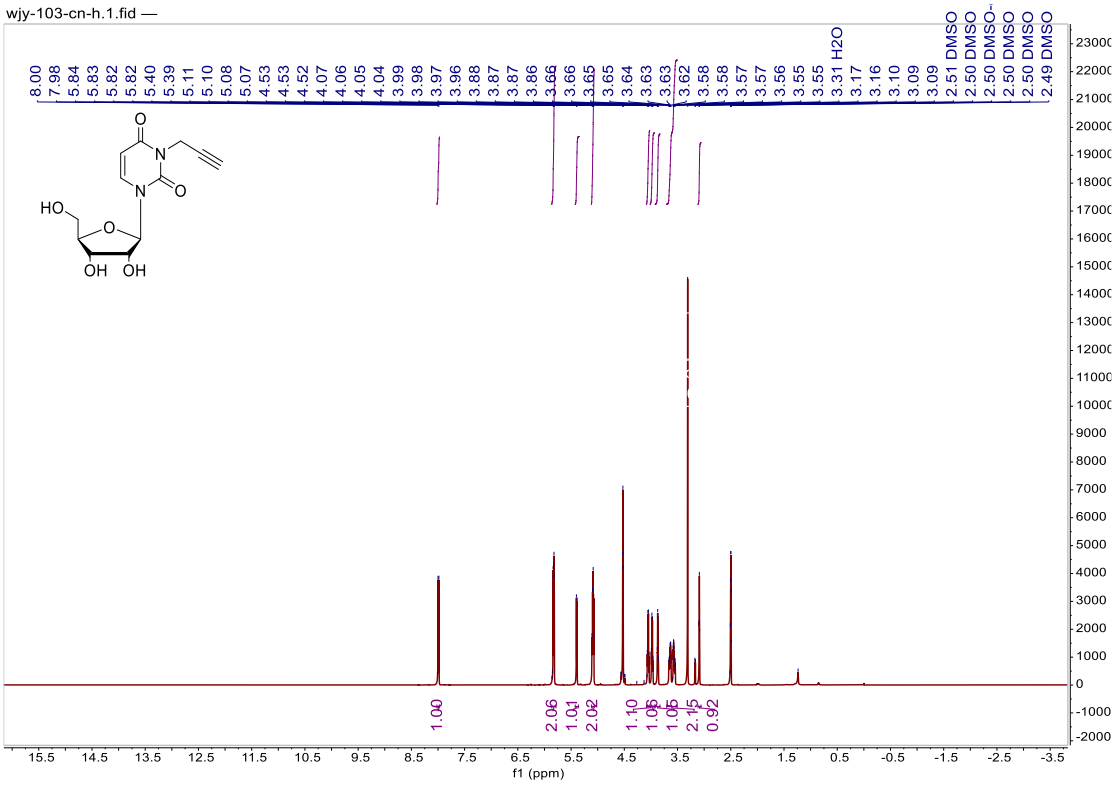
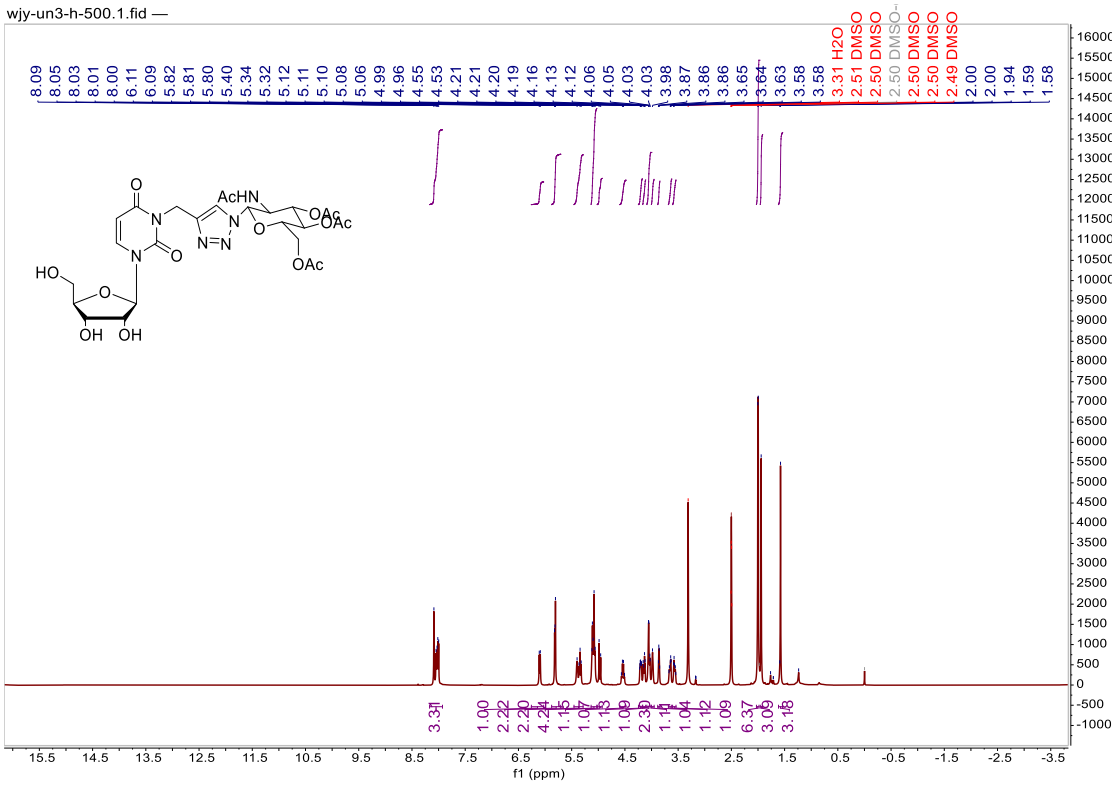
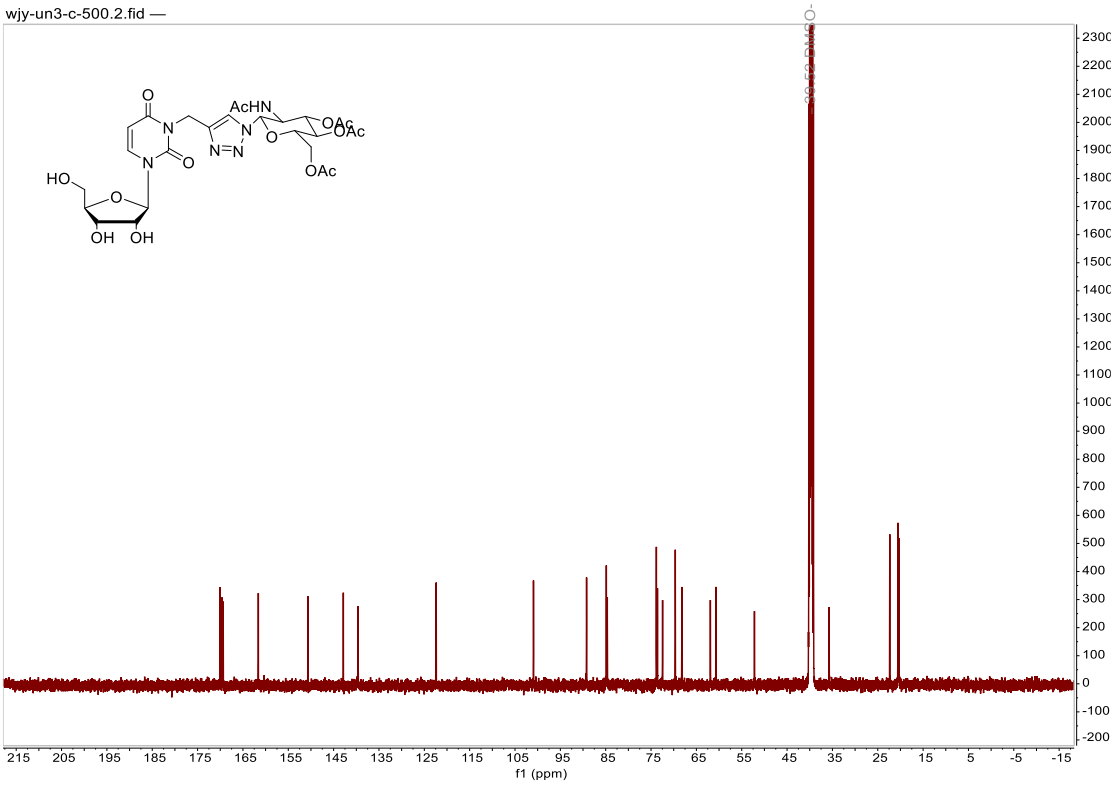


Figure S4: Mass spectrum of 5'-CAUG(Up-Glu)UGCA-3'

Section S5: NMR spectra



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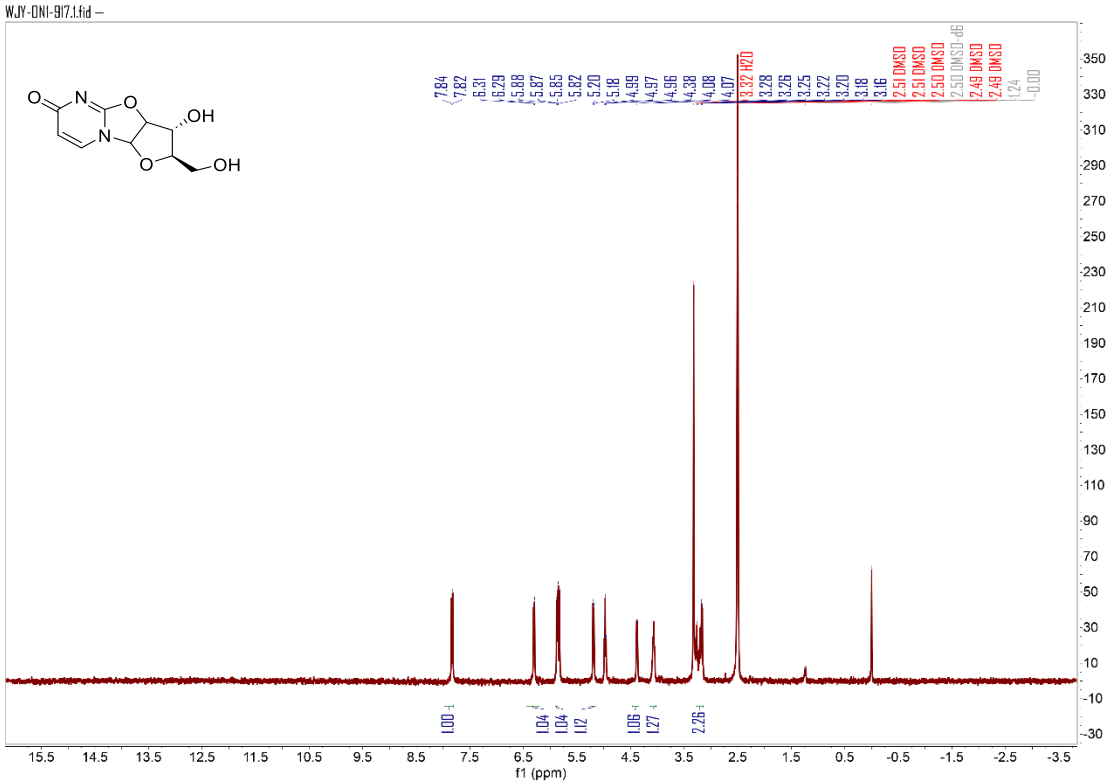
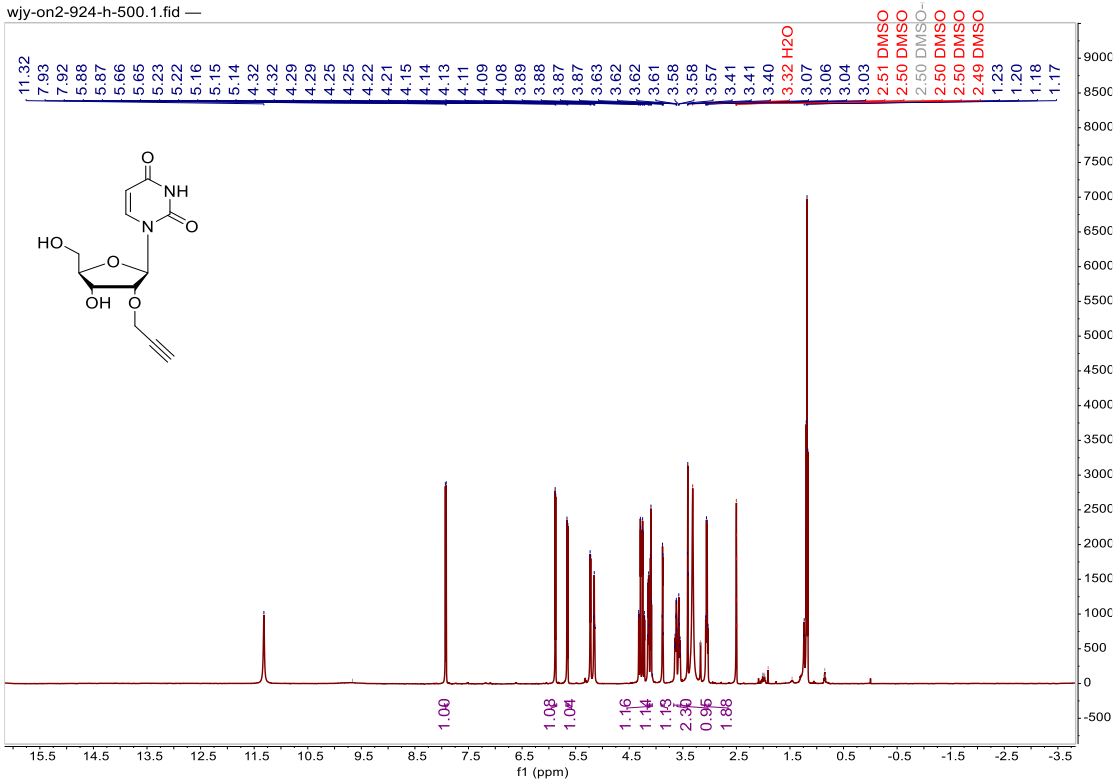
OAc
OAc
OAc
HO
OH
OH
f1 (ppm)


WJY-DNI-917.1.fid
OH
OH
7.84
7.82
6.31
6.29
5.88
5.87
5.85
5.82
5.20
5.18
4.99
4.97
4.96
4.38
4.08
4.07
3.32 H2O
3.28
3.26
3.25
3.22
3.20
3.18
3.16
2.51 DMSO
2.50 DMSO
2.49 DMSO
1.24
0.00
1.00
1.04
1.04
1.12
1.06
1.27
2.26
f1 (ppm)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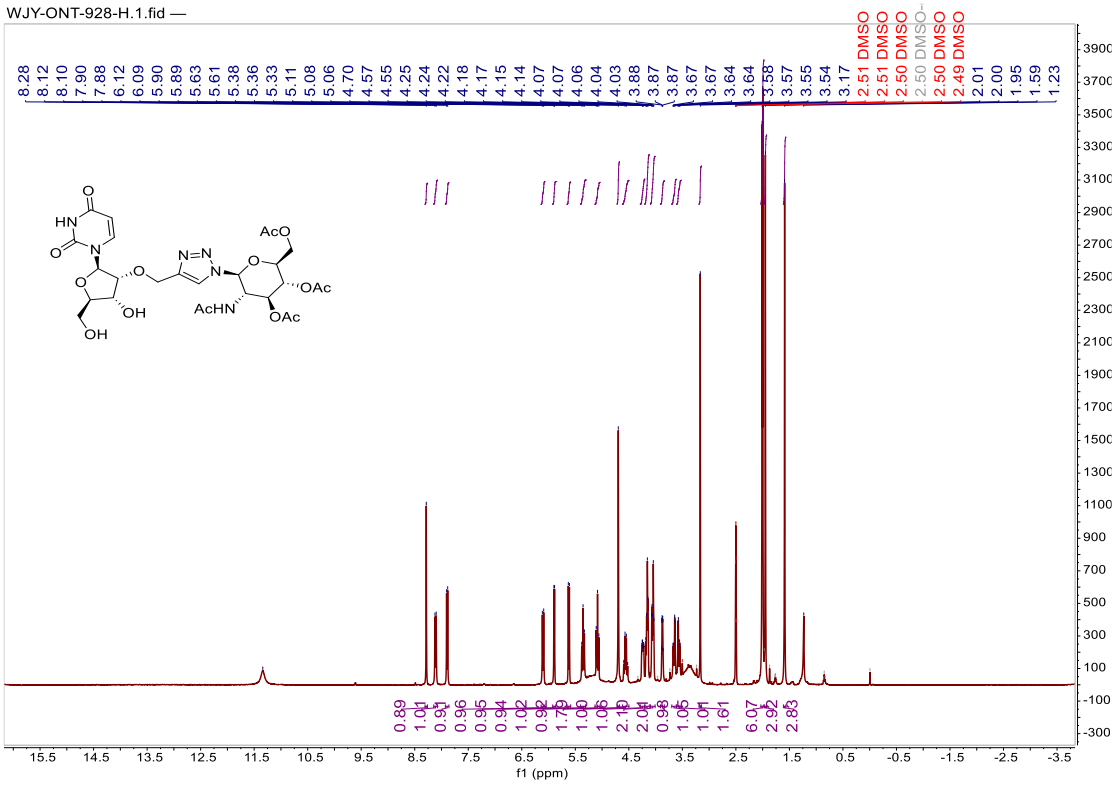
f1 (ppm)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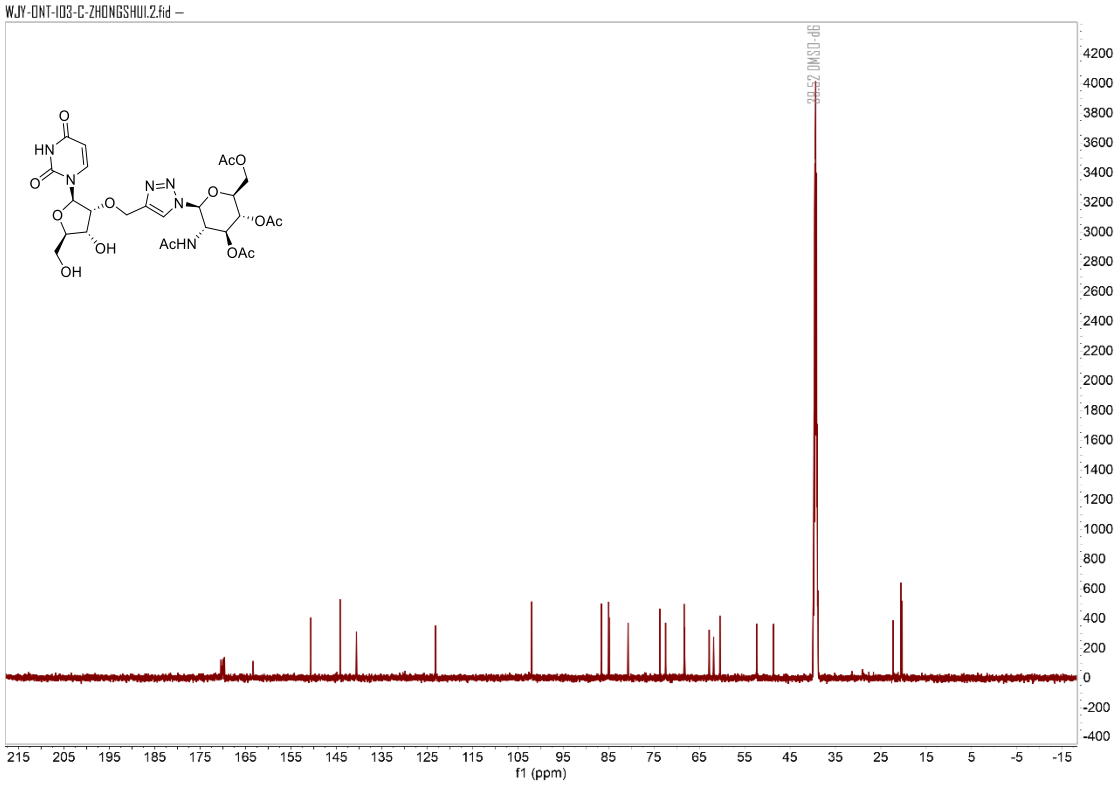
f1 (ppm)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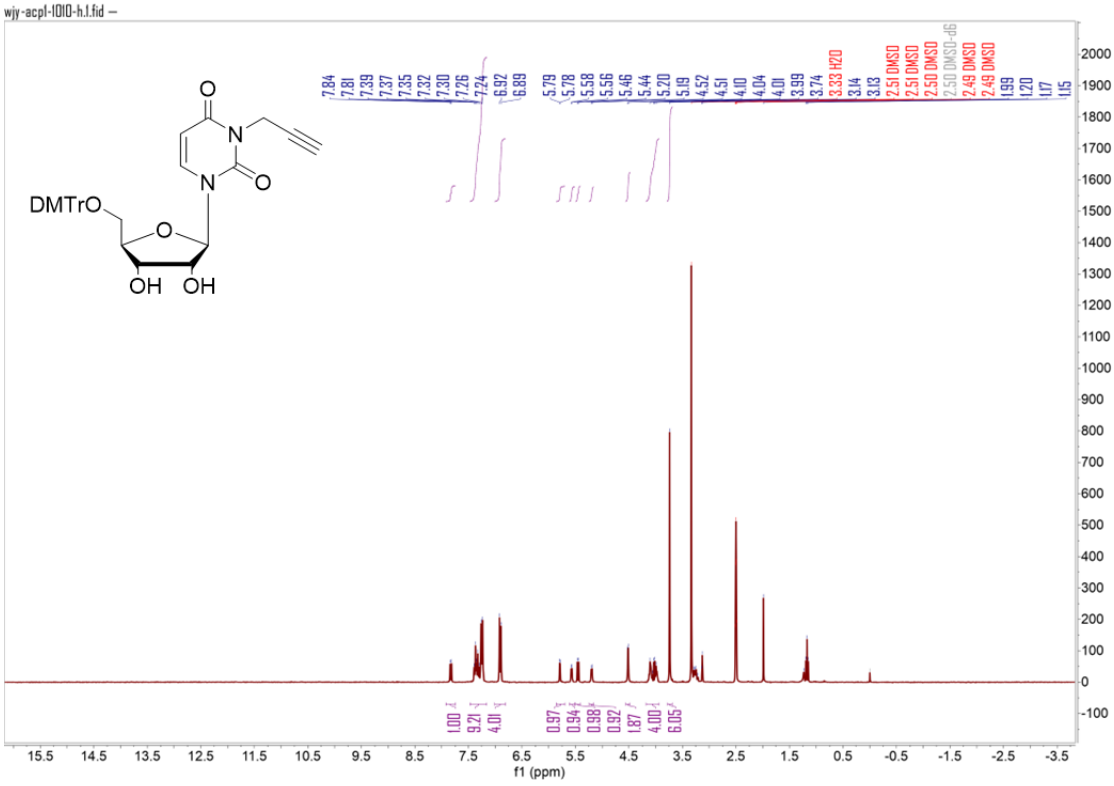
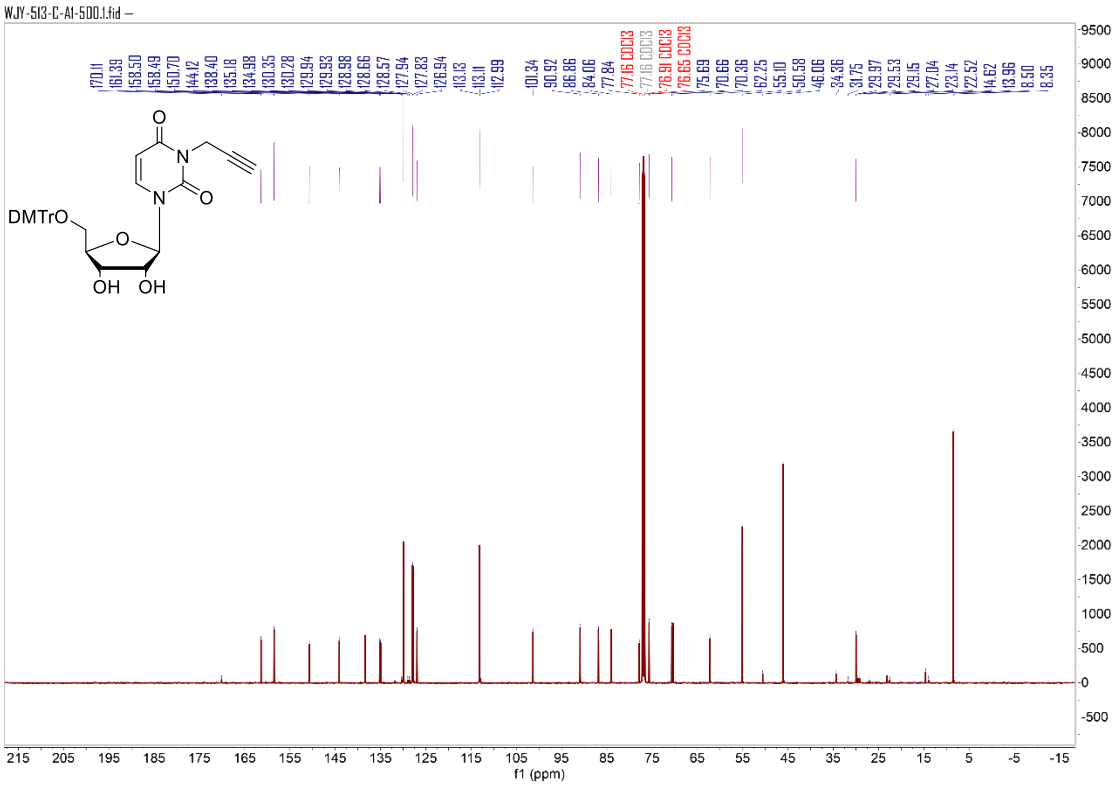
39.52 DMSO-d6
f1 (ppm)


wjy-acpl-1010-h.1.fid
DMTrO
OH
OH
3.33 H2O
2.51 DMSO
2.50 DMSO-d6
2.49 DMSO
f1 (ppm)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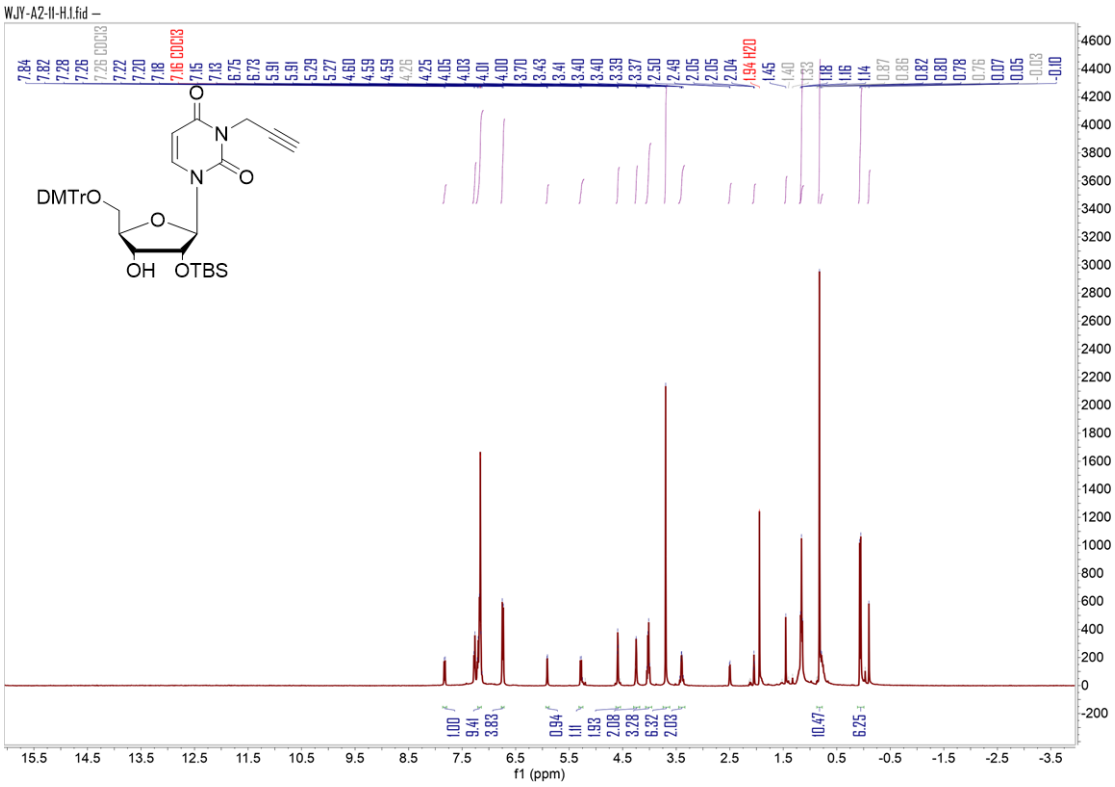
DMTrO
O
N
N
O
O
OH
OH
f1 (ppm)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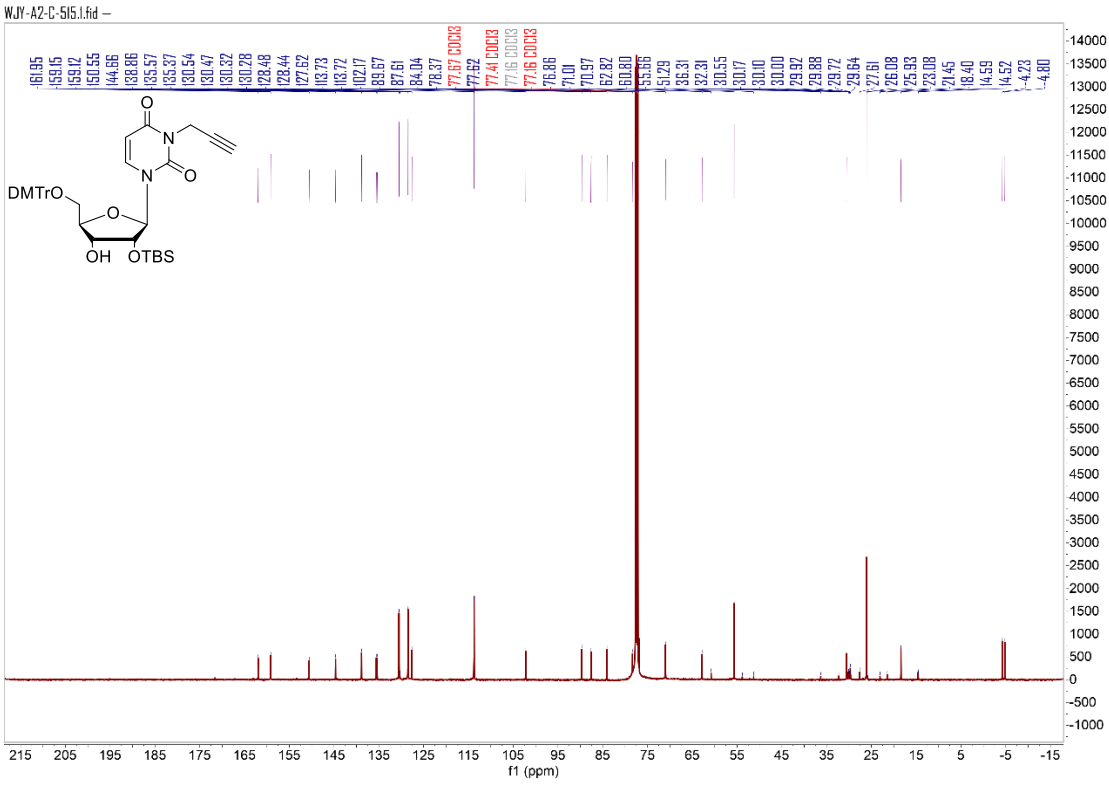
DMTrO
OH
OTBS
f1 (ppm)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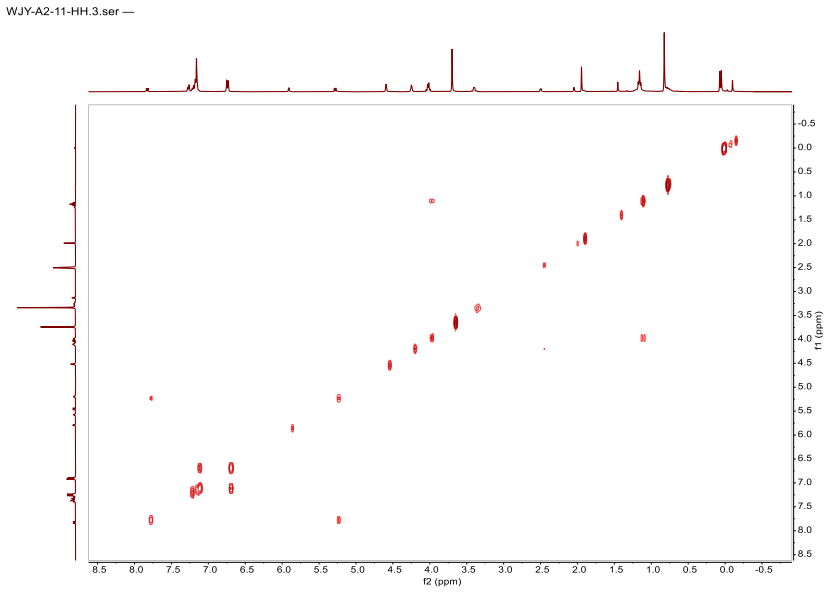
DMTrO
OH
OTBS
f1 (ppm)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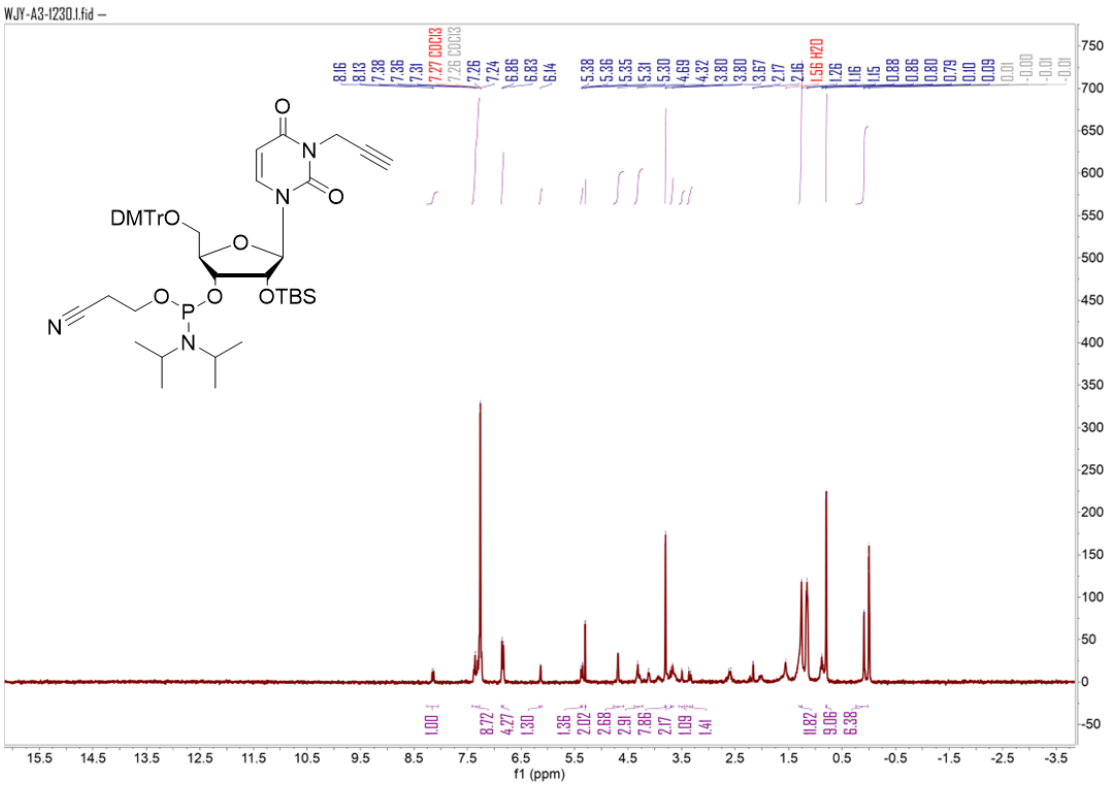
f2 (ppm)
f1 (ppm)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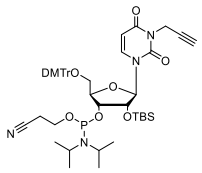
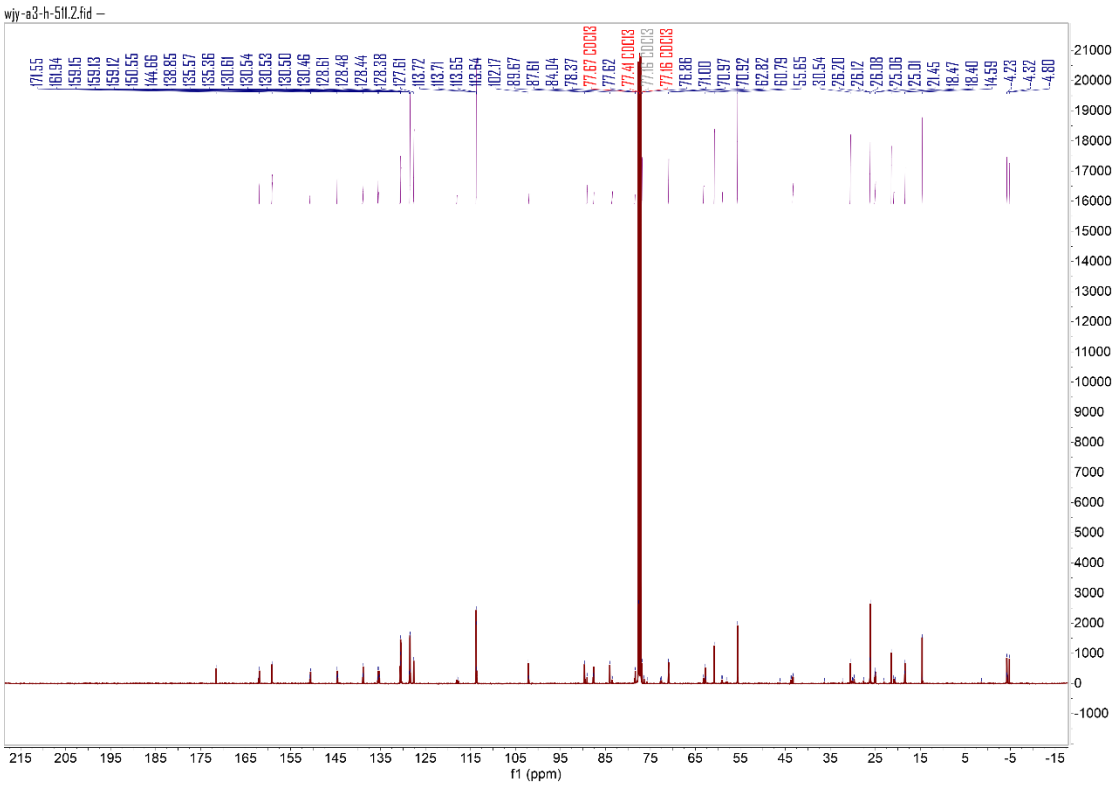
DMTrO
OTBS
N
f1 (ppm)


DMTrO
OTBS
N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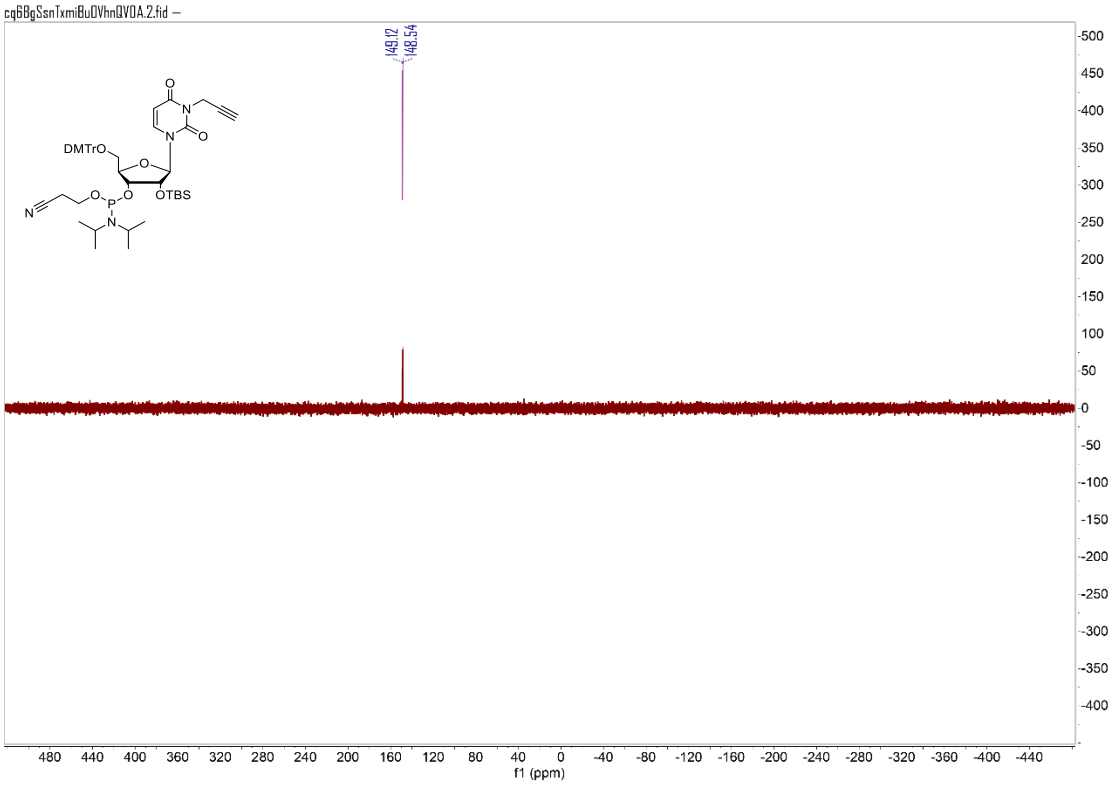
161.94
158.15
158.13
158.12
150.55
144.66
138.85
135.57
135.36
130.61
130.54
130.53
130.50
130.46
128.61
128.48
128.44
128.38
127.61
113.72
113.71
113.65
102.17
89.67
87.61
84.04
78.37
77.87 CDCl3
77.62
77.41 CDCl3
77.16 CDCl3
76.88
71.00
70.97
70.92
62.82
60.79
55.65
31.54
26.20
26.12
26.08
25.06
25.01
21.45
18.47
18.40
14.58
-4.23
-4.32
-4.80
f1 (ppm)


cqGBgSsnTxmiBuDVhnQVDA.2.fid
148.12
148.54
DMTrO
OTBS
N
f1 (ppm)

References

[1] Li-Jun Xie, Cui-Lian Lin, Li Liu, Liang Cheng,A chemical labeling of N6-formyl adenosine (f6A) RNA,*Chinese Chemical Letters,Volume 33, Issue 3,***2022***,Pages 1563-1566.*

[2] Szlenkier, Maurycy; Kamel, Karol; Boryski, Jerzy ,Regioselective Mitsunobu Reaction of Partially Protected Uridine.*Nucleosides, Nucleotides & Nucleic Acids* (**2016**)*, 35(8), 410-425*

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