Supporting Information

Squaryl group-modified UDP analogs as inhibitors of the ER resident folding sensor enzyme UGGT

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Contents

(4) Conditions for transformation of 5’-OH of **S3**

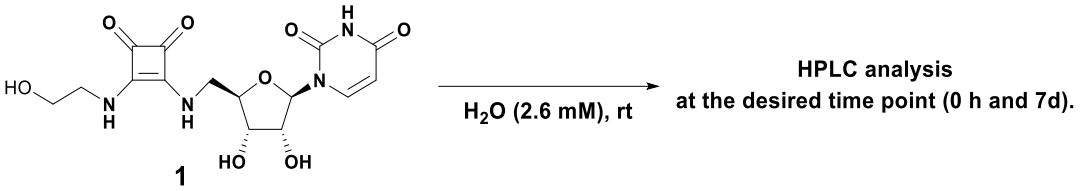
Biological experiments

1. General and abbreviation

Air and/or moisture sensitive reactions were carried out under argon atmosphere with dried solvents. Reactions were monitored by thin-layer chromatography (TLC) (TLC Silica gel 60 $F_{254,}$ Merk). Compounds were detected by UV light (254 nm) and/or Hanessian's stainand or 5% H_2SO_4 / MeOH and purified by column chromatography (Silica gel 60, 0.040-0.063 mm, 230-400 mesh, Merk). Solvent was removed lower than 40 ºC under reduced pressure. Synthesized compounds were analyzed by ^{1}H NMR and ^{13}C NMR (Bruker Avance 400MHz spectrometer). All NMR signals were assigned by COSY, HSQC and HMBC. ^{1}H NMR analyses were conducted with $CDCl_3$ or CD_3OD referenced to $CHCl_3$ at 7.26 ppm or to CHD_2OD at 3.31 ppm, respectively. ^{13}C NMR analyses were referenced to $CDCl_3$ at 77.0 ppm or CD_3OD at 49.00. Synthesized compounds were assigned by standard 2D experiments. High-resolution mass spectrometry determinations were performed with Thermo Fisher Scientific Orbitrap XL. All reagents were purchased from Kanto Chemicals Co. Inc., Tokyo Chemical Industries Co., Ltd., FUJIFILM Wako Pure Chemical Co., Aldrich Chemical Co. An accurate balance (Sartorius, QUINTIX125D-1SJP) that can be applicable for >50 μg scales was used to quantify materials. RP-UHPLC analyses were performed on a Dionex UltiMate 3000 UHPLC system equipped with a multi wavelength detector. RP-column (Proteonavi, C4, 4.6 × 250 nm, OSAKA SODA Co., Ltd., Osaka, Japan) was used for analytical HPLC using 0.1% aq. TFA and 0.1% TFA in MeCN.

DIPEA (N, N-diisoproylethylamine), DIAD (diisopropylazodicarboxylate), THF (tetrahydrofuran), DMF (N, N-dimethylformamide), TFA (trifluoroacetic acid), DPPA (diphenylphosphoryl azide), MeOH (methanol), EtOH (ethanol), MeCN (acetonitrile), $CHCl_3$ (chloroform), $NaHCO_3$ (sodium bicarbonate), Pd/C (palladium on carbon).

2. Stability of SQ-UDP analog (1)



To a 5 mL vial containing SQ-UDP analog (**1**) (0.5 mg, 0.00131 mmol) was added dist. H_2O (0.5 mL). The vial was left at ambient temperature until the desired time point (0h, 7d). At the desired time point, the mixture was analyzed by analytical HPLC of which conditions was shown below.

Analytical HPLC conditions

Analytical HPLC was performed by the HPLC systems (a DIONEX UltiMate 3000 UHPLC system) equipped with a variable wavelength detector. Analytical HPLC was carried out using a C4 reversed-phase column (4.6 × 250 mm; proteonavi (C4); OSAKA SODA Co., Ltd., Osaka, Japan) and an isocratic of 99 / 1 = 0.1% aq. TFA / 0.1%TFA in MeCN for 10 min, followed by a linear gradient of 1−100% of 0.1%TFA in MeCN in 0.1% aq TFA over 40 min at ambient temperature. The flow rate was 1 mL/min. The compound was detected at 218, 254 and 278 nm.

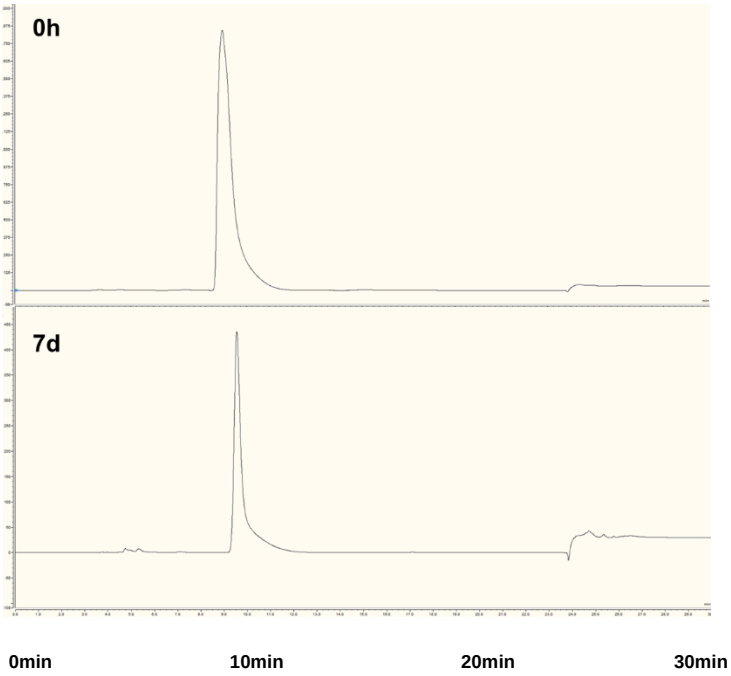


Fig. S1 HPLC profile of SQ-UDP analog (**1**) at the desired time point (0 h and 7 d).

3. Glc transfer reaction by UGGT under various concentration of M9-Asn-BODIPY

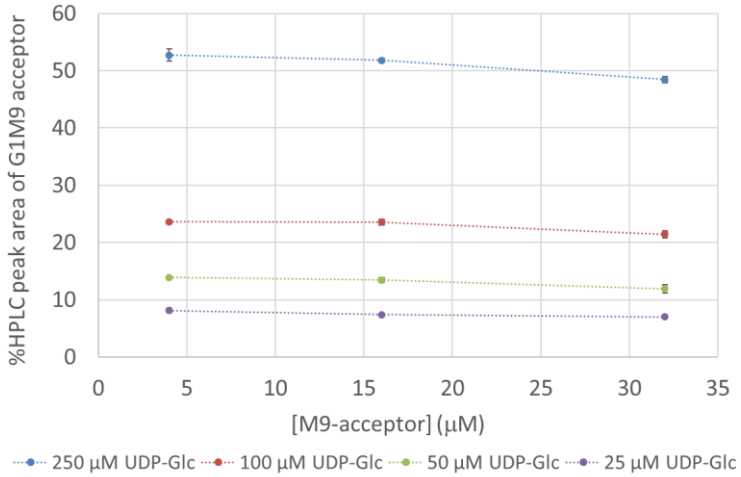


Fig. S2 Glc transfer reaction of UGGT under various concentration of M9-Asn-BODIPY. Assay conditions: 0.07 mg/mL UGGT1 (expressed in Sf9 cells), 4 mM Tris-HCl (pH 7.6), 0.05% TritonX-100, 1 mM 2-mercaptoethanol, 2 mM $CaCl_2$, UDP-Glc (25, 50, 100 or 250 μM), M9-Asn-BODIPY (M9-acceptor) (4, 16 or 32 μM).

4. Kinetic analysis of Glc transfer reaction by UGGT

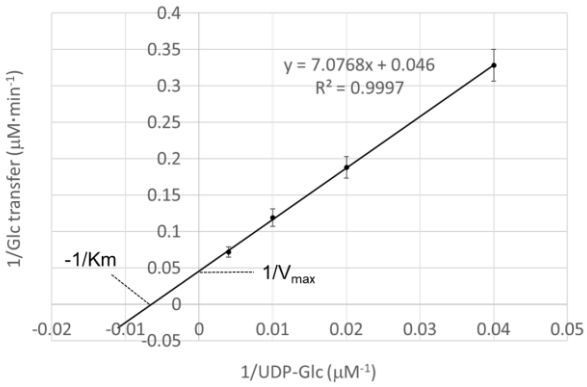
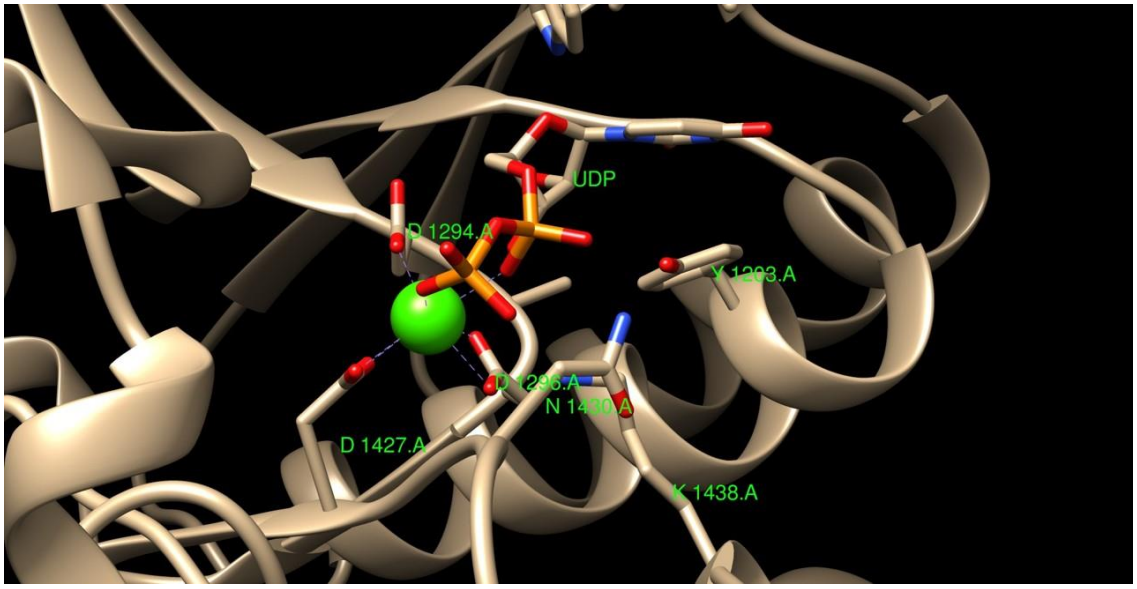


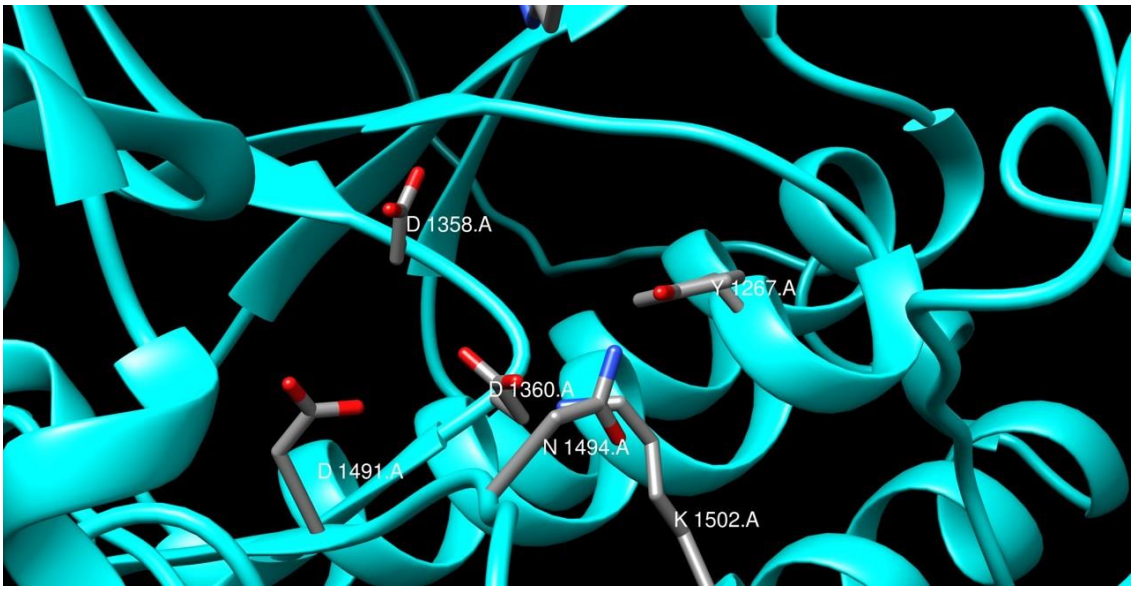
Fig. S3 Lineweaver-Burk plot of UDP-Glc. K_m = 154 [μM], V_{max} = 21.7 [μM/min]. The Km value was higher than the reported one derived from other UGGT species[1].

5. The similarity of the structure between Td-UGGT and HUGT1

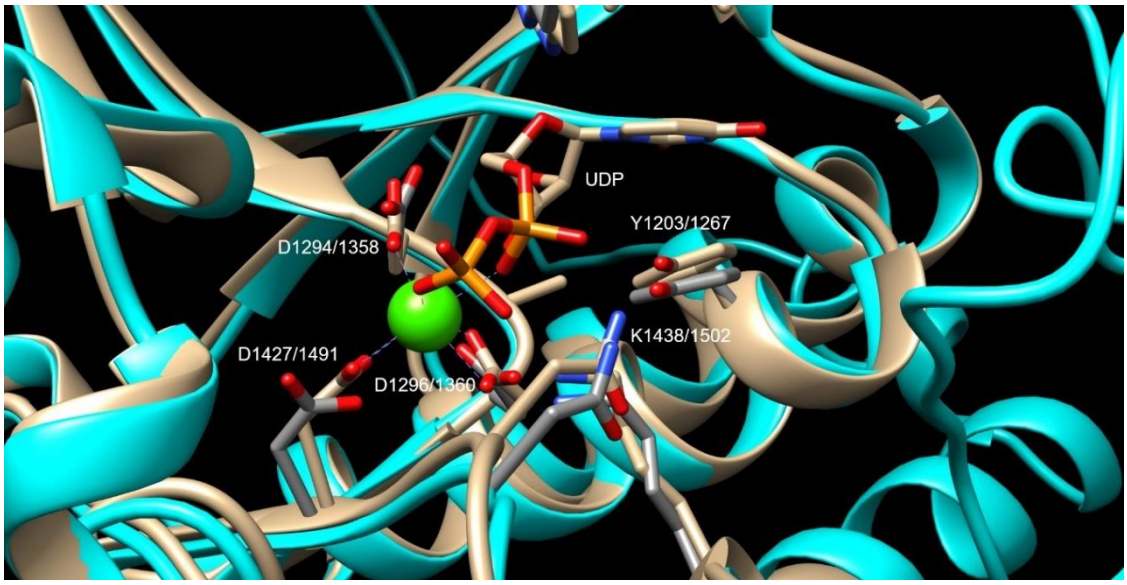
(A) Td-UGGT complexed with UDP (Satoh et al., Sci Rep 7: 12142, 2017; PDB:5Y7F)



(B) HUGT1 (Acquired from AlphaFold DB: AF-Q9NYU2)



(C) Aligned (Td-UGGT + HUGT1)



(D) The conserved key amino acids responsible for UDP binding in Td-UGGT and HUGT1

UGGT	AA1	AA2	Distance (Å)
Td UGGT (+UDP)*	Y1203	D1294	9.2
		D1296	6.6
	K1438	Y1203	9.7
		D1294	13.1
UGGT1 (human)**	Y1267	D1358	9.4
		D1360	7.0
	K1502	Y1267	10.0
		D1358	13.7

***Satoh et al., Sci Rep 7: 12142 (2017) PDB:5Y7F**

****AF-Q9NYU2**

Fig. S4 The substrate binding sites of Td-UGGT and Human UGGT1 (HUGT1) (A, B, C) and distances between selected amino acids (D). Models were created by UCSF Chimera (https://www.cgl.ucsf.edu/chimera/).

6. The reported cocrystal structure of Td-UGGT and UDP

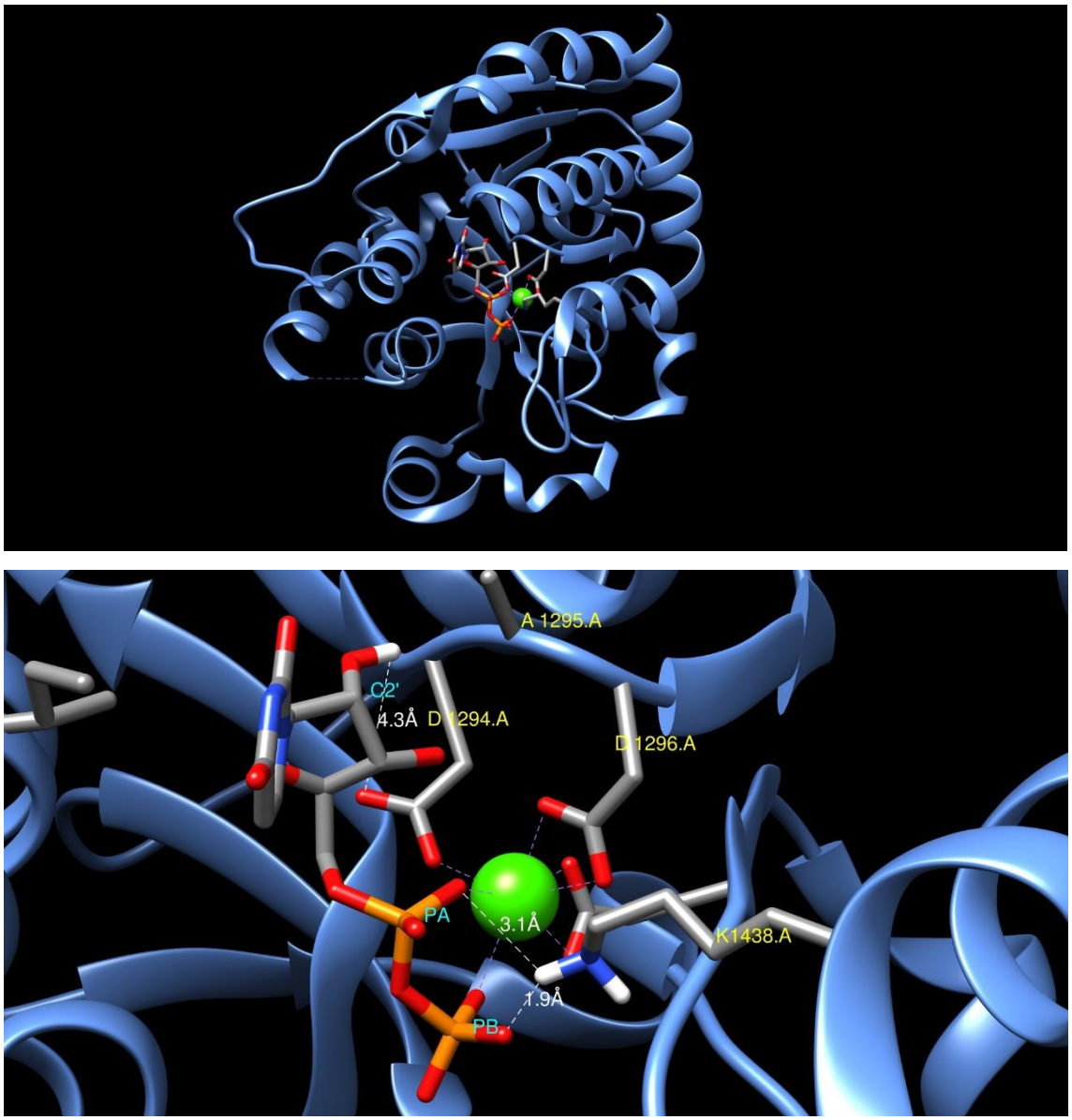


Fig. S5 The catalytic subunit (AA1188-1462) of Td-UGGT (UDP-bound form). The structure acquired from PDB (5Y7F) was visualized by UCSF Chimera (https://www.cgl.ucsf.edu/chimera/).

7. Initial velocity and Dixon plot of 3a

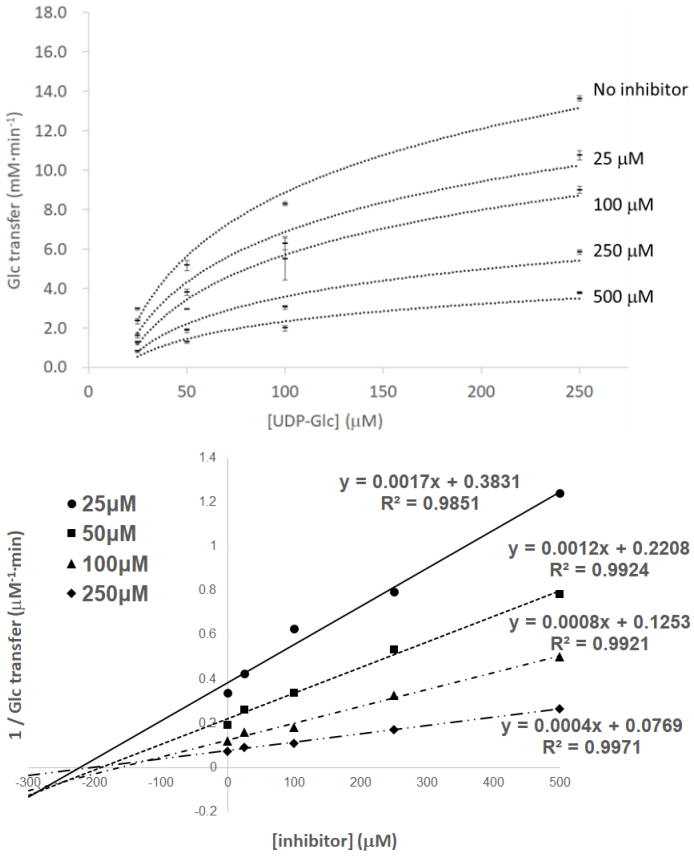
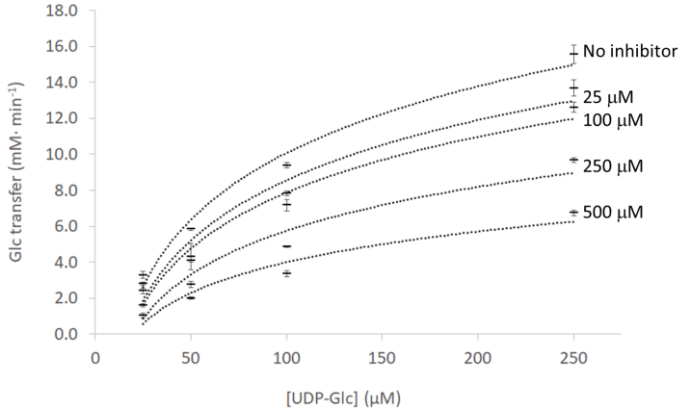


Fig. S6 Initial velocity of **3a** (upper), dixon plot of **3a** (lower)

8. Initial velocity and Dixon plot of UDP



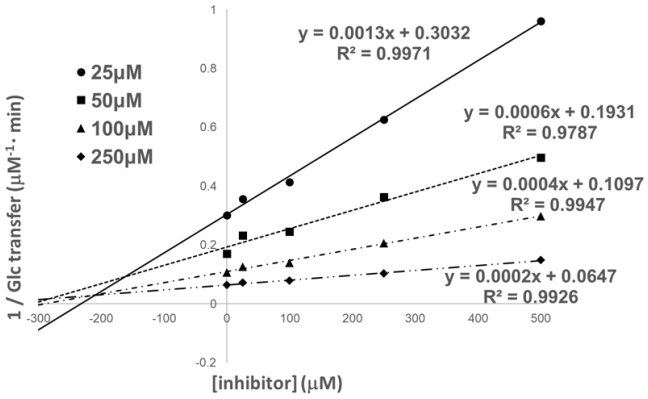


Fig. S7 Initial velocity of UDP (upper), dixon plot of UDP (lower)

9. Different binding mode of UDP compared to UDP-Glc

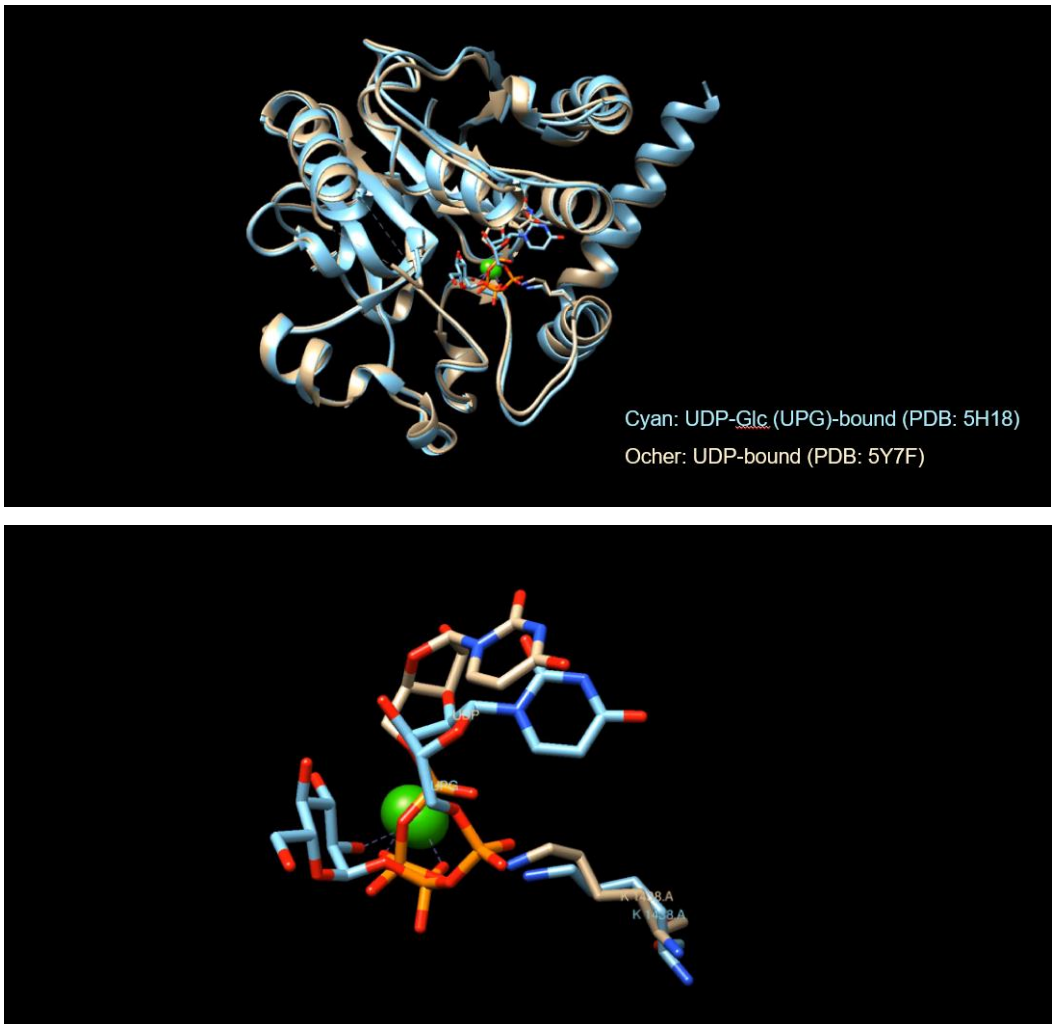


Fig. S8 Crystal structures of Td-UGGT's catalytic domain complexed with UDP/UDP-Glc (upper) and the structure of UDP/UDP-Glc in the binding site of Td-UGGT (Satoh et al., Sci Rep 7: 12142 (2017); PDB: 5Y7F)

10. Caluculation of IC_{50} for 3a and UDP

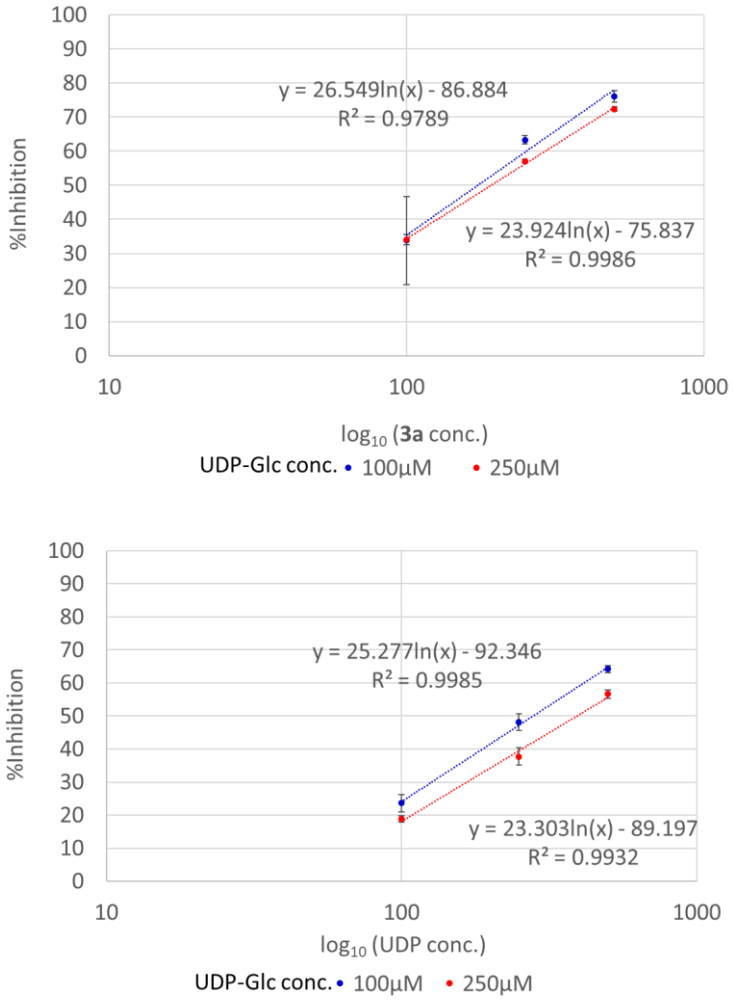
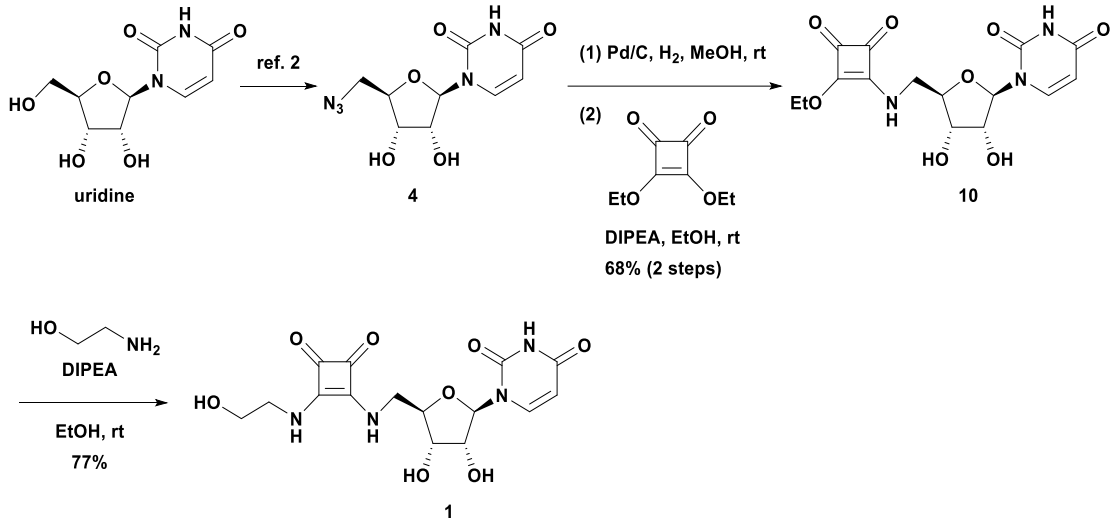


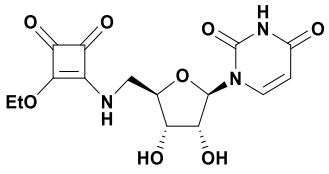
Fig. S9 Approximate formula of %inhibition by **3a** (upper) and UDP (lower). The plot at 25 μM was not considered because this point might be affected largely by the generated UDP in the Glc transfer.

11. Synthesis and characterization of SQ-UDP analogs



Scheme S1 Synthesis of SQ-UDP analog (**1**)

1-((2R,3R,4S,5R)-5-(((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)-3,4-dihydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (**10**)

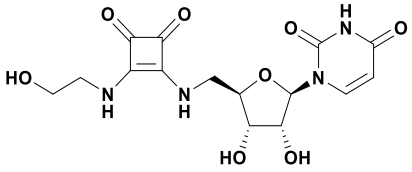


5'-azido-5'-deoxyuridine was synthesized by the reported method.[2]

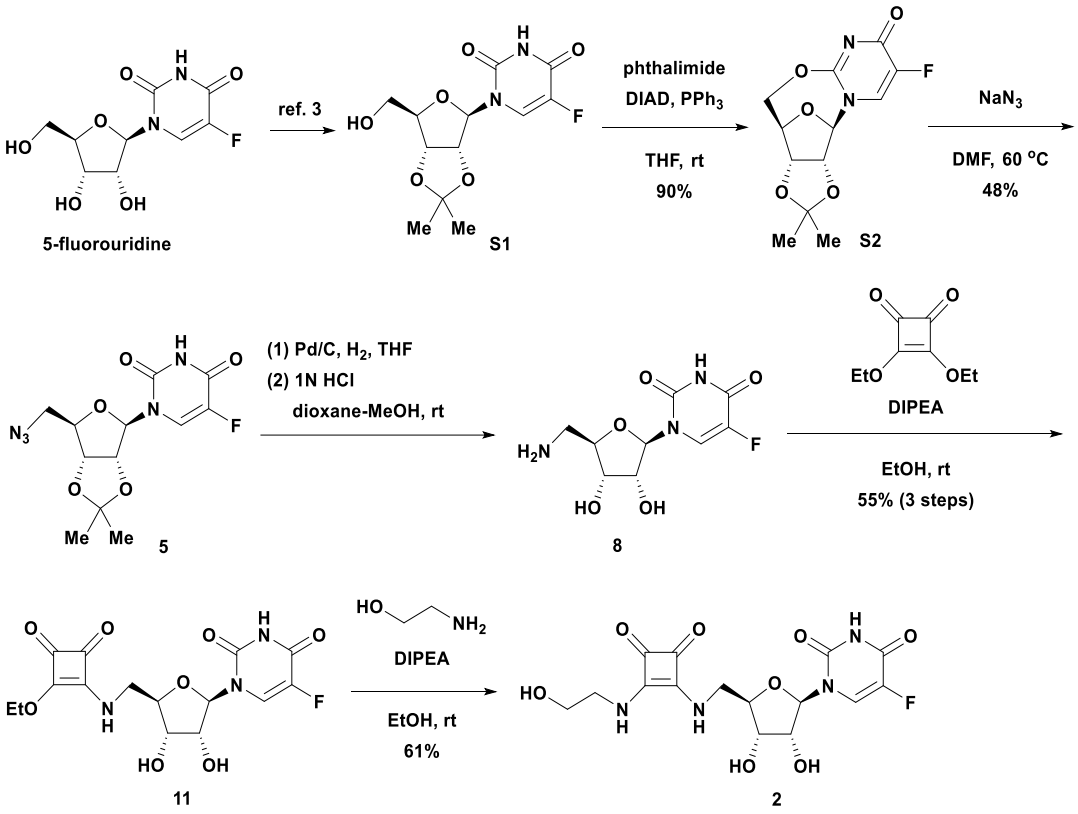
To a solution of 5'-azido-5'-deoxyuridine (**4**) (20.0 mg, 0.0742 mmol) in MeOH (1 mL) was added 10% Pd/C (2.0 mg) under argon atmosphere. The argon atmosphere of the flask was exchanged into hydrogen atmosphere. The mixture was stirred at ambient temperature for 18 h. The reaction mixture was filtered by celite filtration, followed by washing the residue with MeOH. After evaporation under reduced pressure, 5'-amino-5'-deoxyuridine (**9**) was obtained (18.0 mg, 0.0742 mmol), which was used for the next step without further purification.

To a solution of **9** (18.0 mg, 0.0742 mmol) and diethylsquarate (16.5 μL, 0.111 mmol) in EtOH (0.7 mL) was added DIPEA (12.9 μL, 0.0742 mmol). The mixture was stirred at ambient temperature for 14 h. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, $CHCl_3$ / MeOH = 10 / 1) to afford **10** (67% yield, 18.0 mg, colorless oil). $[\alpha]_D^{26}$ = 42.3 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.63 (d, *J* = 8.1 Hz, 0.5H), 7.56 (d, *J* = 8.2 Hz, 0.5H), 5.78 (d, 0.5H), 5.70 (m, 1.5H), 4.71 (q, *J* = 7.0 Hz, 2H), 4.25 (m, 1H), 4.11-3.88 (m, 3H), 3.81-3.66 (m, 1H), 1.44 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 190.16, 189.69, 185.10, 184.87, 178.53, 178.034, 175.44, 175.07, 166.06, 152.23, 152.11, 143.49, 143.06, 102.91, 93.38, 92.29, 83.99, 83.82, 74.63, 74.41, 72.10, 71.92, 70.84, 47.09, 46.98, 16.14, 16.06; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{15}H_{17}N_3O_8Na$ 390.0908 found 390.0908.

1-((2R,3R,4S,5R)-3,4-dihydroxy-5-(((2-((2-hydroxyethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (**1**)

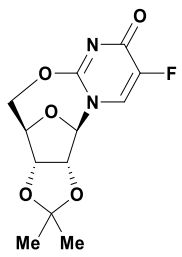


To a solution of **10** (20.0 mg, 0.0544 mmol) and 2-aminoethanol (3.3 μL, 0.0544 mmol) in EtOH (0.54 mL) was added DIPEA (9.5 μL, 0.0544 mmol). The mixture was stirred at ambient temperature for 19 h. After evaporation, the crude was purified by column chromatography (silicagel, $CHCl_3$ / MeOH = 10 / 1 to 5 / 1) to afford **1** (77% yield, 16 mg, white solid). $[\alpha]_D^{26}$ = 39.1 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.64 (d, *J* = 8.0 Hz, 1H), 5.76 (d, *J* = 4.5 Hz, 1H), 5.71 (d, *J* = 8.0 Hz, 1H), 4.28 (dd, *J* = 5.0, 4.5 Hz, 1H), 4.10-3.93 (m, 4H), 3.69 (br s, 4H); ^{13}C NMR (CD_3OD, 100 MHz): δ 184.08, 183.89, 169.61, 166.04, 152.28, 143.47, 103.01, 92.85, 84.29, 74.43, 71.96, 62.59, 47.51, 46.80; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{15}H_{18}N_4O_8Na$ 405.1017, found 405.1016.



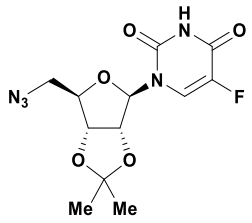
Scheme S2 Synthesis of SQ-UDP analog (**2**)

(3aR,4R,12R,12aR)-9-fluoro-2,2-dimethyl-3a,4,12,12a-tetrahydro-5H,8H-4,12-epoxy[1,3]dioxolo[4,5-e]pyrimido[2,1-b][1,3]oxazocin-8-one (**S2**)



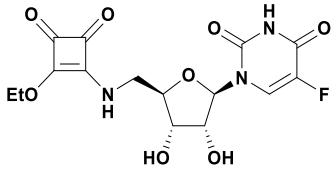
S1 was synthesized by the reported method.[3] To a flask containing **S1** (90.0 mg, 0.298 mmol), phthalimide (65.7 mg, 0.447 mmol), triphenylphosphine (117 mg, 0.447 mmol) under argon atmosphere was added THF (2.9 mL), followed by addition of DIAD (1.9 M in toluene, 235 μL, 0.447 mmol). The mixture was stirred at ambient temperature for 12h. The mixture was stirred under reflux for 2h. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, hexane / ethylacetate = 1 / 1 to 1 / 3) to afford **S2** (78 % yield, 66 mg, white solid). The analytical data was identical with the reported one.[4]

1-((3aR,4R,6R,6aR)-6-(azidomethyl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-5-fluoropyrimidine-2,4(1H,3H)-dione (**5**).



To a solution of **S2** (36.0 mg, 0.127 mmol) in DMF (1.3 mL) was added sodium azide (24.7 mg, 0.380 mmol). The suspension was stirred at ambient temperature for 12 h and 60 °C for 4.5 h. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, hexane / ethylacetate = 2 / 1 to 1 / 1) to afford **5** (48% yield, 20.0 mg, colorless oil). The analytical data was identical with the reported one.[5]

1-((2R,3R,4S,5R)-5-(((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)-3,4-dihydroxytetrahydrofuran-2-yl)-5-fluoropyrimidine-2,4(1H,3H)-dione (**11**)



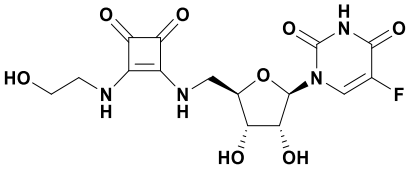
To a solution of **5** (18.0 mg, 0.0550 mmol) in MeOH (0.55 mL) was added 10% Pd/C (1.8 mg) under argon atmosphere. The argon atmosphere of the flask was exchanged into hydrogen atmosphere. The mixture was stirred at ambient temperature for 1.5 h. The reaction mixture was filtered through celite pad, followed by washing the reside with MeOH. After evaporation under reduced pressure, the crude containing 5'-amino-5'-deoxy-2′,3′-*O*-(1-methylethylidene)-5-fluorouridine was obtained and used next step without further purification.

To the crude in MeOH (1.5 mL) was added 4*N* HCl-dioxane (0.5 mL) on ice bath. The mixture was stirred at ambient temperature for 2 h. After evaporation under reduced pressure, **8** was obtained and used for the next step without further purification.

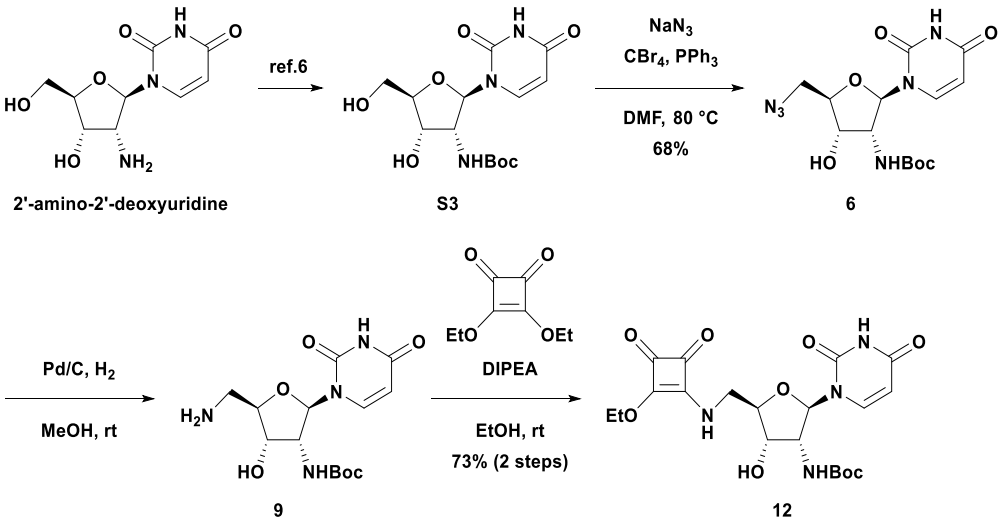
To a solution of **8** and diethylsquarate (12.2 μL, 0.0825 mmol) in EtOH (0.55 mL) was added DIPEA (9.6 μL, 0.0550 mmol). The mixture was stirred at ambient temperature for 17 h. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, $CHCl_3$ / MeOH = 10 / 1 to 5 /1) to afford **11** (55% (3 steps), 11.7 mg, colorless oil). $[\alpha]_D^{26}$ = 9.20 (c

1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.78 (d, J = 6.6 Hz, 0.5H), 7.74 (d, J = 6.5 Hz, 0.5H), 5.77 (d, J = 3.5 Hz, 0.5H), 5.73 (d, J = 3.2 Hz, 0.5H), 4.72 (q, J = 7.1 Hz, 2H), 4.21 (m, 1H), 4.06-4.01 (m, 2H), 3.93 (m, 1H), 3.82-3.64 (m, 1H), 1.45 (t, J = 7.1 Hz, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 190.17, 184.93, 178.61, 177.94, 175.39, 175.15, 159.69, 151.02, 143.08, 140.75, 127.08, 126.77, 126.44, 92.85, 92.01, 84.08, 83.91, 74.59, 74.41, 71.85, 70.85, 47.13, 46.91, 16.11, 16.03; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{15}H_{18}FN_3O_8Na$ 408.0814, found 408.0815.

1-((2R,3R,4S,5R)-3,4-dihydroxy-5-(((2-((2-hydroxyethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)tetrahydrofuran-2-yl)-5-fluoropyrimidine-2,4(1H,3H)-dione (**2**)

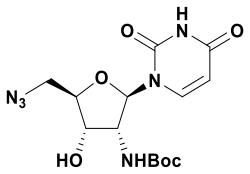


To a solution of **11** (10.0 mg, 0.0259 mmol) and 2-aminoethanol (1.6 μL, 0.0259 mmol) in EtOH (0.25 mL) was added DIPEA (4.5 μL, 0.0259 mmol). The mixture was stirred at ambient temperature for 16.5 h. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, $CHCl_3$ / MeOH = 10 / 1 to 5 / 1) to afford **2** (61% yield, 5.1 mg, colorless oil). $[\alpha]_D^{26}$ = -13.7 (c 0.30 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.81 (d, J = 6.2 Hz, 1H), 5.73 (d, J = 4.2 Hz, 1H), 4.26 (dd, J = 5.0, 5.0 Hz, 1H), 4.08-4.01 (m, 2H), 4.00-3.90 (m, 2H), 3.69 (br s, 4H); ^{13}C NMR (CD_3OD, 100 MHz): δ 184.11, 183.90, 170.04, 169.60, 163.10, 160.27, 151.69, 143.23, 140.89, 127.18, 126.83, 92.72, 84.41, 74.28, 71.82, 62.58, 47.50, 46.70; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{15}H_{17}FN_4O_8Na$ 423.0923, found 423.0923.



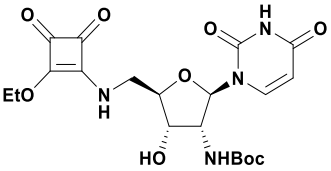
Scheme S3 Synthesis of **12**

tert-butyl ((2R,3R,4S,5R)-5-(azidomethyl)-2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-hydroxytetrahydrofuran-3-yl)carbamate (**6**)



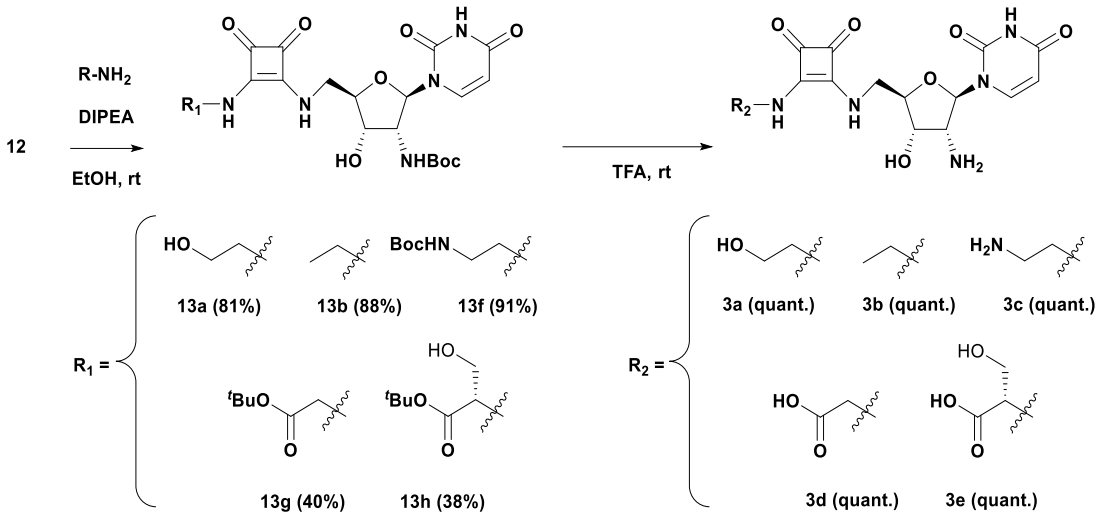
S3 was synthesized by the reported method.[6] To a solution of **S3** (200 mg, 0.583 mmol), triphenylphosphine (157 mg, 0.597 mmol) and sodium azide (177 mg, 2.72 mmol) in dry DMF (3.6 mL) was added tetrabromomethane (198 mg, 0.597 mmol). The mixture was stirred at ambient temperature for 5 h and 80 ℃ for 18.5 h. After addition of sat. $NaHCO_3$ aq. (0.1 mL) to the mixture on ice bath, the mixture was stirred at the same temperature for 10 min. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, $CHCl_3$ / MeOH = 15 / 1 to 10 / 1 to 5 / 1) to afford **6** (68% yield, 146 mg, white solid). $[\alpha]_D^{26}$ = 44.4 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.72 (d, *J* = 8.1 Hz, 1H), 5.92 (d, *J* = 7.9 Hz, 1H), 5.76 (d, *J* = 8.1 Hz, 1H), 4.24 (dd, *J* = 7.9, 6.4 Hz, 1H), 4.18 (br m, 1H), 4.07 (br m, 1H), 3.64 (m, 2H), 1.41 (s, 9H); ^{13}C NMR (CD_3OD, 100 MHz): δ 165.99, 157.76, 152.36, 142.36, 103.42, 89.04, 85.70, 80.96, 71.16, 57.21, 53.64, 28.56; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{14}H_{20}N_6O_6Na$ 391.1337, found 391.1338.

tert-butyl ((2R,3R,4S,5R)-2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-5-(((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)-4-hydroxytetrahydrofuran-3-yl)carbamate (**12**)



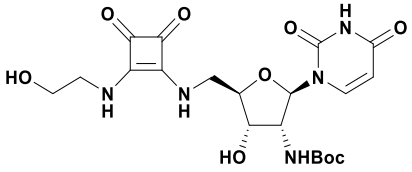
To a solution of **6** (123 mg, 0.334 mmol) in MeOH (3.3 mL) was added 10% Pd/C (12 mg) under argon atmosphere. The argon atmosphere of the flask was exchanged into hydrogen atmosphere. The mixture was stirred at ambient temperature for 14 h. The reaction mixture was filtered through celite pad, followed by washing the reside with MeOH. After evaporation under reduced pressure, **9** was obtained (123 mg, colorless oil) and used for the next step without further purification.

To a solution of some amount of **9** (67 mg, 0.196 mmol) and diethylsquarate (43.5 μL, 0.294 mmol) in EtOH (2.0 mL) was added DIPEA (34 μL, 0.196 mmol). The mixture was stirred at ambient temperature for 13.5 h. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, $CHCl_3$ / MeOH = 15 / 1 to 10 / 1) to afford **12** (91% yield, 82.8 mg, white solid). $[\alpha]_D^{26}$ = 10.9 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.64 (d, *J* = 8.0 Hz, 0.5H), 7.60 (*J* = 8.0 Hz, 0.5H), 5.83 (m, 1H), 5.72 (d, *J* = 8.0 Hz, 1H), 4.72 (q, *J* = 7.1 Hz, 2H), 4.30-4.13 (m, 2H), 4.04 (m, 1H), 3.97-3.64 (m, 2H), 1.45 (t, *J* = 7.1 Hz, 3H), 1.40 (s, 9H); ^{13}C NMR (CD_3OD, 100 MHz): δ 188.80, 188.25, 183.73, 183.52, 177.28, 176.61, 173.96, 173.62, 164.58, 156.35, 150.97, 141.71, 101.80, 89.08, 88.25, 84.58, 84.19, 79.56, 69.71, 69.53, 55.41, 55.21, 45.67, 27.17, 14.66; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{20}H_{26}N_4O_9Na$ 489.1592, found 489.1591.



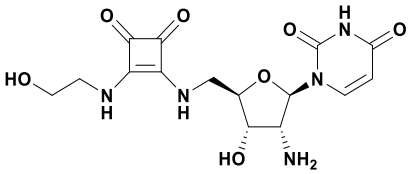
Scheme S4 Synthesis of **13a**, **b**, **f-h** and SQ-UDP analogs (**3a-e**)

tert-butyl ((2R,3R,4S,5R)-2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-hydroxy-5-(((2-((2-hydroxyethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)tetrahydrofuran-3-yl)carbamate (**13a**)



To a solution of **12** (9.0 mg, 0.0193 mmol) and 2-aminoethanol (1.2 μL, 0.0193 mmol) in EtOH (0.19 mL) was added DIPEA (3.4 μL, 0.0193 mmol). The mixture was stirred at ambient temperature for 12.5 h. The reaction mixture was purified directly by column chromatography (silicagel, $CHCl_3$ / MeOH = 10 / 1 to 5 / 1) to afford **13a** (81%, 7.5 mg, colorless oil). $[\alpha]_D^{26}$ = 23.7 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.65 (d, *J* = 8.0 Hz, 1H), 5.80 (d, *J* = 7.6 Hz, 1H), 5.73 (d, *J* = 8.0 Hz, 1H), 4.30 (m, 1H), 4.18 (m, 1H), 4.08 (m, 1H), 3.95 (m, 2H), 3.69 (br s, 4H), 1.40 (s, 9H); ^{13}C NMR (CD_3OD, 100 MHz): δ 184.14, 183.90, 170.06, 169.44, 166.04, 157.77, 152.54, 143.47, 103. 25, 90.80, 86.25, 80.96, 71.13, 62.59, 56.56, 47.52, 46.82, 28.57; HRMS (ESI) m/z [M + Na]$^+$ calculated for $C_{20}H_{27}N_5O_9Na$ 504.1701, found 504.1700.

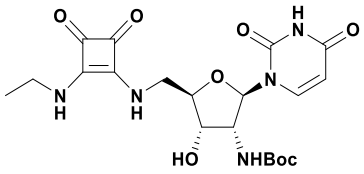
1-((2R,3R,4S,5R)-3-amino-4-hydroxy-5-(((2-((2-hydroxyethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (**3a**)



To a flask containing **13a** (11.0 mg, 0.0228 mmol) was added TFA (0.5 mL) under argon atmosphere. The mixture was stirred on ice bath for 20 min. Removing TFA by argon flow, the mixture was diluted with H_2O (4.0 mL). After lyophilization, **3a** was obtained (quant. (as TFA salt), 11.3 mg, white solid). $[\alpha]_D^{26}$ = 29.2 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.65 (d, *J* = 8.1 Hz, 1H),

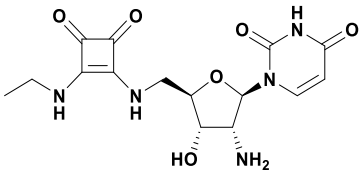
6.01 (d, J = 6.4 Hz, 1H), 5.75 (d, J = 8.1 Hz, 1H), 4.41 (m, 1H), 4.15 (m, 1H), 4.08-3.95 (m, 3H), 3.69 (br s, 4H); ^{13}C NMR (CD_3OD, 100 MHz): δ 184.08, 183.91, 169.32, 165.71, 152.29, 142.59, 103.64, 89.27, 86.30, 70.17, 62.53, 56.15, 47.54, 46.16; HRMS (ESI) m/z $[M + H]^+$ calculated for $C_{15}H_{20}N_5O_7$ 381.1357, found, 382.1356.

tert-butyl ((2R,3R,4S,5R)-2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-5-(((2-(ethylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)-4-hydroxytetrahydrofuran-3-yl)carbamate (**13b**)



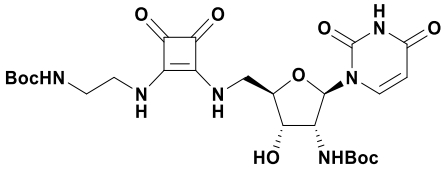
To a solution of **12** (20.0 mg, 0.0429 mmol) and ethylamine hydrochloride (3.5 mg, 0.0429 mmol) was added DIPEA (14.9 μL, 0.0858 mmol). The mixture was stirred at ambient temperature for 22.5 h. The reaction mixture was purified directly by column chromatography (silicagel, $CHCl_3$ / MeOH = 10 / 1) to afford **13b** (88% yield, 17.6 mg, colorless oil). $[\alpha]_D^{26}$ = 40.2 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.65 (d, J = 8.0 Hz, 1H), 5.81 (d, J = 7.7 Hz, 1H), 5.72 (d, J = 8.0 Hz, 1H), 4.29 (dd, J = 7.7, 6.9 Hz, 1H), 4.18 (br s, 1H), 4.08 (m, 2H), 3.63 (m, 2H), 1.25 (t, J = 7.2 Hz, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 183.99, 183.64, 169.61, 169.36, 165.96, 157.73, 152.50, 143.38, 103.26, 90.65, 86.20, 80.94, 71.18, 56.55, 46.91, 40.34, 28.57, 16.89; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{20}H_{27}N_5O_8Na$ 488.1752, found 488.1752.

1-((2R,3R,4S,5R)-3-amino-5-(((2-(ethylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)-4-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (**3b**)



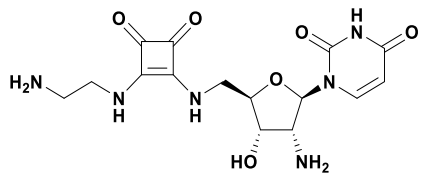
To a flask containing **13b** (8.0 mg, 0.0172 mmol) was added TFA (0.5 mL). The mixture was stirred at ambient temperature for 20 min. Removing TFA by argon flow, the mixture was diluted with H_2O (5.0 mL). After lyophilization, **3b** was obtained (quant. (as TFA salt), 8.3 mg, white solid). $[\alpha]_D^{26}$ = 44.5 (c 1.0 in H_2O). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.65 (d, J = 8.1 Hz, 1H), 6.03 (d, J = 6.4 Hz, 1H), 5.75 (d, J = 8.1 Hz, 1H), 4.42 (m, 1H), 4.17 (m, 1H), 4.07-3.95 (m, 3H), 3.64 (m, 2H), 1.25 (t, J = 7.2 Hz, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 183.66, 169.79, 165.69, 152.29, 142.44, 103.67, 89.01, 86.33, 70.17, 56.10, 46.24, 40.35, 16.86; HRMS (ESI) m/z $[M + H]^+$ calculated for $C_{15}H_{20}N_5O_6$ 366.1408, found 366.1408.

tert-butyl ((2R,3R,4S,5R)-5-(((2-((2-((*tert*-butoxycarbonyl)amino)ethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)-2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-hydroxytetrahydrofuran-3-yl)carbamate (**13f**)



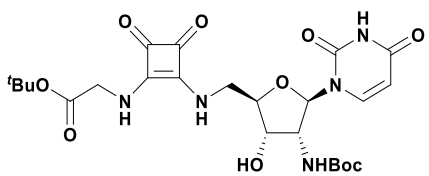
1-(*tert*-Butyloxycarbonyl)ethyldiamine (**S4**) was synthesized by the reported method.[7] To a solution of **12** (20.0 mg, 0.0429 mmol) and **S4** (6.9 mg, 0.0429 mmol) in EtOH (0.4 mL) was added DIPEA (7.5 μL, 0.0429 mmol). The mixture was stirred at ambient temperature for 22.5 h. The reaction mixture was purified directly by column chromatography (silicagel, $CHCl_3$ / MeOH = 10 / 1) to afford **13c** (91% yield, 22.7 mg, white solid). $[\alpha]_D^{26}$ = 17.4 (c 1.0 in MeOH) ^{1}H NMR (CD_3OD, 400 MHz): δ 7.67 (d, *J* = 8.0 Hz, 1H), 5.78 (d, *J* = 7.8 Hz, 1H), 5.73 (d, *J* = 8.0 Hz, 1H), 4.32 (dd, *J* = 7.8, 6.8 Hz, 1H), 4.18 (br s, 1H), 4.09 (br s, 1H), 3.94 (br s, 2H), 3.64 (m, 2H), 3.25 (t, *J* = 5.6 Hz, 4H), 1.40 (s, 18H) ; ^{13}C NMR (CD_3OD, 100 MHz): δ 184.01, 170.11, 169.35, 165.96, 158.68, 157.75, 152.58, 143.63, 103.29, 91.04, 86.35, 80.96, 80.37, 71.22, 56.39, 46.93, 45.29, 42.36, 28.73, 28.58; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{25}H_{36}N_6O_{10}Na$ 603.2385, found 603.2384.

1-((2R,3R,4S,5R)-3-amino-5-(((2-((2-aminoethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)methyl)-4-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (**3c**)



To a flask containing **13c** (10.0 mg, 0.0172 mmol) was added TFA (0.5 mL). The mixture was stirred at ambient temperature for 20 min. Removing TFA by argon flow, the mixture was diluted with H_2O (5.0 mL). After lyophilization, **3c** was obtained (quant. (as TFA salt), 10.4 mg, white solid) $[\alpha]_D^{26}$ = 22.6 (c 1.0 in H_2O). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.67 (d, *J* = 8.0 Hz, 1H), 6.02 (d, *J* = 6.5 Hz, 1H), 5.73 (d, *J* = 8.0 Hz, 1H), 4.42 (m, 1H), 4.17 (m, 1H), 4.04 (dd, *J* = 6.7, 6.5 Hz, 1H), 3.89 (m, 2H), 3.87 (t, *J* = 6.0 Hz, 2H), 3.20 (t, *J* = 6.0 Hz, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ 184.32, 184.14, 170.29, 169.66, 165.70, 152.30, 142.41, 103.65, 88.76, 86.26, 70.13, 56.17, 46.33, 42.47, 41.44; HRMS (ESI) m/z $[M + H]^+$ calculated for $C_{15}H_{21}N_6O_6$ 381.1517, found 381.1518.

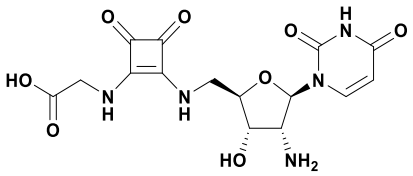
tert-butyl (2-((((2R,3S,4R,5R)-4-((*tert*-butoxycarbonyl)amino)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl)amino)-3,4-dioxocyclobut-1-en-1-yl)glycinate (**13g**)



To a solution of **12** (15.0 mg, 0.0322 mmol) and glycine *tert*-butylester hydrochloride (5.4 mg, 0.0322 mmol) in EtOH (0.3 mL) was added DIPEA (11.2 μL, 0.0644 mmol). The mixture was stirred at ambient temperature for 2 h and

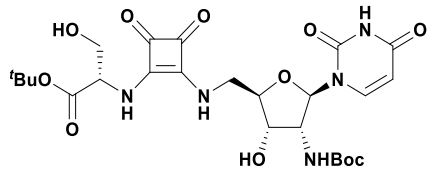
refluxed for 22h. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, $CHCl_3$ / MeOH = 12 / 1) to afford **13d** (40% yield, 17.8 mg, colorless oil). $[\alpha]_D^{26}$ = 33.2 (c 0.30 in MeOH) ^{1}H NMR (CD_3OD, 400 MHz): d 7.64 (d, *J* = 8.0 Hz, 1H), 5.81 (d, *J* = 7.2 Hz, 1H), 5.73 (d, *J* = 8.0 Hz, 1H), 4.29 (br s, 3H), 4.20 (m, 1H), 4.08 (m, 1H), 3.94 (m, 2H), 1.48 (s, 9H), 1.41 (s, 9H); ^{13}C NMR (CD_3OD, 100 MHz): d 184.56, 184.38, 170.42, 169.78, 166.01, 157.74, 152.55, 143.34, 103.32, 90.65, 86.07, 83.50, 82.40, 80.94, 71.09, 56.59, 46.86, 46.76, 28.57, 28.275; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{24}H_{34}N_5O_{10}Na$ 574.2120, found, 574.2120.

(2-((((2R,3S,4R,5R)-4-amino-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl)amino)-3,4-dioxocyclobut-1-en-1-yl)glycine (**3d**)



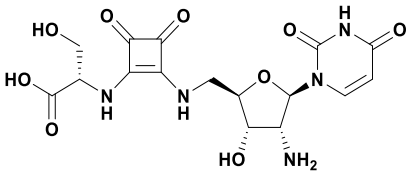
To a flask containing **13d** (7.0 mg, 0.0127 mmol) was added TFA (0.5 mL). The mixture was stirred at ambient temperature for 20 min. Removing TFA by argon flow, the mixture was diluted with H_2O (5.0 mL). After lyophilization, **3d** was obtained (quant. (as TFA salt), 6.5 mg, white solid). $[\alpha]_D^{26}$ = -85.4 (c 1.0 in H_2O) ^{1}H NMR (CD_3OD, 400 MHz): δ 7.63 (d, *J* = 8.0 Hz, 1H), 6.02 (d, *J* = 6.0 Hz, 1H), 5.76 (d, *J* = 8.0 Hz, 1H), 4.44-4.38 (m, 3H), 4.16 (m, 1H), 4.09-4.06 (m, 2H), 3.92 (m, 1H); ^{13}C NMR (CD_3OD, 100 MHz): δ 184.55, 184.32, 172.85, 169.93, 169.62, 165.70, 152.30, 142.46, 103.73, 89.12, 86.30, 70.10, 56.11, 46.14, 45.83; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{15}H_{17}N_5O_8Na$ 418.0969, found 418.0970.

tert-butyl (2-((((2R,3S,4R,5R)-4-((*tert*-butoxycarbonyl)amino)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl)amino)-3,4-dioxocyclobut-1-en-1-yl)-L-serinate (**13h**)



L-serine *tert*-butylester (**S5**) was synthesized by the reported method.[8] To a solution of **12** (20.0 mg, 0.0429 mmol) and **S5** (6.9 mg, 0.0429 mmol) in EtOH (0.4 mL) was added DIPEA (7.5 μL, 0.0429 mmol). The mixture was stirred at ambient temperature for 20h and refluxed for 16h. After evaporation under reduced pressure, the crude was purified by column chromatography (silicagel, $CHCl_3$ / MeOH = 12 / 1) to afford **13e** (38% yield, 9.6 mg, colorless oil). $[\alpha]_D^{26}$ = 53.5 (c 0.40 in MeOH) ^{1}H NMR (CD_3OD, 400 MHz): d 7.65 (d, *J* = 8.1 Hz, 1H), 5.83 (d, *J* = 7.6 Hz, 1H), 5.74 (d, *J* = 8.1 Hz, 1H), 4.73 (br s, 1H), 4.27 (dd, *J* = 7.6, 7.4 Hz, 1H), 4.17 (m, 1H), 4.08-3.87 (m, 5H), 1.49 (s, 9H), 1.40 (s, 9H); ^{13}C NMR (CD_3OD, 100 MHz): d 184.47, 184.12, 170.48, 169.78, 169.19, 165.98, 157.75, 152.49, 143.26, 103.34, 90.37, 86.23, 83.80, 80.97, 71.17, 64.02, 60.17, 56.67, 46.90, 28.57, 28.25; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{25}H_{35}N_5O_{11}Na$ 604.2225, found 604.2227.

(2-((((2R,3S,4R,5R)-4-amino-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl)amino)-3,4-dioxocyclobut-1-en-1-yl)-L-serine (**3e**)



To a flask containing **13e** (8.0 mg, 0.0138 mmol) was added TFA (0.5 mL). The mixture was stirred at ambient temperature for 20 min. Removing TFA by argon flow, the mixture was diluted with H_2O (5.0 mL). After lyophilization, **3e** was obtained (quant. (as TFA salt), 7.4 mg, white solid). $[\alpha]_D^{26}$ = -40.6 (c 1.0 in H_2O) ^{1}H NMR (CD_3OD, 400 MHz): δ 7.63 (d, *J* = 8.0 Hz, 1H), 6.03 (d, *J* = 6.2 Hz, 1H), 5.77 (d, *J* = 8.0 Hz, 1H), 4.84 (br s, 1H), 4.41 (m, 1H), 4.16 (m, 1H), 4.15-4.01 (m, 3H), 3.95-3.88 (m, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ 184.47, 184.08, 172.79, 169.66, 169.35, 165.70, 152.26, 142.44, 103.74, 89.13, 86.34, 70.14, 64.03, 59.62, 56.13, 46.19; HRMS (ESI) m/z $[M + Na]^+$ calculated for $C_{16}H_{20}N_5O_9Na$ 448.1075, found 448.1074.

12. References

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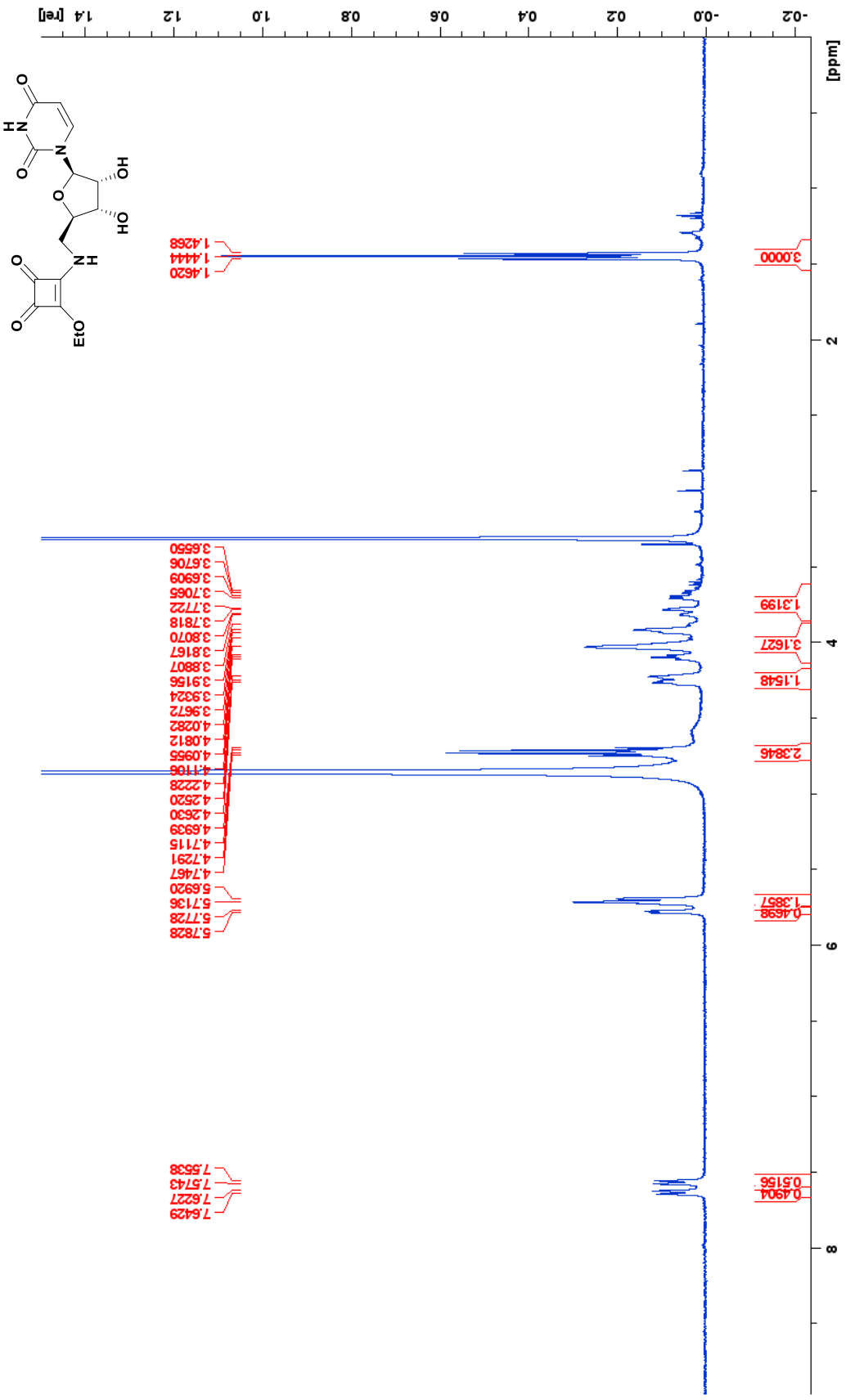
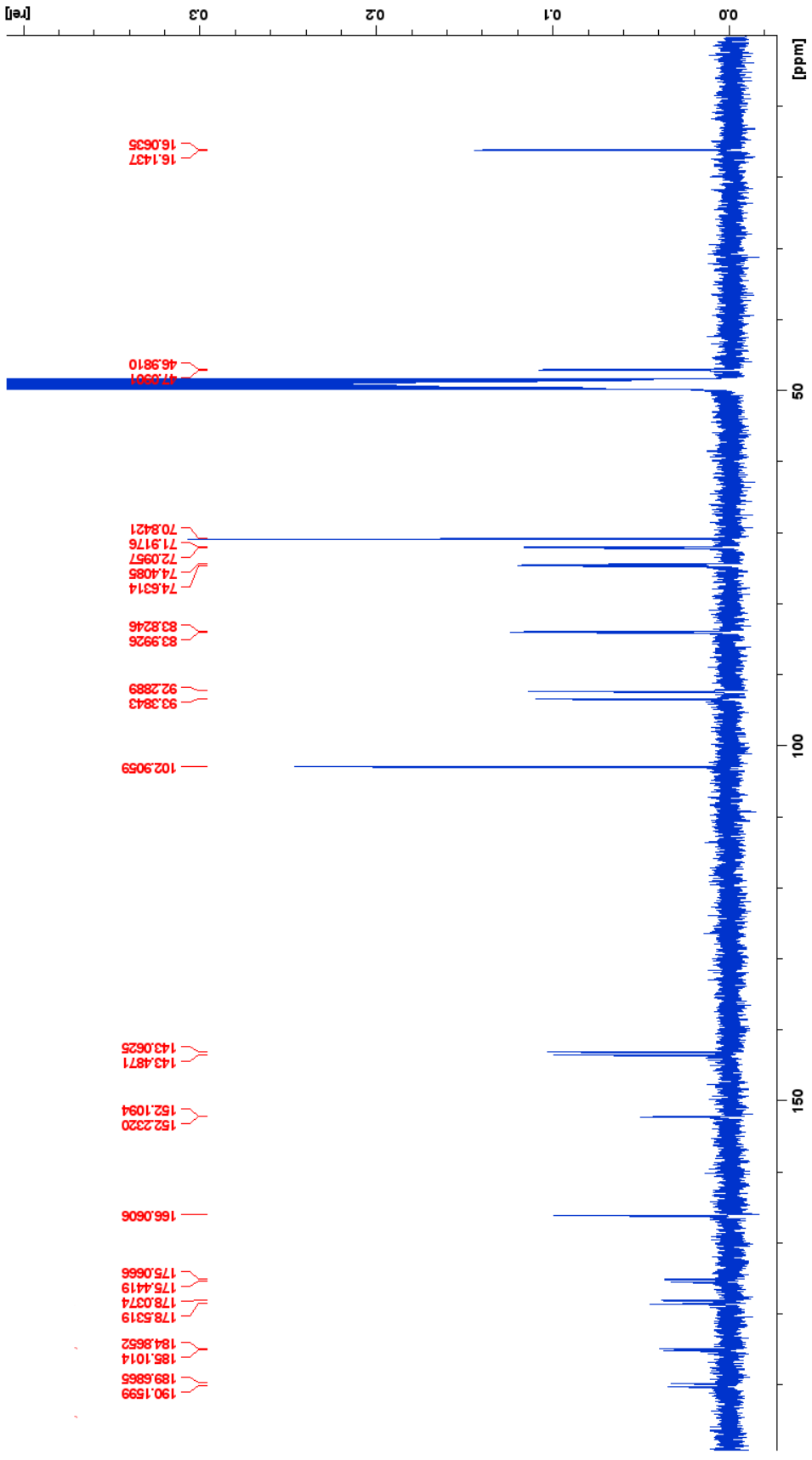
13. ^{1}H and ^{13}C NMR spectra

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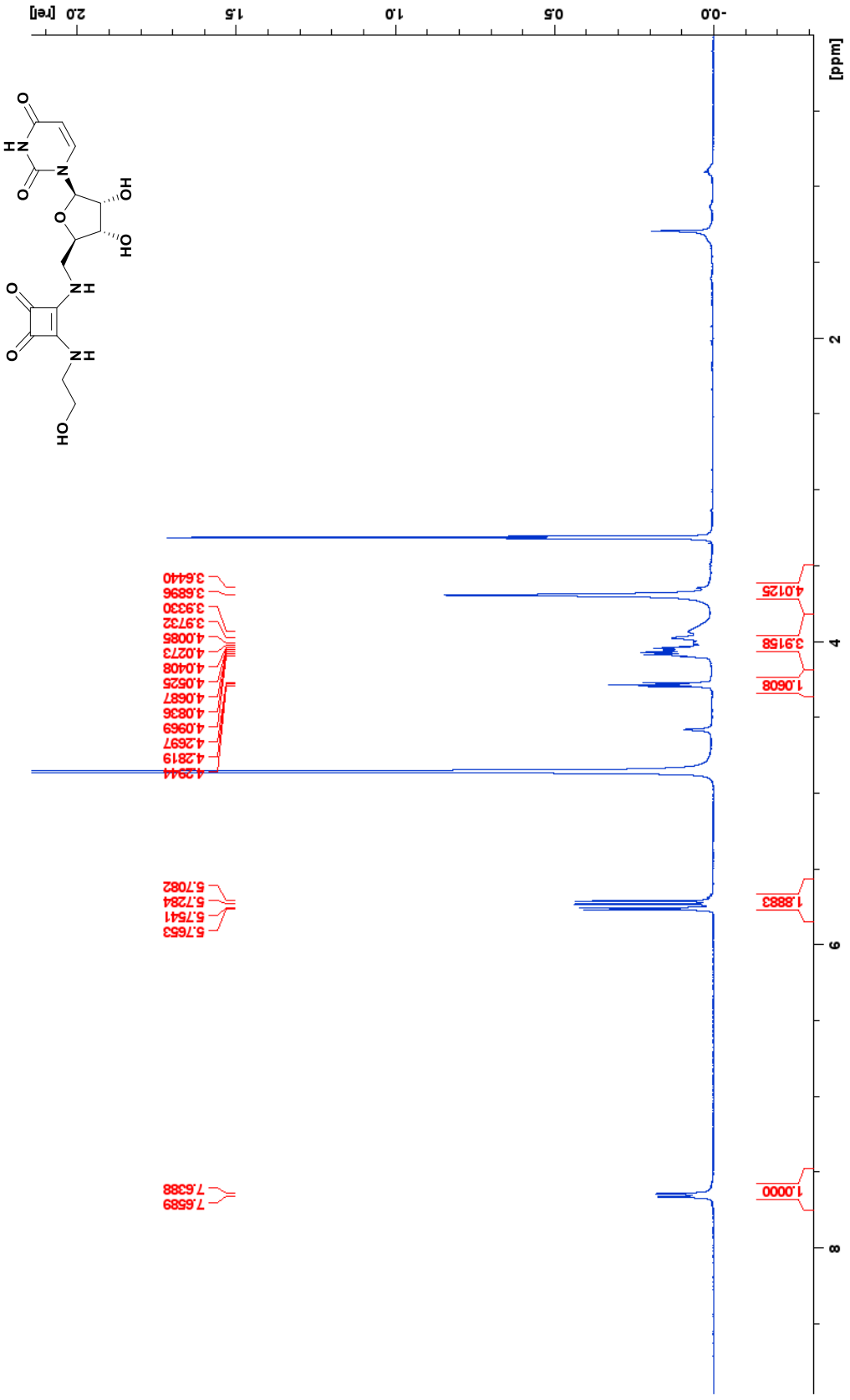
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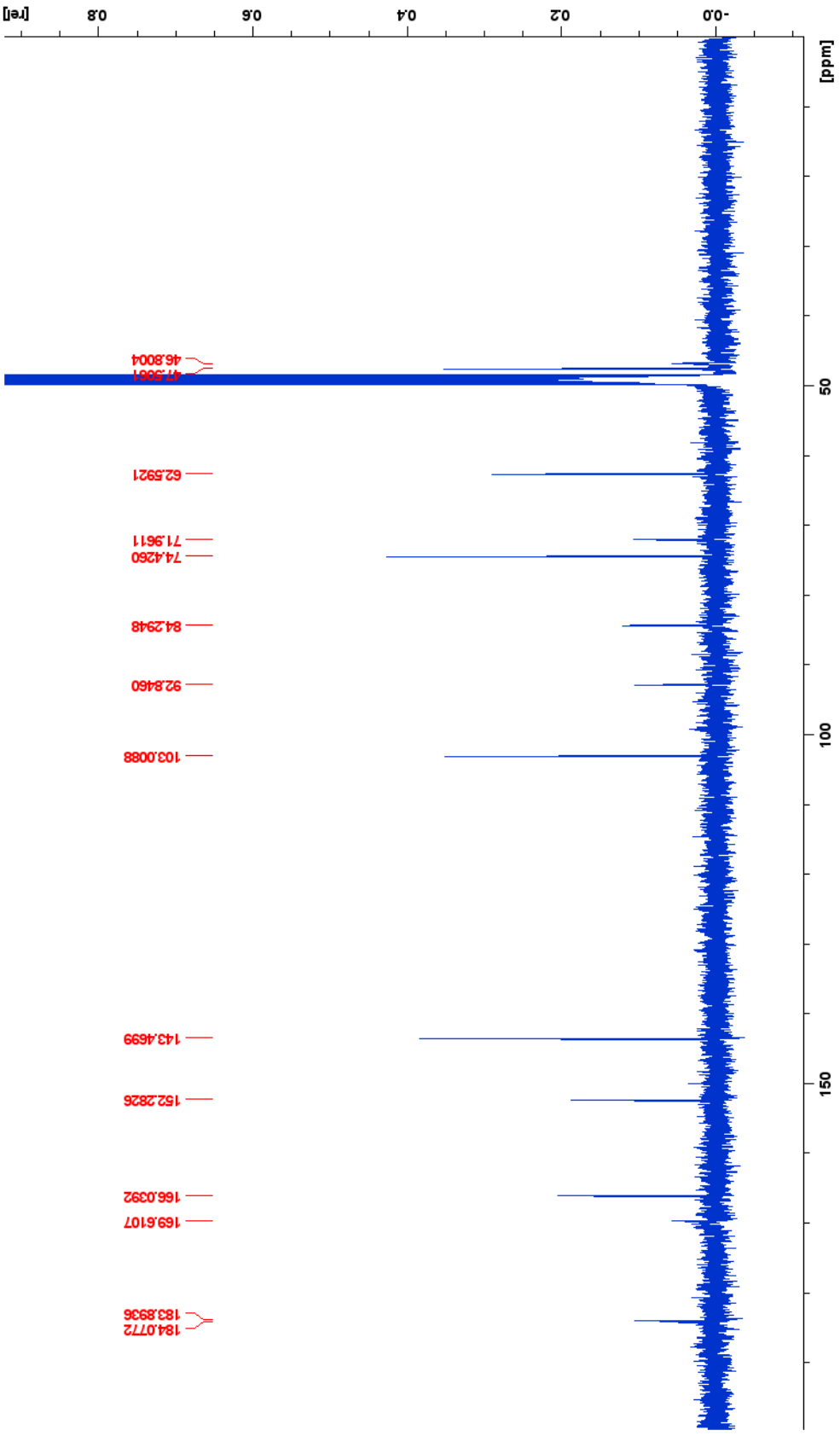
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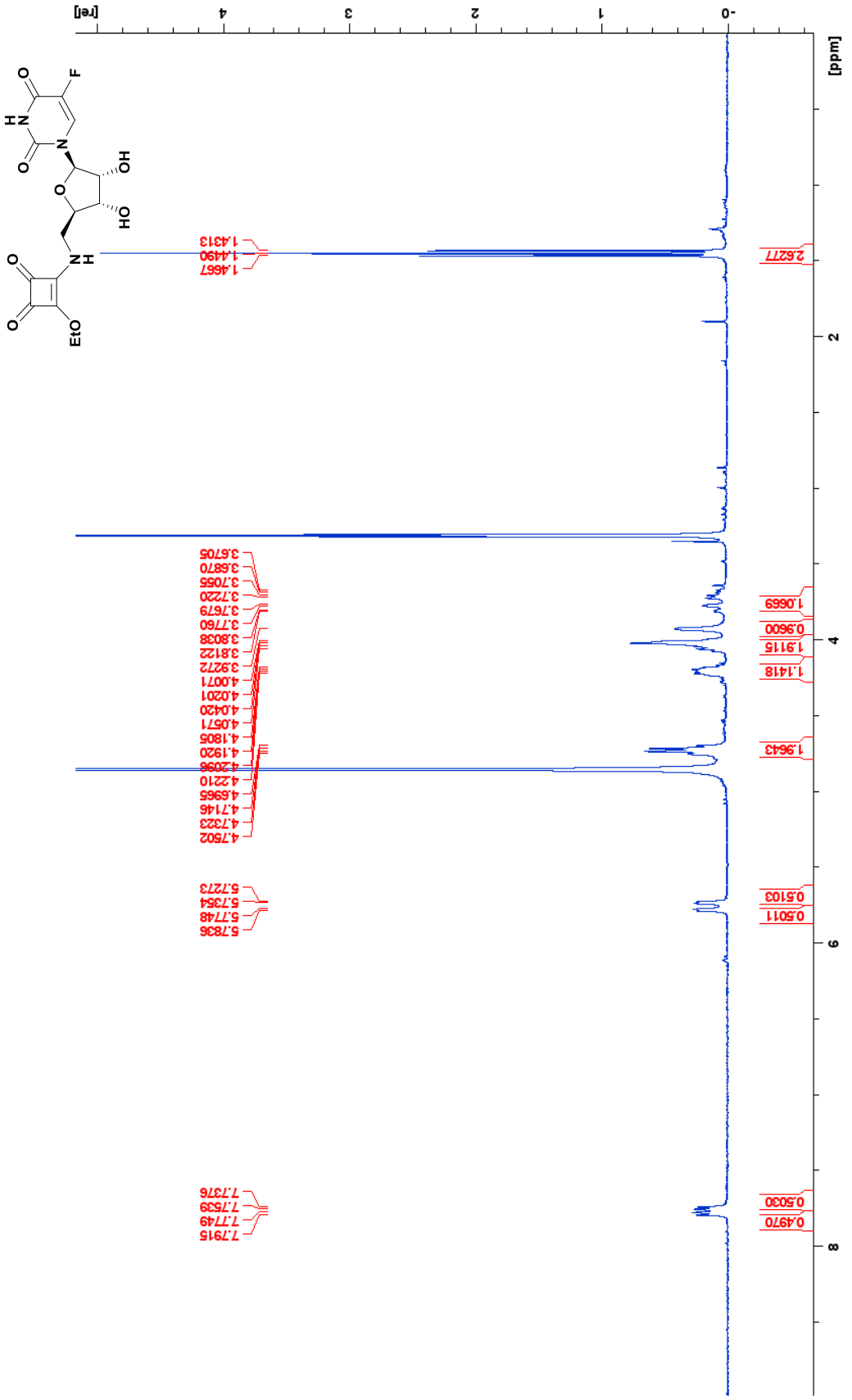
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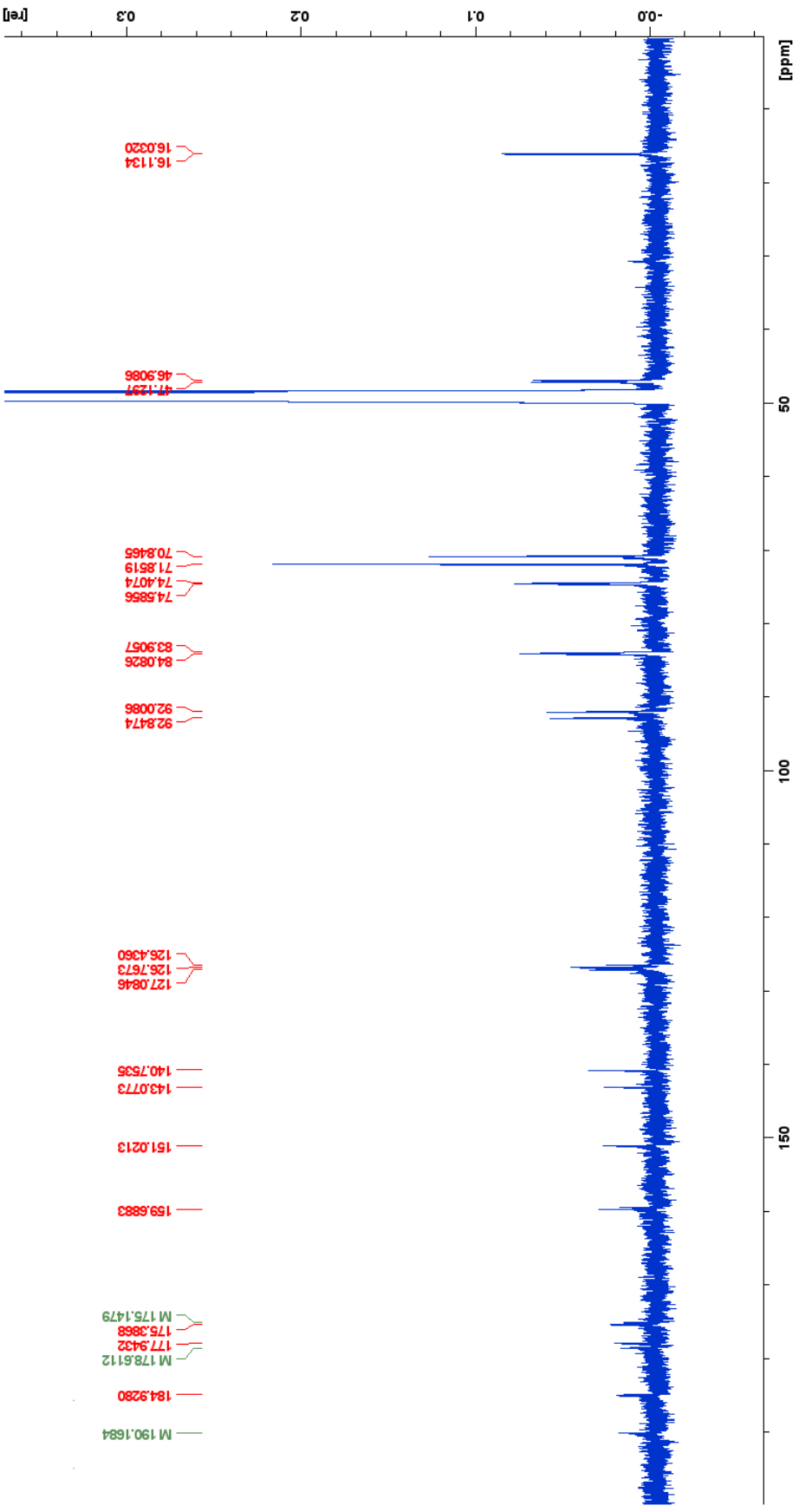
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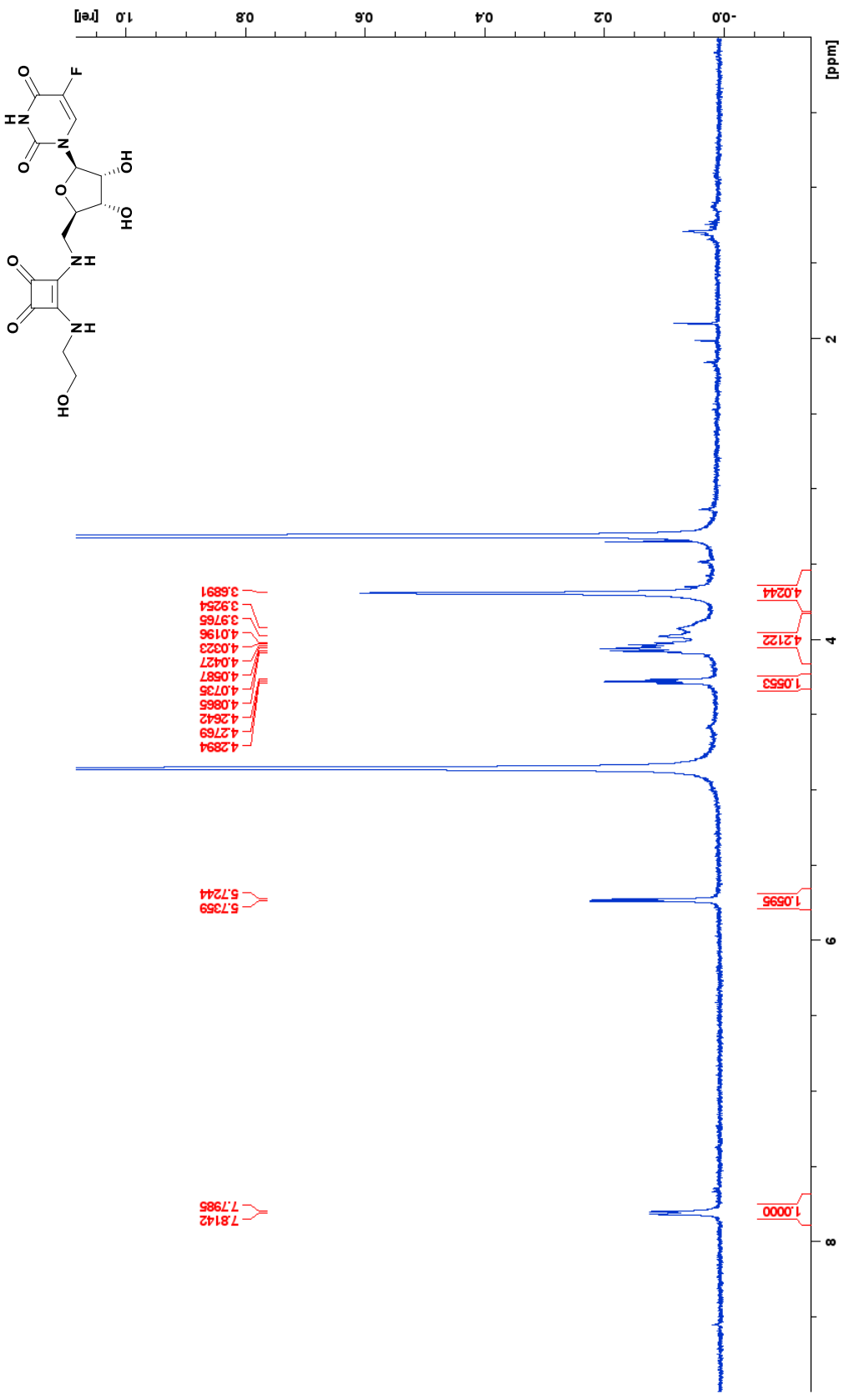
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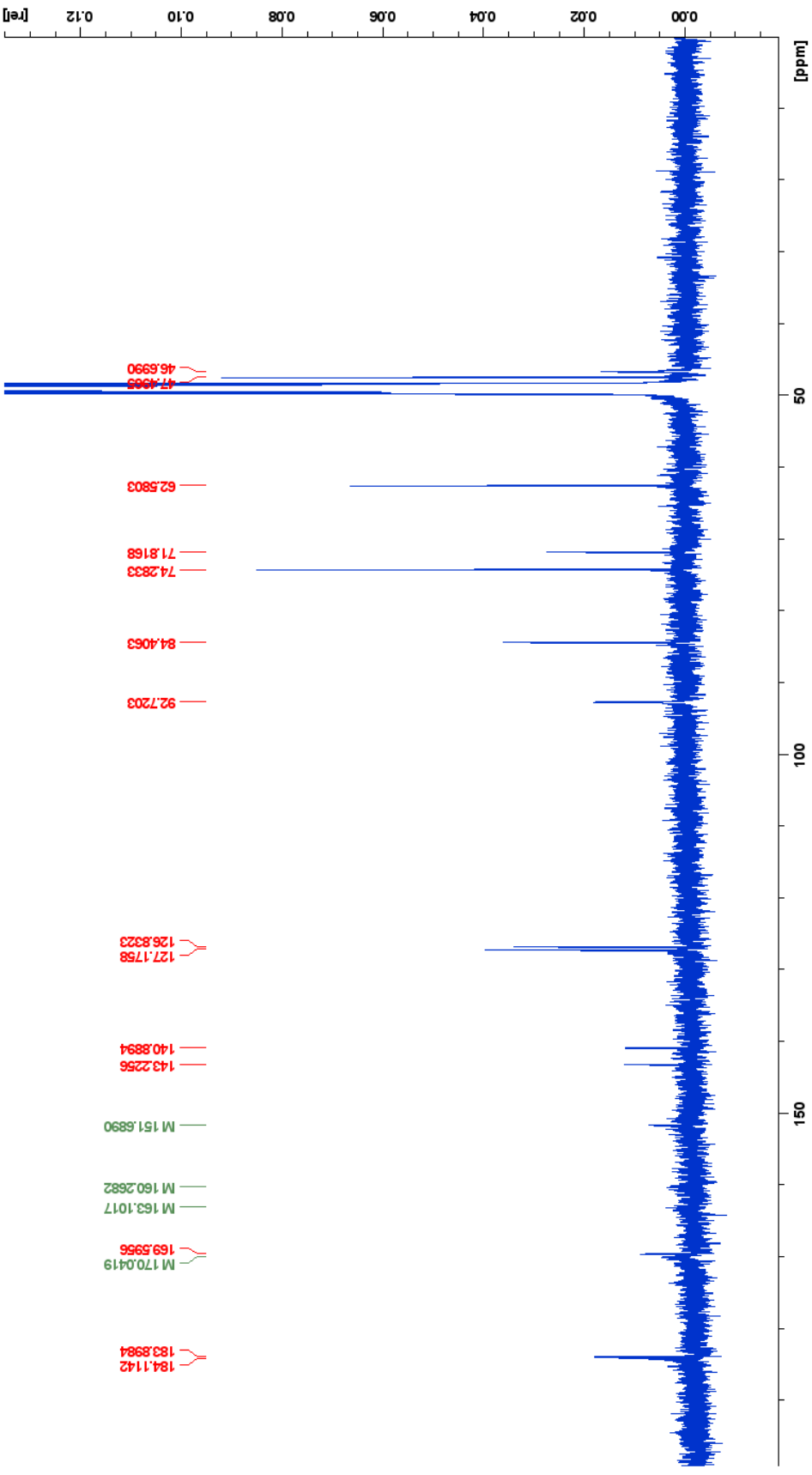
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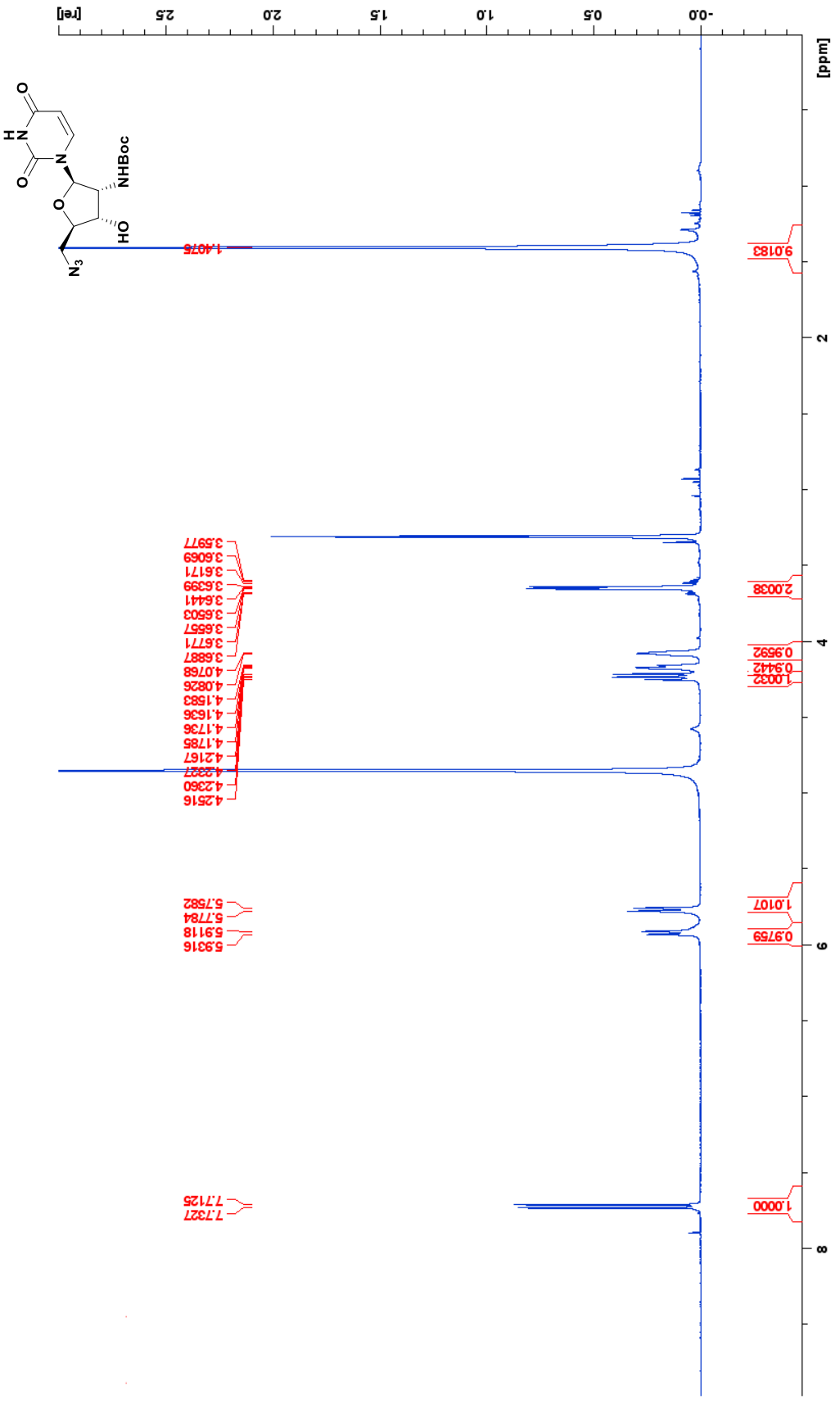
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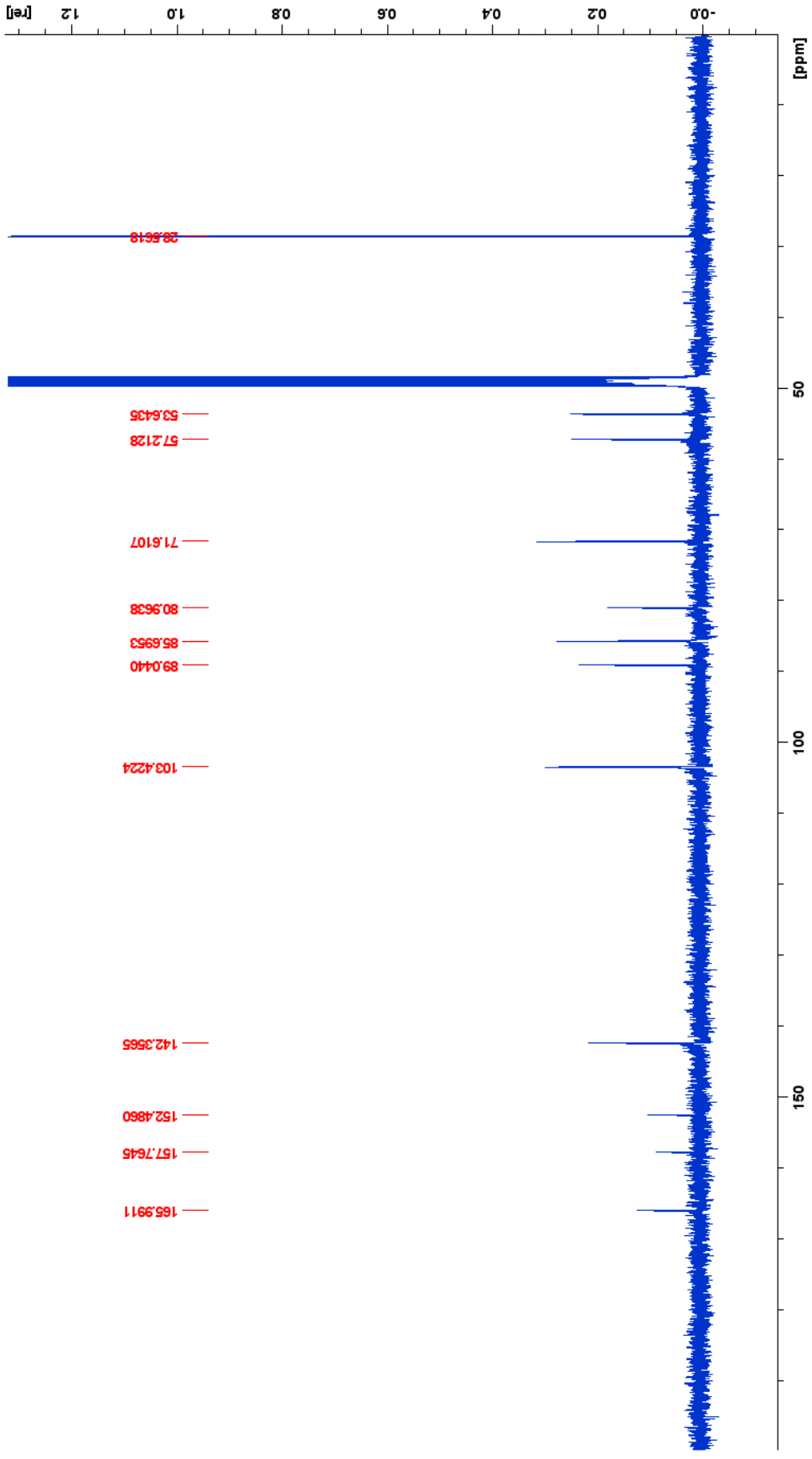
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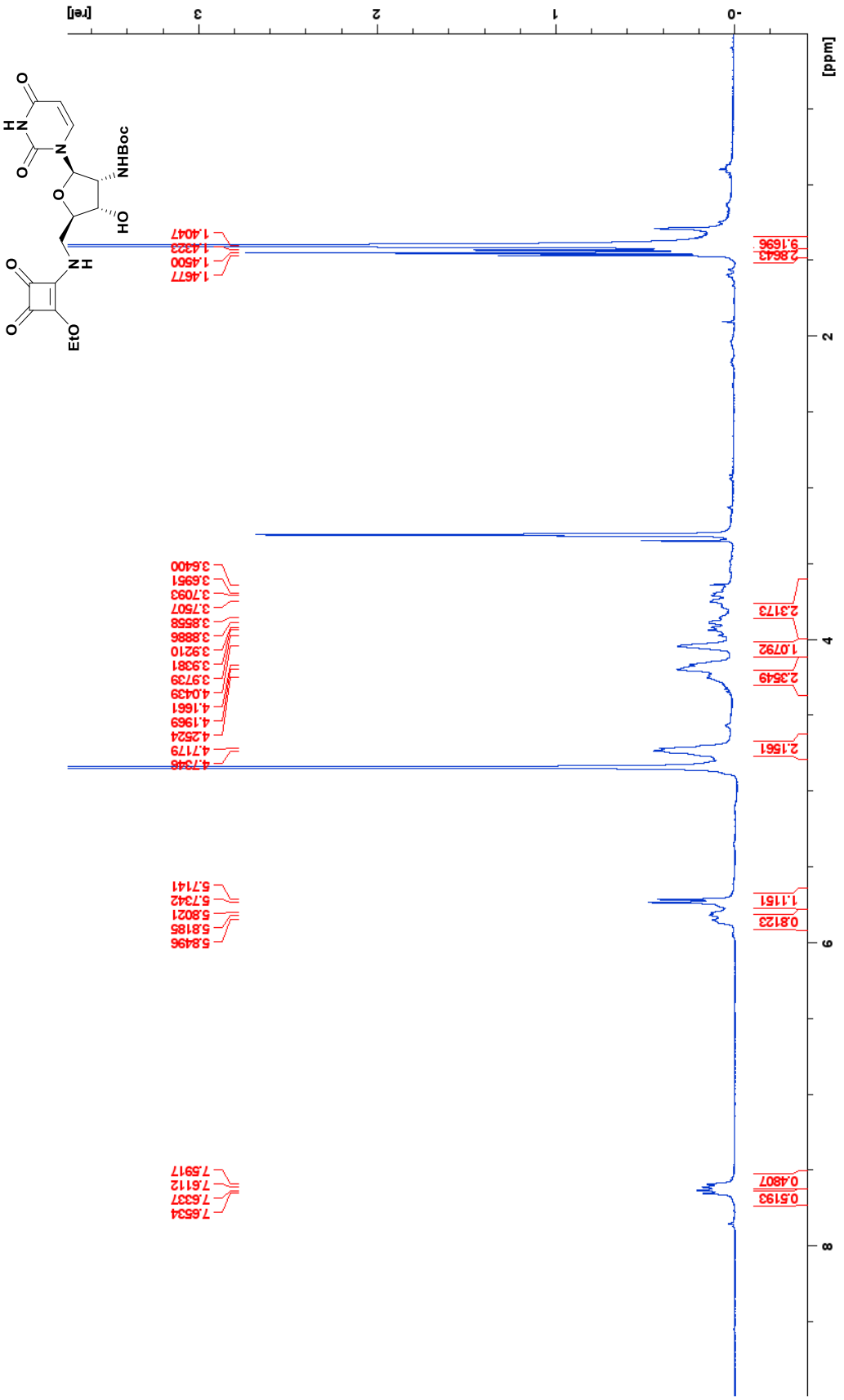
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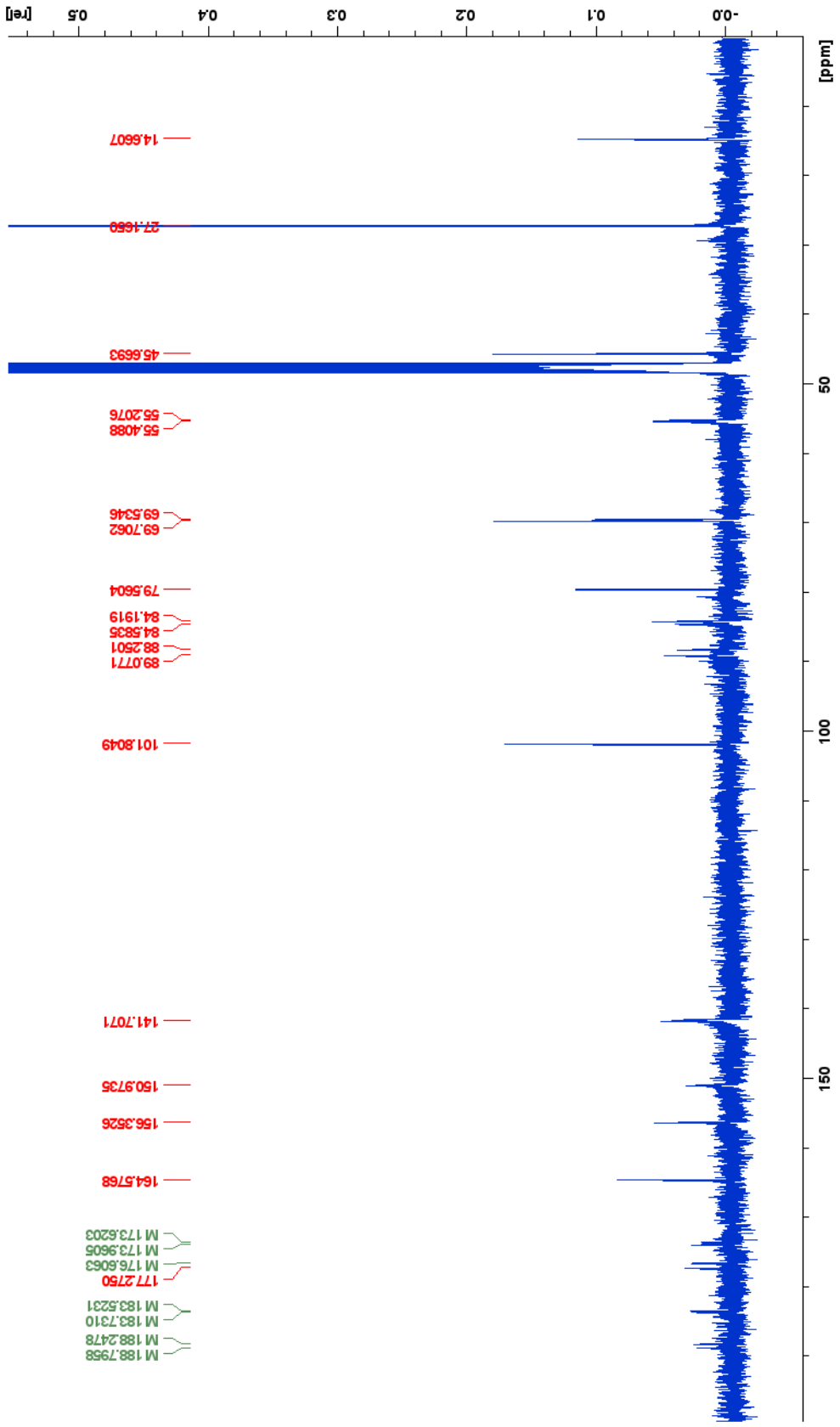
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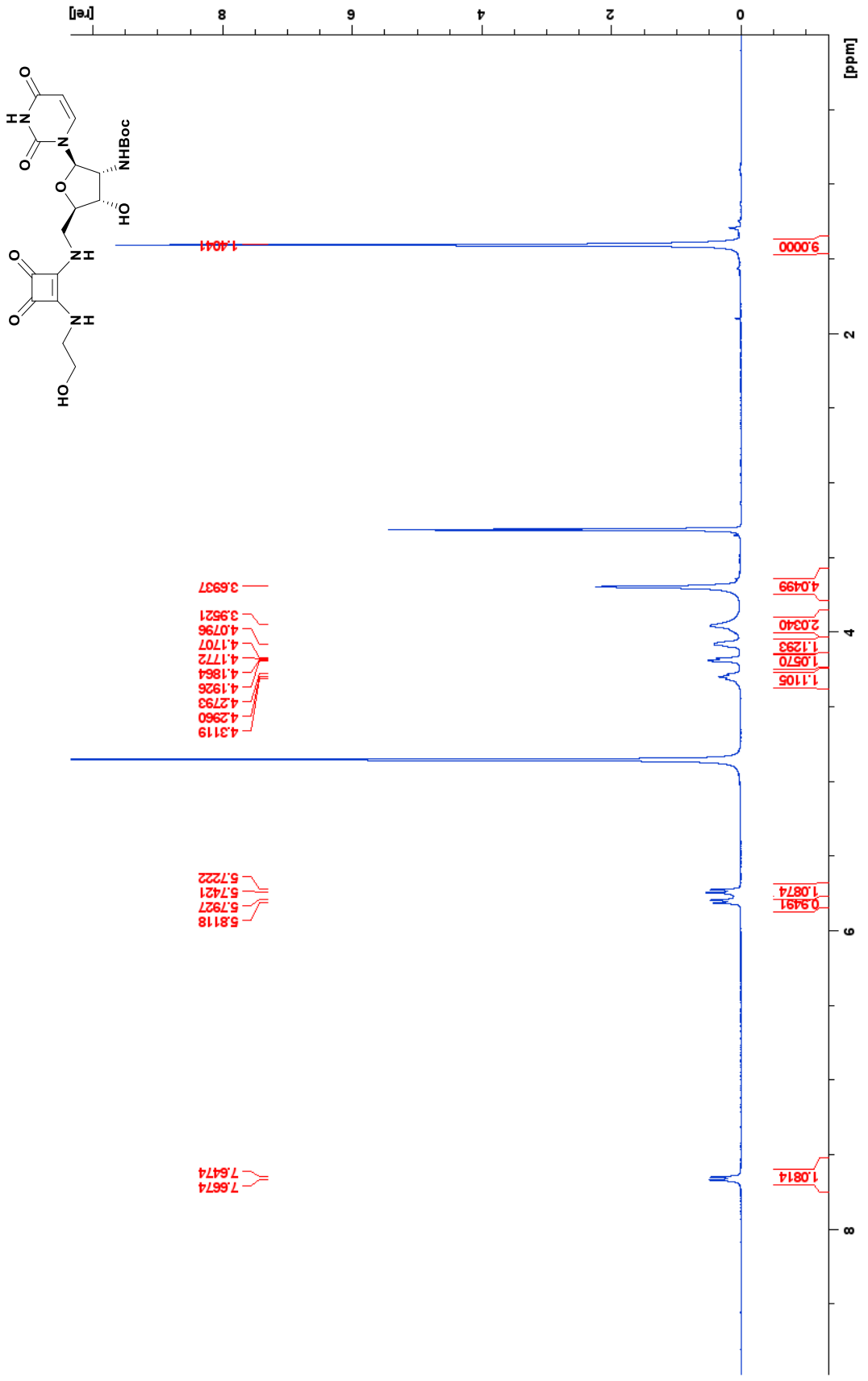
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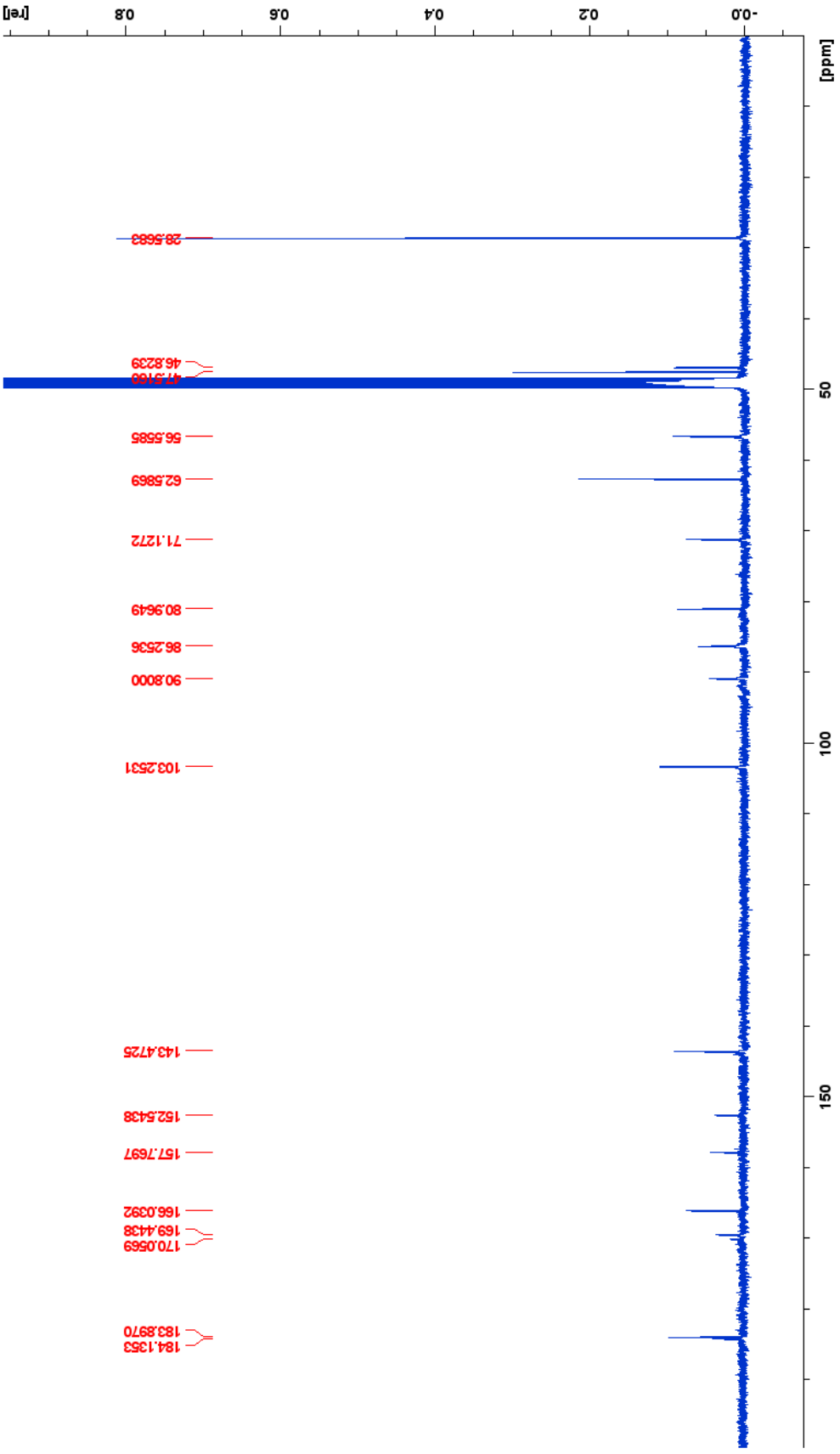
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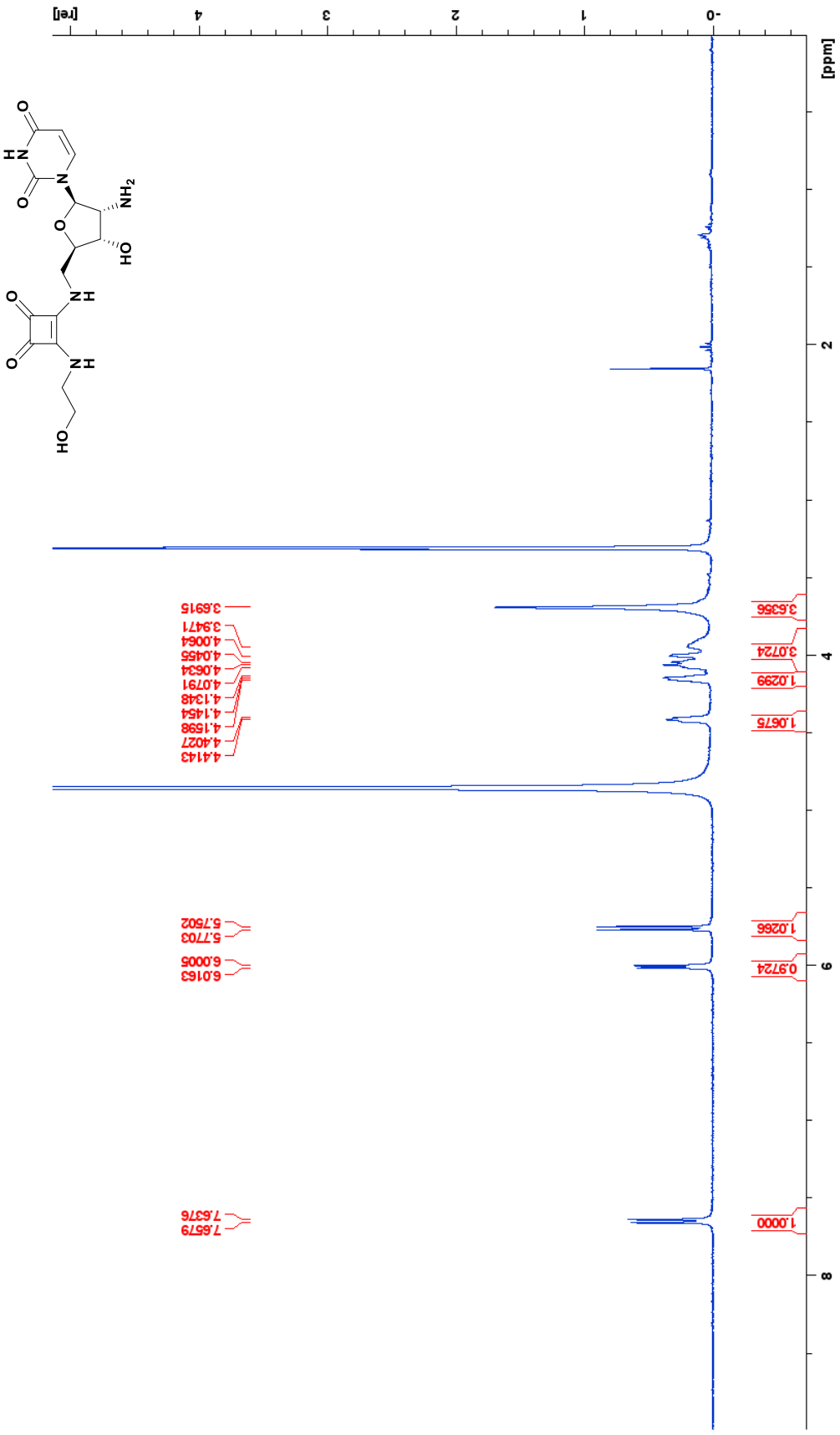
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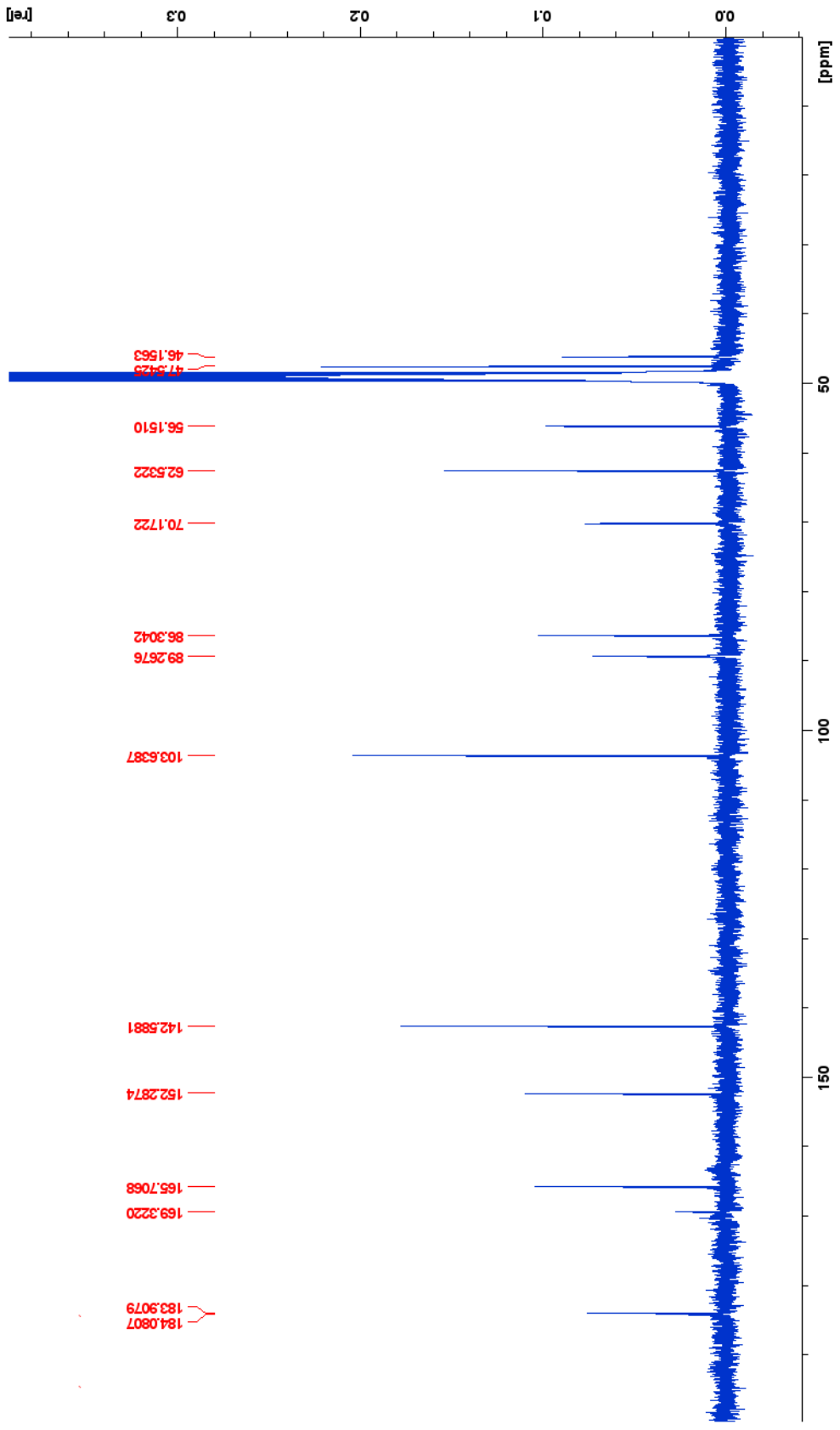
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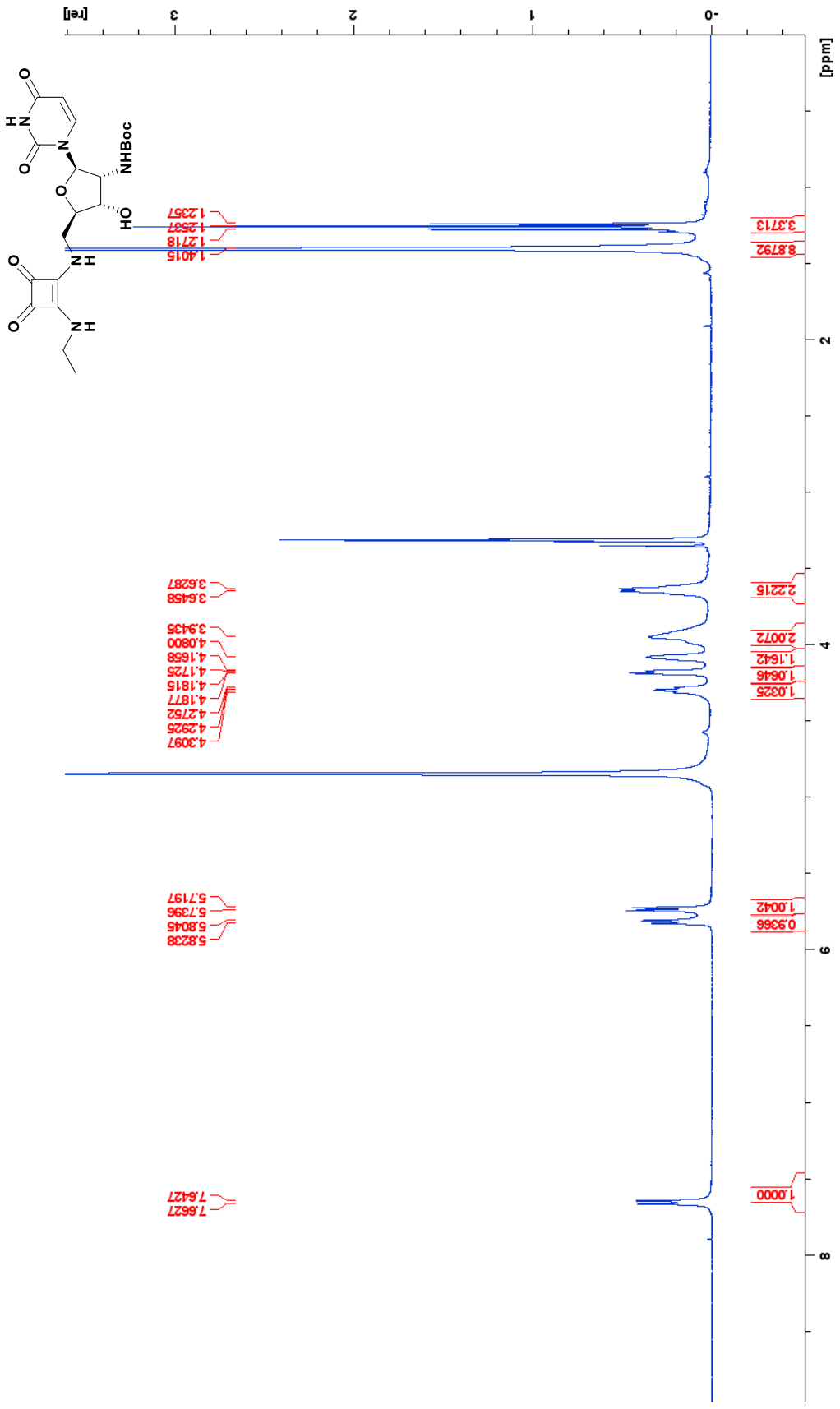
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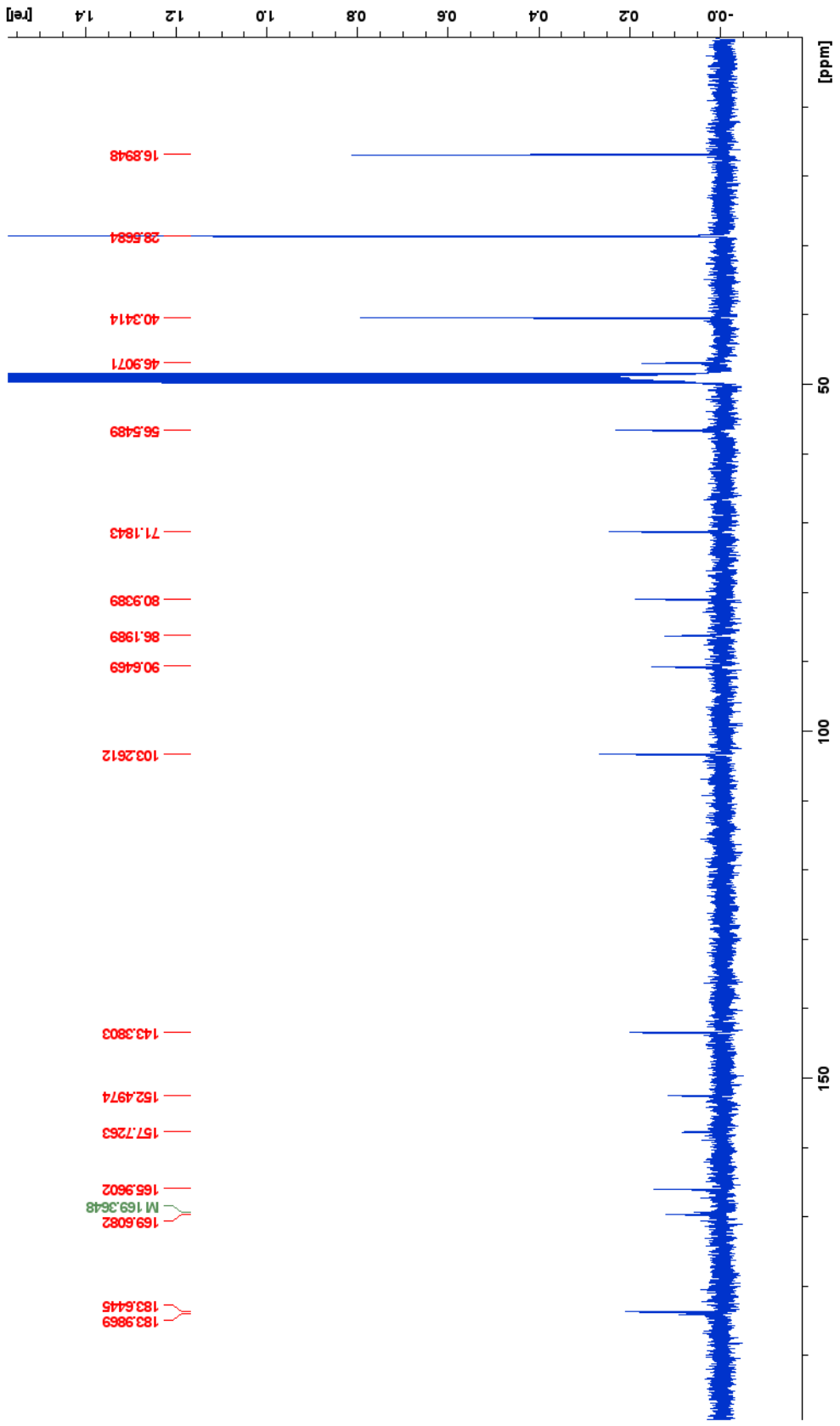
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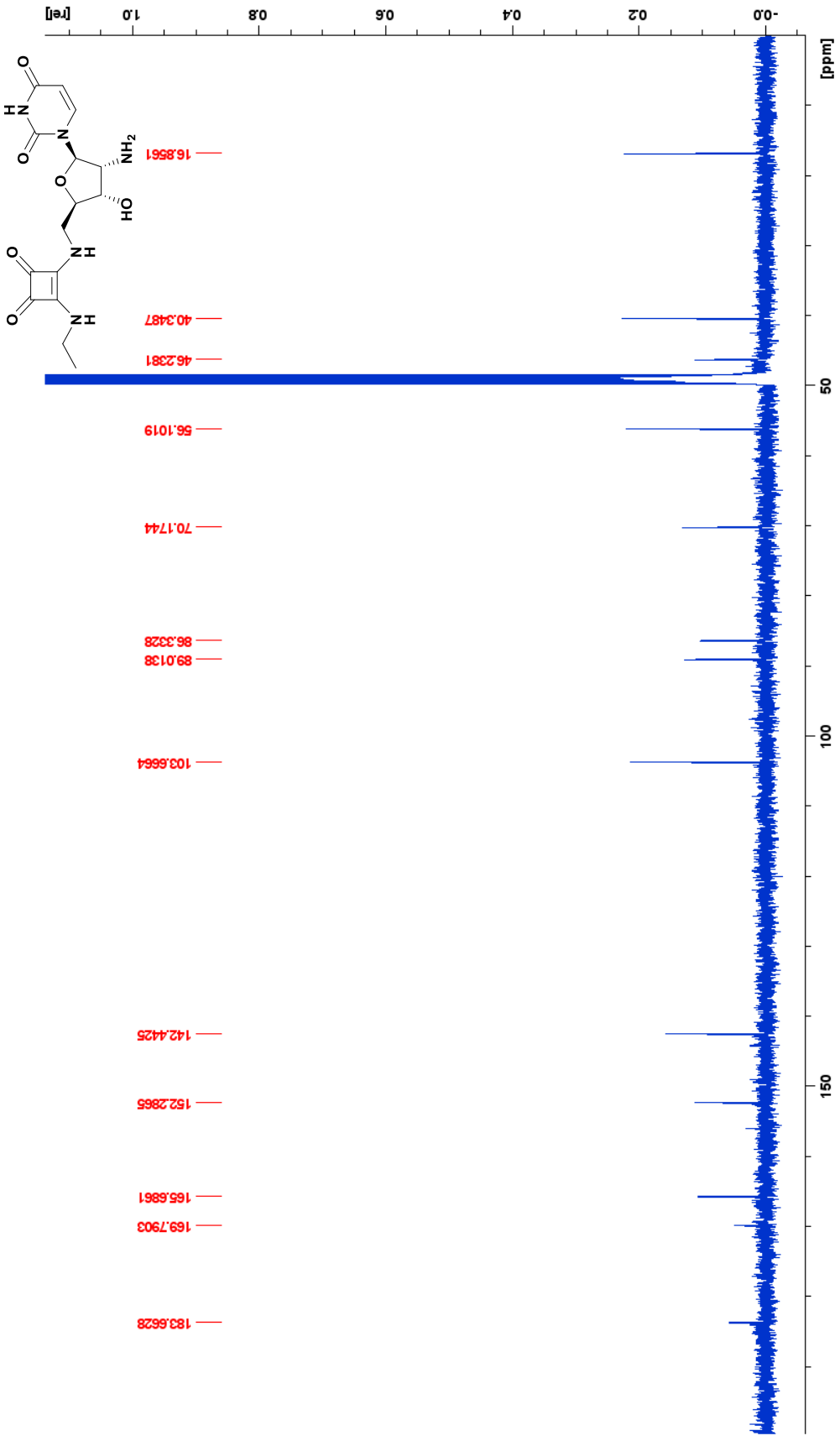
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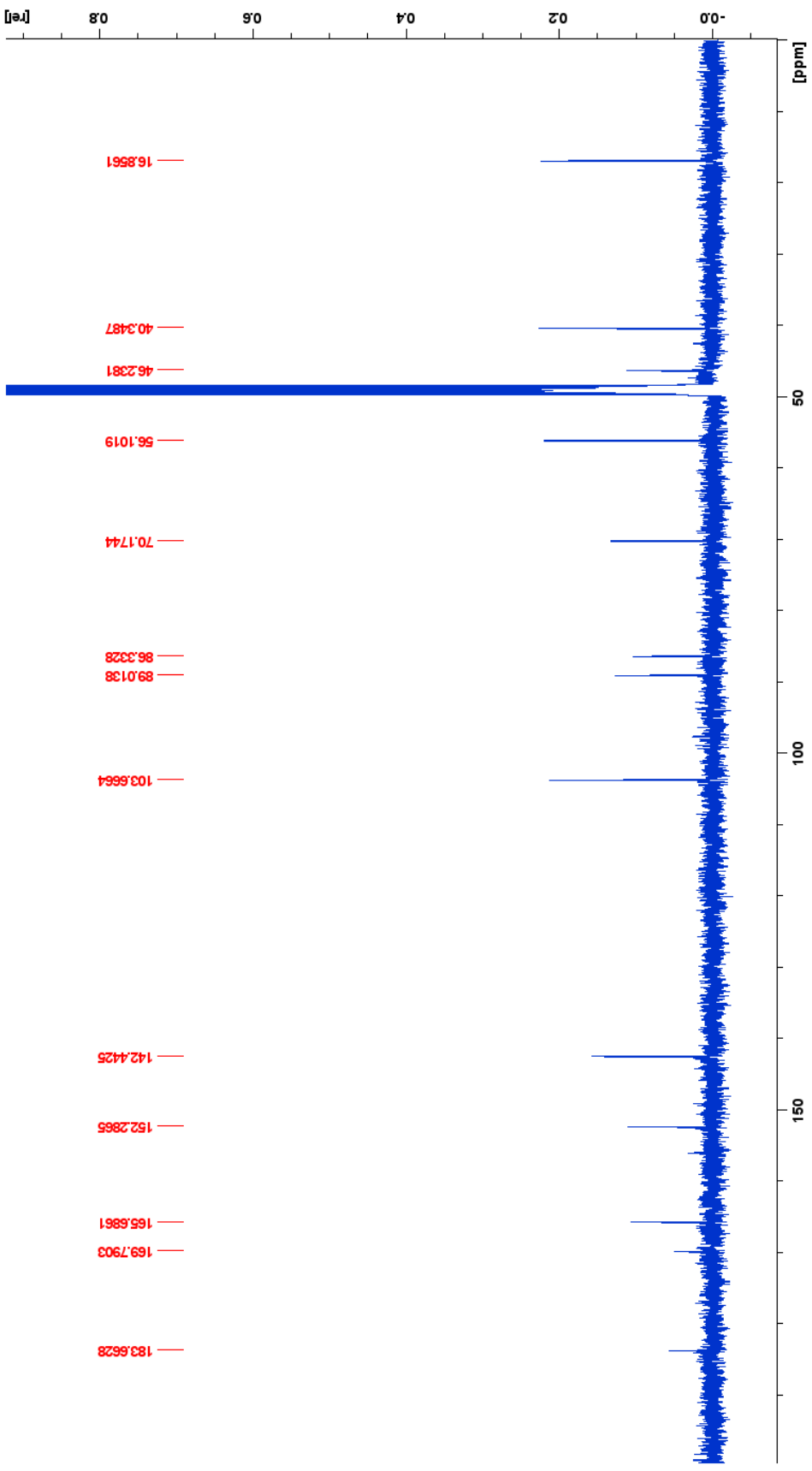
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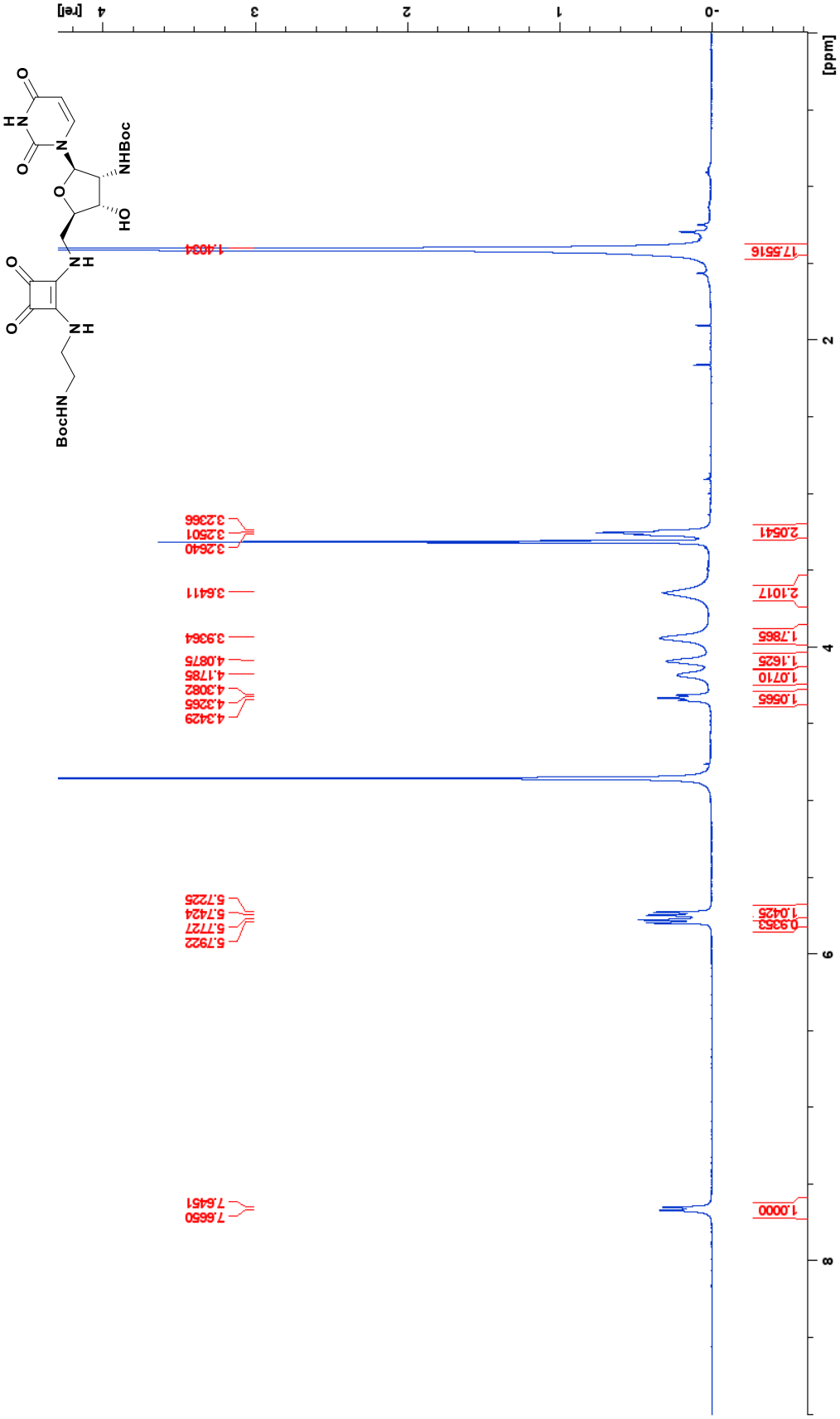
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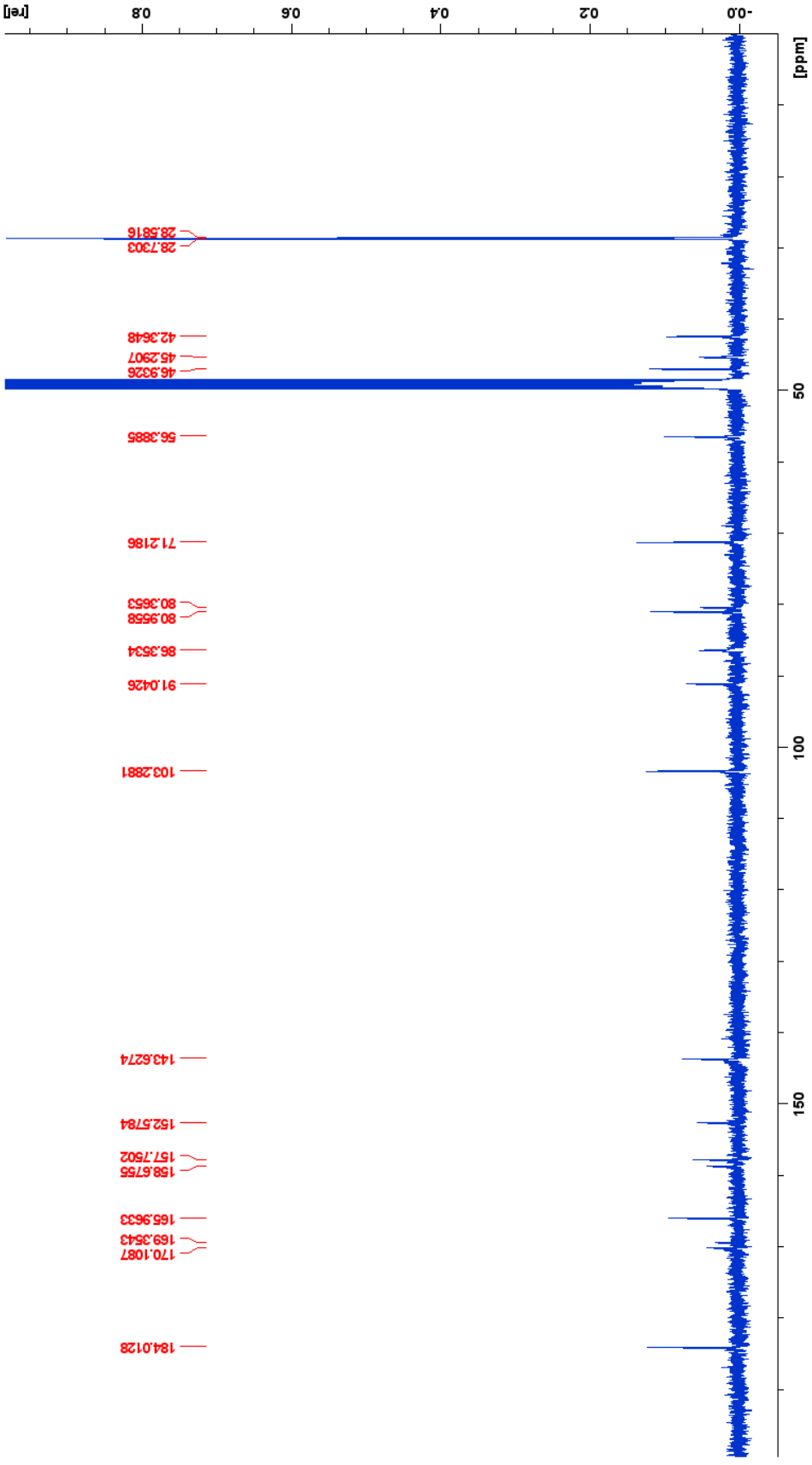
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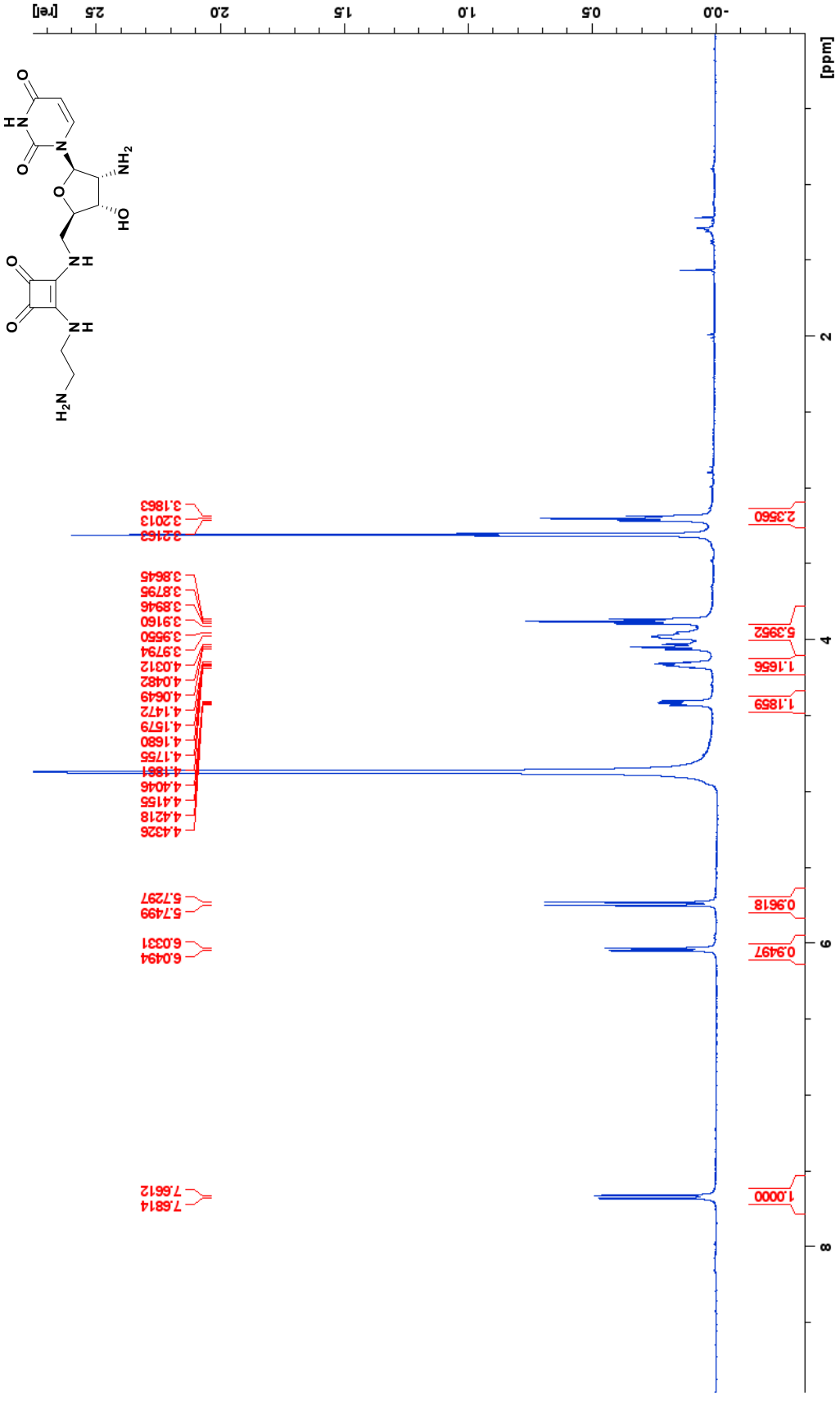
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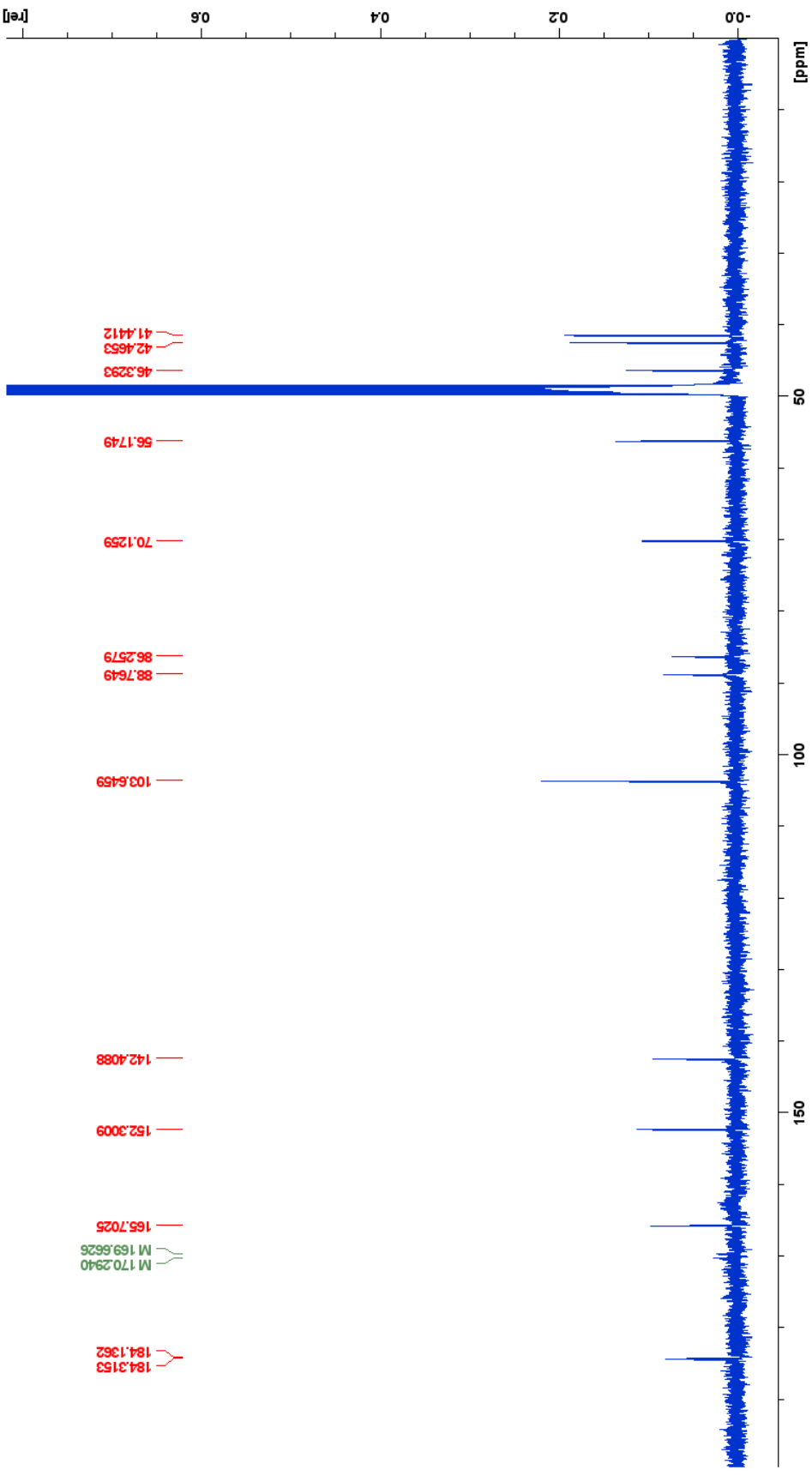
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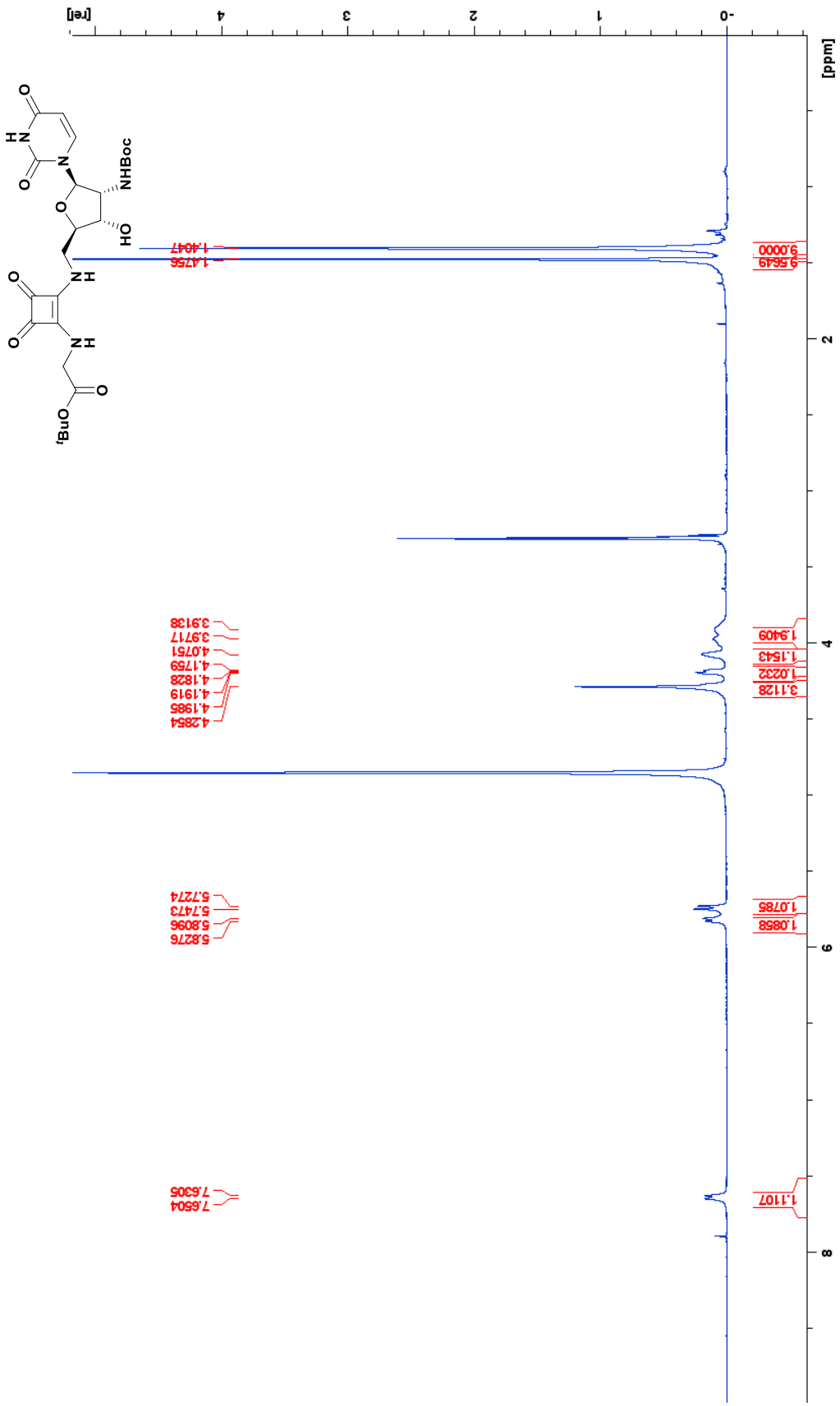
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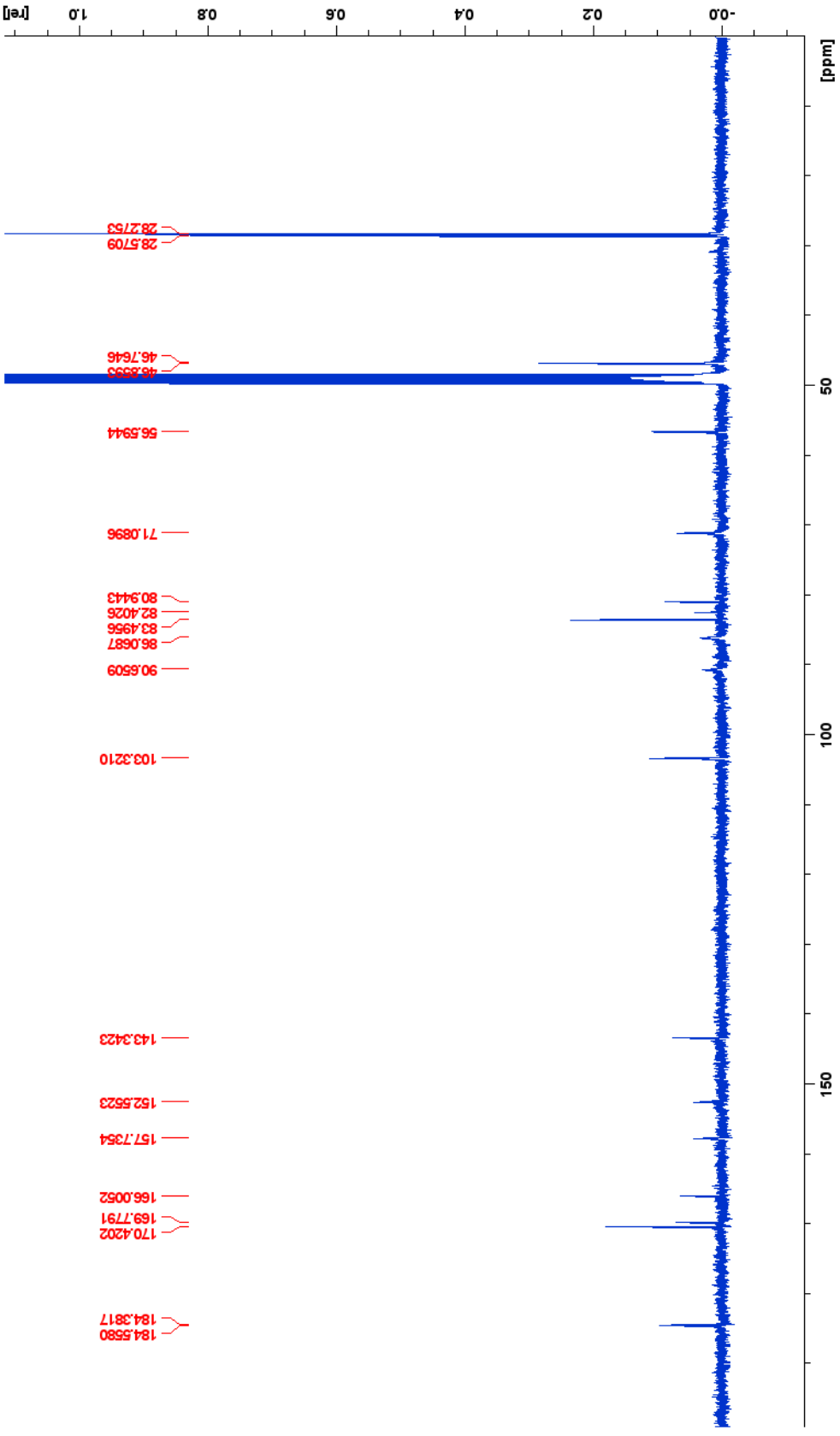
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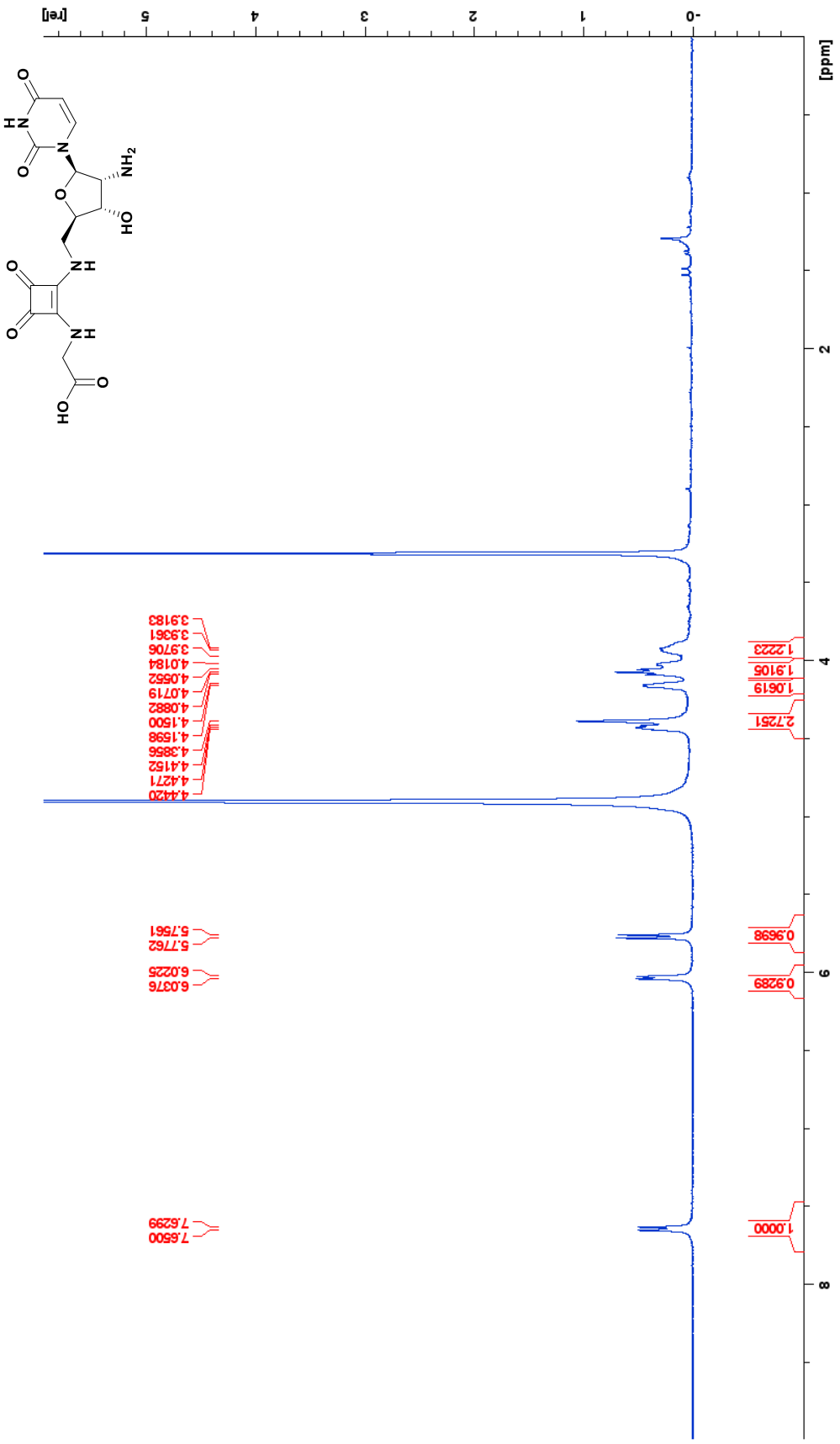
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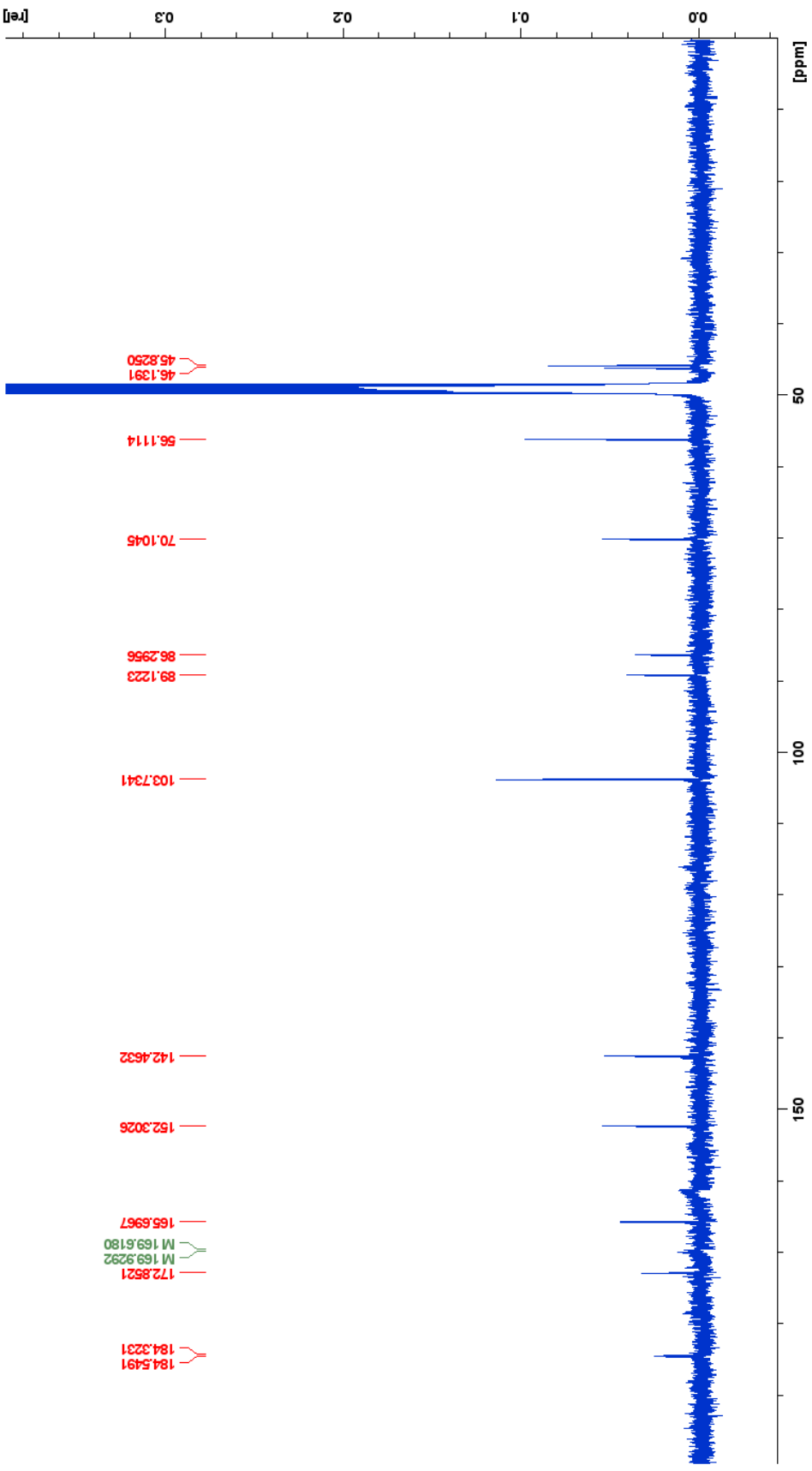
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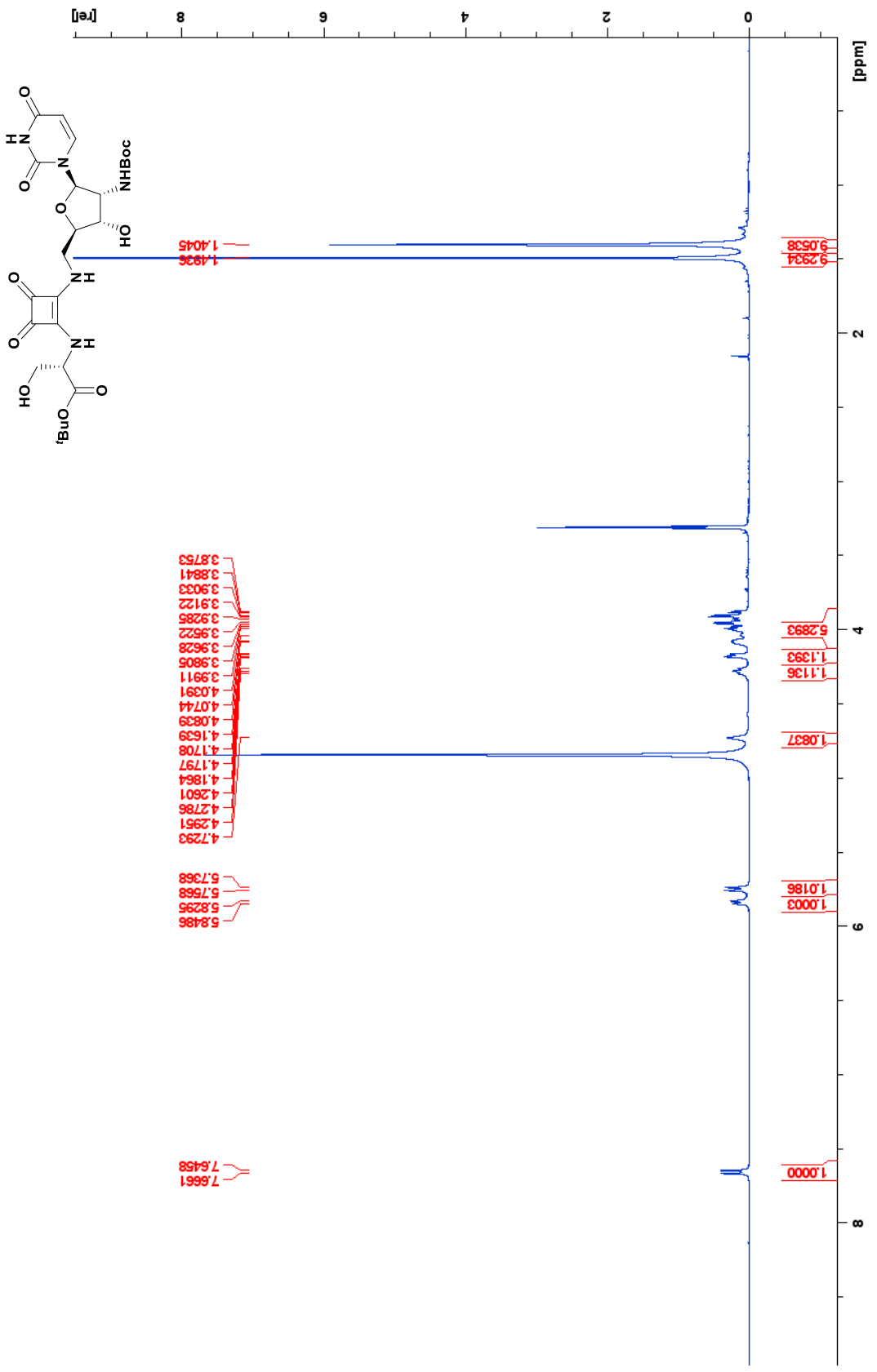
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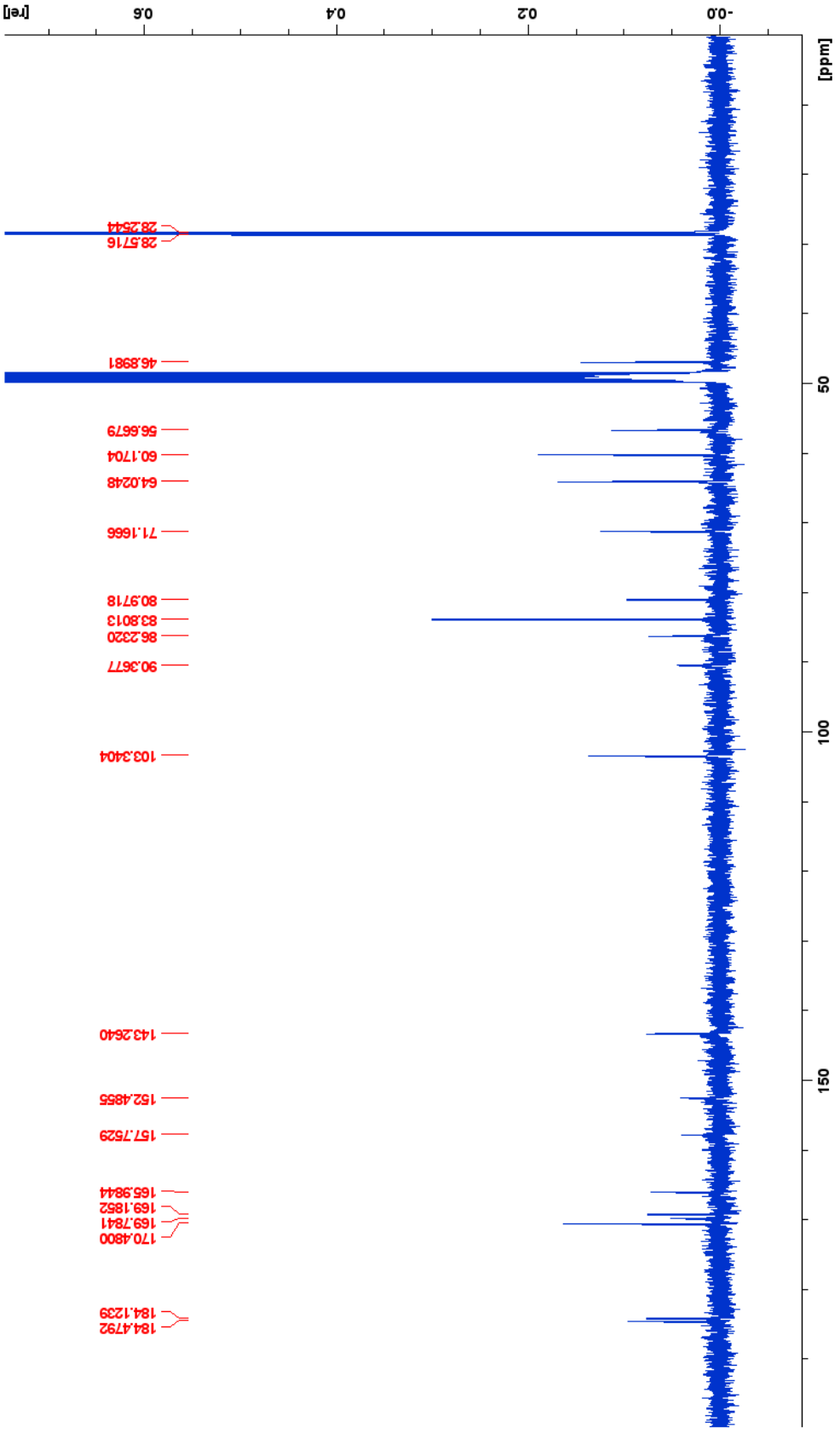
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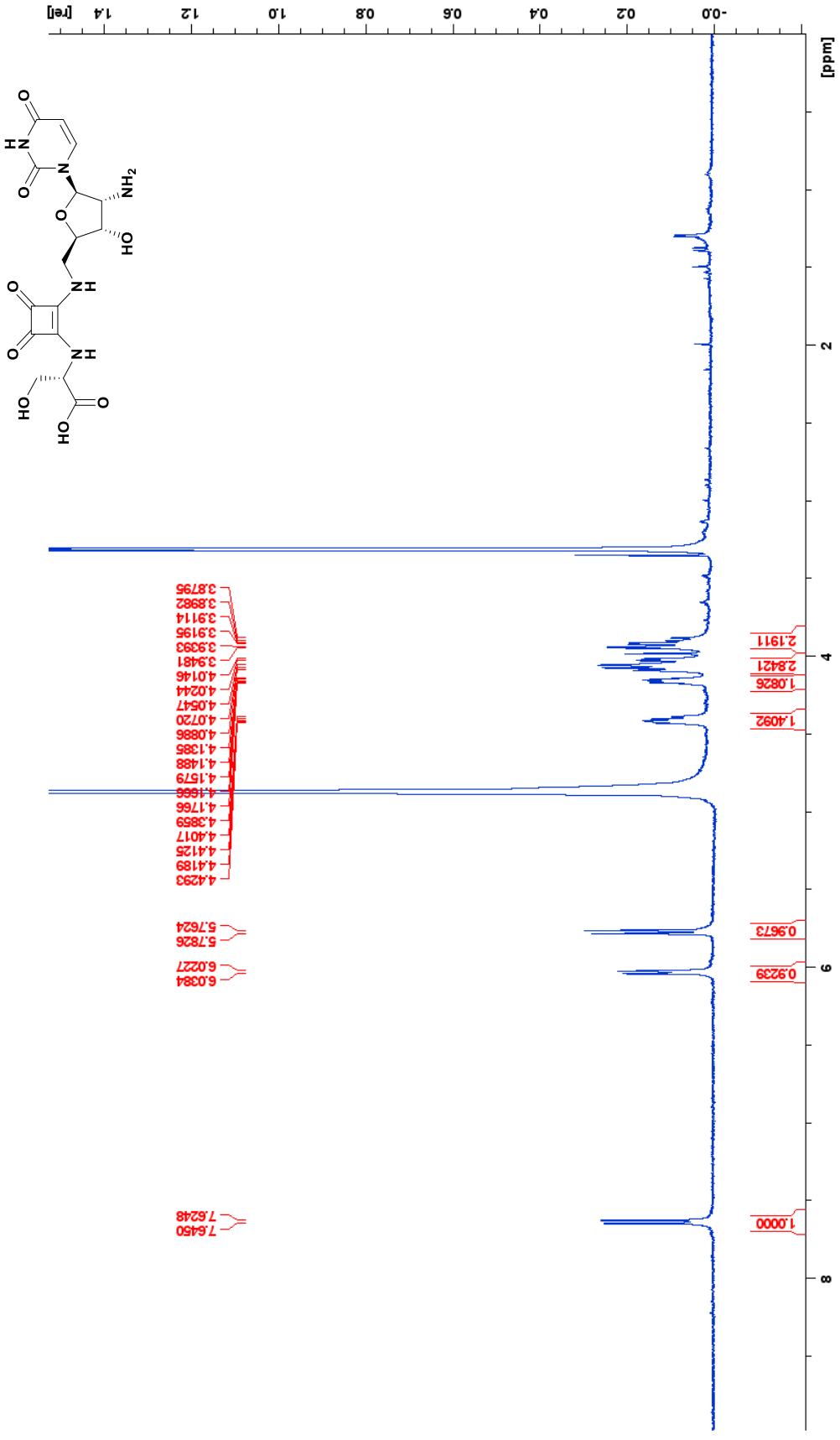
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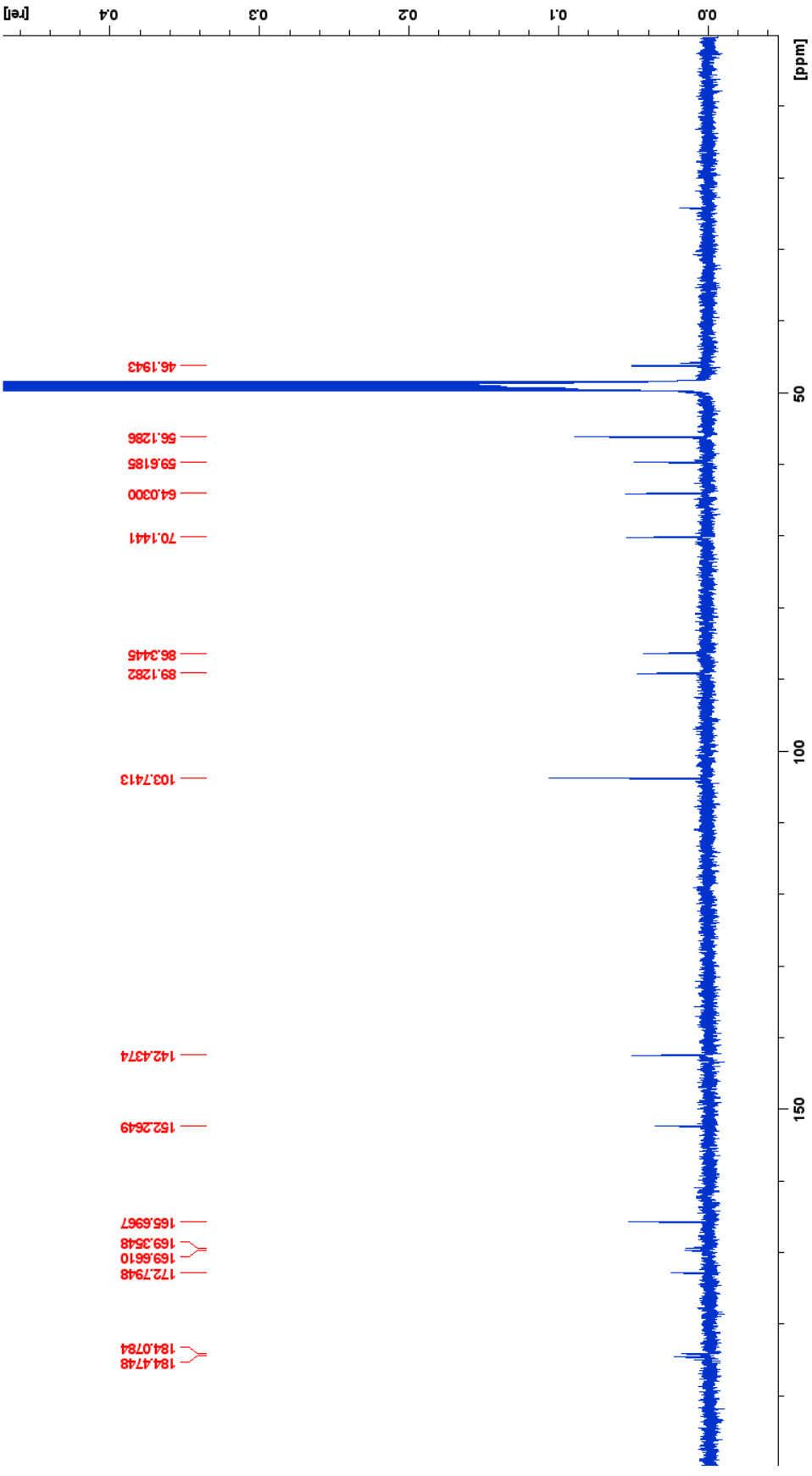
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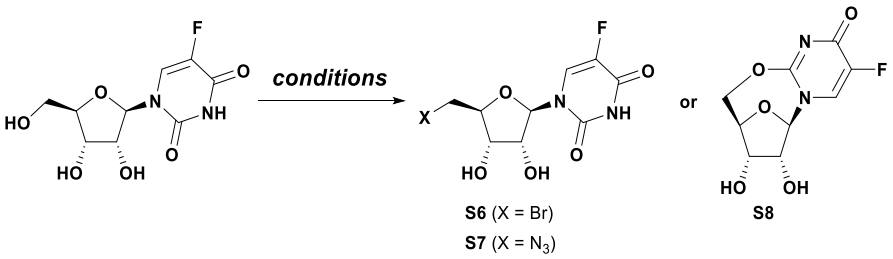
14. Investigation of reaction conditions

(1) Conditions for transformation of 5'-OH of 5-fluorouridine

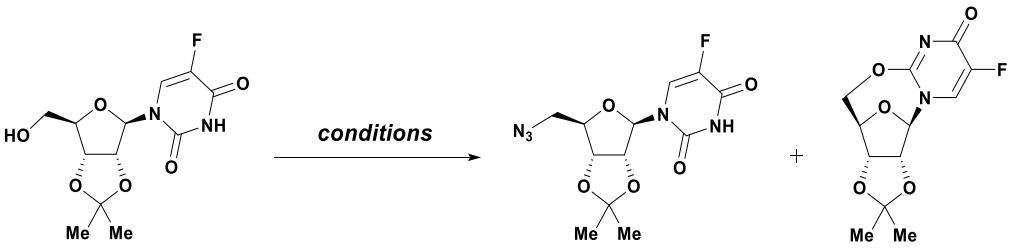


S6 (X = Br)
S7 (X = N_3)

S8

entry	conditions	solvent (SM conc.)	temp.	results
1	CBr_4,PPh_3 (1.5 eq. each) (4 h); CBr_4, PPh_3 (1.5 eq. each more) (19 h)	dry DMF (0.4 M)	rt	S6, 24%
2	NaN_3 (5.0 eq.), CBr_4 (1.1 eq.), PPh_3 (1.1 eq.)	dry DMF (0.15 M)	80 °C	N.R.
3	DIAD (1.5 eq.), PPh_3 (1.5 eq.)	dry MeCN (0.2 M)	rt	almost N.R.

(2) Conditions for azidation or cyclization of S1

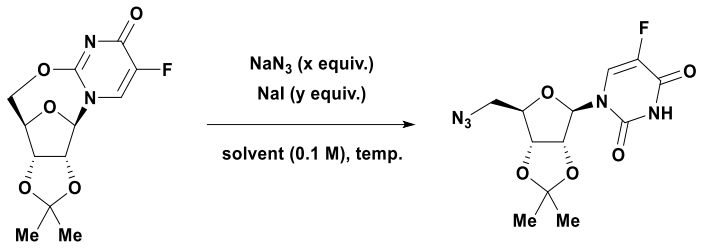


S1 **5** **S2**

entry	conditions	solvent (SM conc.)	temp. (C)	results
1	DPPA (1.5 equiv.), DIAD (1.5 equiv.), PPh_3 (1.5 equiv.)	THF (0.4 M)	rt	5 (0%), S2 ([a]NMR y. 38%)
2	DIAD (1.5 equiv.), PPh_3 (1.5 euiv.)	THF (0.1 M)	rt	S2 ([b]i.y. 59%)
3	phthalimide (2.5 eq.), DIAD (2.5 eq.), PPh_3 (2.5 eq.)	THF (0.4 M)	rt	S2 ([b]i. y. 77%)
4	phthalimide (3.0 eq.), DIAD (1.5 eq.), PPh_3 (1.5 eq.)	THF (0.4 M)	rt	S2 ([b]i. y. 90%)

[a]NMR y. = NMR yield. 1,1,2,2-tetrachloroethane was used as internal standard. [b]i.y. = isolated yield.

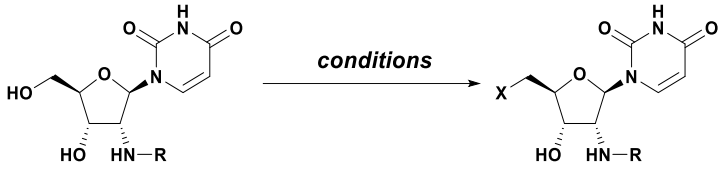
(3) Azidation at C5' of S2 via ring opening



S2 5

entry	x	y	temp.	solv.	TM (%)
1	3.0	1.0	90 °C	DMF	35
2	3.0	0	60 °C	THF	N.R.
3	3.0	0	60 °C	DMF	48

(4) Conditions for transformation of 5'-OH of S3



S3 (R = Boc)

6 (X = N_3, R = Boc)
S9 (X = Br, R = Boc)

entry	conditions	solvent (SM conc.)	temp. (°C)	results
1	CBr_4 (2.0 eq.), PPh_3 (2.0 eq.)	DMF (0.1 M)	rt	**S9** (19%), 28% (X = Br, R = H)
2	CBr_4 (2.0 eq.), PPh_3 (2.0 eq.), imidazole (2.0 eq.)	DMF (0.1 Mr)	rt	**S9** (56%)
4	DPPA (1.5 eq.), DIAD (1.5 eq.), PPh_3 (1.5 eq.)	dry THF (0.1 M)	rt	**6** (37%)
5	NaN_3 (4.7 eq.), CBr_4 (1.0 eq.), PPh_3 (1.0 eq.)	dry DMF (0.15 M)	rt → 80	**6** (68%)

Biological experiments

1. Insect cell expression of human UGGT (HUGT1)

To construct the plasmid for expressing His and Flag-tagged HUGT1 (His-Flag-HUGT1), the coding region of HUGT1 was amplified by PCR using the primer pair: 5'-CATCGGGCGCGGATCCCATCATCACCATCACCATGACTACAAAGACCATGACGG -3' and 5'-ACTTCTCGACAAGCTTTCATAATTCTTCACGTTTCTGAGG -3', and pXXXX[9] was used as a template. The amplified fragments were cloned into BamHI-digested pFastBac1 vector (Invitrogen, Carlsbad, CA) with a gp67 secretion in the 5'-upstream region of BamHI siteusing the In-Fusion HD Cloning Kit (Takara Bio Inc, Shiga, Japan). The resultant vector pFastBac1-His-FLAG-HUGT1 was introduced into E. coli DH10Bac (Invitrogen) and E. coli colonies with recombinant Bacmid was selected following by manufacture's protocol. The recombinant Bacmid DNA was isolated using a Plasmid Midi Kit (QIAGEN, Chatsworth, CA) and then transfected into Spodoptera frugiperda (Sf9) cells using Lipofectamine (ThemoFisher Scientific, Waltham, MA). After the preparation of recombinant P1 baculovirus, HUGT1 production was analyzed by Western blotting using anti-FLAG M2 antibody (Sigma, St. Louis, MO) and anti-mouse antibody as primary and secondary antibodies, respectively. The specific signals were visualized using Luminate Forte western HRP Substrate (MILLIPORE, Billerica,MA) and iBright system (TermoFisher Scientific). The baculovirus producing the largest amount of HUGT1 was used for the preparation of P2 baculovirus and the P2 baculovirus was used for further study.

2. Conditions of Glc transfer reaction from UDP-Glc to M9-Asn-BODPIY by UGGT (HUGT1)

Method of the HUGT1 activity assay

To a solution of HUGT1 (0.07 mg/mL) and M9-Asn-BODIPY[10] (4 μM) in 4 mM Tris-HCl (pH 7.6) containing 0.05% TritonX-100, 1 mM 2-mercaptoethanol and 2 mM $CaCl_2$ was added an inhibitor candidate (the concentration was adjusted to 25, 50, 100 or 250 μM, respectively). After incubation of the mixture at 25 ℃ for 10 minutes, to the mixture was added UDP-Glc (final conc. 25, 100, 250 or 500 μM for the each concentration of the inhibitor candidate). The mixture was incubated at 37 ℃ for 4 h and then 50 μL acetonitrile was added to quench the reaction. The percentage of Glc transfer in each reaction was analyzed using high-performance liquid chromatography (HPLC) under the following conditions: TSK-gel Amide 80 column (Tosoh) (4.6 mm × 150 mm); mobile phase: CH_3CN/100 mM ammonium formate (pH 4.5); linear gradient from 65:35 to 55:45 for 20 min; flow rate: 1.0 mL/min; temperature: 40 ℃. BODIPY-labeled glycans were quantified by fluorescence intensities (λ_{ex} = 488 nm, λ_{em} = 520 nm) using a Waters 2475 fluorescence detector.

3. **GlcNAc transfer reaction from UDP-GlcNAc to an acceptor substrate by GnTs (GnT-I, GnT-II, GnT-III, GnT-IVa, GnT-V)**

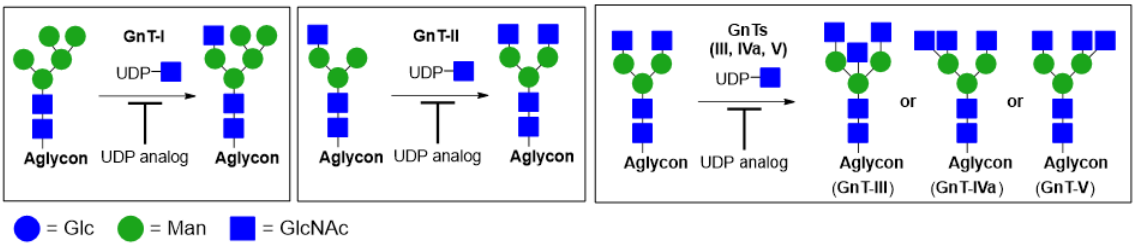


Fig. S10 Diagram of GlcNAc transfer reaction by GnTs (Gn-I~V)

Method of GnTs activity assay

Purification and activity assay of GnTs were performed as described previously.[11] In brief, COS7 cells were transfected with pcDNA-IH/GnTs. After 4–6 h, the medium was changed to Opti-MEM I, followed by culture for 3 days. His-tagged enzymes in the medium were purified through a Ni^{2+}-column. Purified GnTs were incubated at 37°C with the acceptor substrate (Man5-fluorescein for GnT-I, GnMbi-PA (pyridylamine) for GnT-II, and GnGnbi-PA for GnT-III, -IVa, and -V), 0.5 mM UDP-GlcNAc, and a compound (0, 0.15625, 0.625, 2.5 and 10 mM) in 10 μL of buffer containing 125 mM MES (pH 6.25), 20 mM $MnCl_2$, 200 mM GlcNAc, 0.5% (v/v) Triton X-100, and 1 mg/ml BSA. After 60–180 min, the reaction was stopped by boiling and the mixture was diluted with 40 μL water, followed by centrifugation at 15,000 × *g* for 3 min. The supernatant was analyzed by reverse-phase HPLC with an ODS column (TSKgel ODS-80TM, TOSOH Bioscience). The results are shown as values relative to those in the absence of a compound.

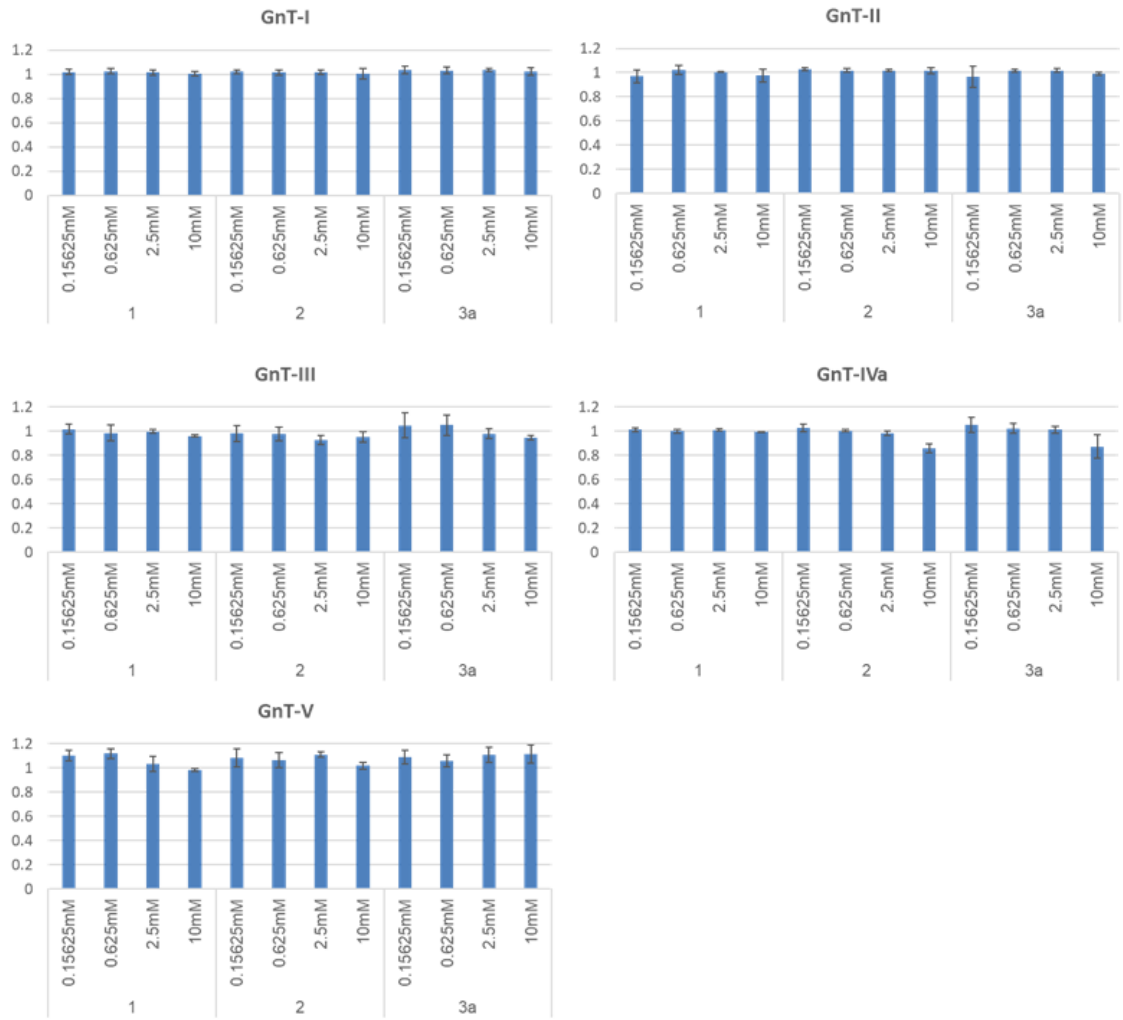


Fig. S11 Investigation of the inhibitory activity with **1**, **2** and **3a** for GlcNAc transfer reaction by GnTs (GnT-I~V). There is no inhibitory activity against GnTs.

4. References

9. Takeda, Y., Seko, A., Fujikawa, K., Izumi, M., Kajihara, Y., Ito, Y. *Glycobiology*, 2016, **26**, 999-1006.
10. Kikuma, T., Ibuki, H., Nakamoto, M., Seko, A., Ito, Y., Takeda, Y. *Biochem. Biophys. Res. Commun*. 2022, **612**, 44-49.
11. Vibhute, A. M., Tanaka, H., Mishra, S. K., Osuka, R. F., Nagae, M., Yonekawa, C., Korekane, H., Doerksen, R. J., Ando, H., Kizuka Y. *Biochim. Biophys. Acta Gen. Subj.* 2022, **1866**, 130118.