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Supporting Information

An Expedient Synthesis of Flexible Nucleosides through Enzymatic Glycosylation of Proximal and Distal Fleximer Bases

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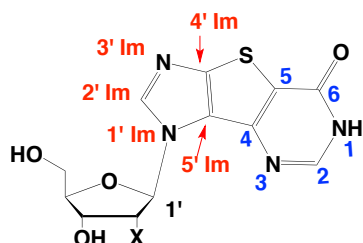
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Only the spectra (as recorded below) are shown.

Compound	Advance 400	Advance 600	Neo 800
2a	^1H	^{13}C	
2b	^1H	^{13}C	
3a	^1H , ^{13}C		
4a	^1H , ^{13}C		
5a			^1H , ^{13}C , HMBC
5b			^1H , ^{13}C , HMBC
5c			^1H , ^{13}C , HMBC
3c	^1H , ^{13}C		
5d		^1H , ^{13}C , HMBC	
5e		^1H , ^{13}C , HMBC	

Nomenclature used for NMR description



General procedures. Canonical nucleobases and nucleosides were purchased from Sigma-Aldrich or Carbosynth. Flex-bases **1-5** were synthesized according to published procedures.^[1,2] Analytical HPLC was carried out on an Agilent system (1200 series) using a C18 reverse phase column (Kromasil, 5 μm , 100 \AA , 4.6x150 mm) at a flow rate of 1 mL/min and a linear gradient of acetonitrile in 10 mM triethylammonium acetate buffer over 20 min (Gradients: G1, 0 to 20 %; G2, 0 to 40%). Purification by preparative HPLC was carried out on an Agilent 1100 Series system on a C18 reverse phase column (Kromasil, 5 μm , 100 \AA , 10x250 mm) using a flow rate of 4.0 mL/min and a linear gradient of acetonitrile in 10 mM triethylammonium acetate buffer over 20 min. ^1H and ^{13}C NMR spectra were recorded on a Bruker Advance 400, Advance 600 or Avance Neo 800. Chemical shifts are given in ppm (δ) relative to residual solvent peak, coupling constants (J) are reported in Hertz and standard abbreviations are used. Assignment of ^1H and ^{13}C signals was performed by analysis of the correlated homonuclear ^1H , ^1H -COSY and heteronuclear ^1H , ^{13}C -HMBC, ^1H , ^{13}C -HSQC spectra. High-resolution mass spectra were recorded on a Waters Q-TOF micro MS instrument under electrospray ionization in positive ionization mode using a mobile phase of acetonitrile/water with 0.1 % formic acid.

NDT-catalyzed transglycosylation reaction. Nucleoside 2'-deoxyribosyltransferases class II from *L. leichmannii* (LINDT) was produced and purified as described previously.^[3] Typically, reaction mixtures (0.1 mL) contained a flex-base (1 μmol) as the acceptor and thymidine (4 μmol) as the donor. A 0.2 M stock solution of flex-base in DMSO was added slowly to 10 mM citrate buffer (pH 6.5) containing thymidine to reach a final volume of 0.1 mL. Reaction was started by adding NDT to the mixture (variable amounts) and incubation was carried out at 37°C. Conversion was monitored by analytical reverse phase HPLC. Optimal conditions leading to all possible glycosylated products were scaled-up to the preparative scale, and the corresponding nucleosides were isolated after purification by reverse phase HPLC and fully characterized.

NDT-catalyzed transglycosylation of flex-base 2. Compound **2** (4.0 mg, 0.02 mmol) and thymidine (20.0 mg, 0.08 mmol) in citrate buffer (4.0 mL, 5% v/v DMSO) were incubated in the presence of NDT (25 μL) at 37°C for 4 h (75% conversion). After purification by reverse phase HPLC, three compounds were isolated, the starting material **2**, and the C4- and C5-pyrimidinyl 2'-deoxyribosylimidazoles derivatives (**2a** and **2b**).

2-Amino-4-methoxy-5-[1-(2'-deoxy- β -D-ribofuranosyl)-1H-imidazol-4-yl]-pyrimidine

(2a): 0.8 mg (13% yield); $t_R = 14.88$ min (G1); ^1H NMR (400 MHz, DMSO- d_6): δ 2.20-2.28 (ddd, $J = 2.9, 5.9, 13.1$ Hz, 1H, H-2'), 2.35-2.42 (ddd, $J = 5.9, 7.3, 13.1$ Hz, 1H, H-2''), 3.49-3.55 (m, 2H, H-5', H-5''), 3.78-3.84 (m, 1H, H-4'), 3.94 (s, 3H, OCH₃), 4.28-4.35 (m, 1H, H-3'), 4.89 (t, $J = 5.2$ Hz, 1H, OH-5'), 5.23 (d, $J = 3.8$ Hz, 1H, OH-3'), 6.03 (t, $J = 6.5$ Hz, 1H, H-1'), 6.50 (br s, 2H, NH₂), 7.47 (d, $J = 1.0$ Hz, 1H, H-5 Im), 7.87 (br s, 1H, H-2 Im), 8.59 (s, 1H, H-6 Pyr); ^{13}C NMR (200 MHz, DMSO- d_6): δ 41.2, 53.1, 62.2, 71.3, 86.4, 88.1, 104.9, 115.1, 134.6, 136.8, 155.8, 162.3, 165.8; HRMS (ESI-TOF) m/z calcd for [C₁₃H₁₇N₅O₄ + H]: 308.1359, found: 308.1355.

2-Amino-4-methoxy-5-[1-(2'-deoxy- β -D-ribofuranosyl)-1H-imidazol-5-yl]-pyrimidine

(2b): 1.0 mg (16% yield); $t_R = 12.77$ min (G1); $^1\text{H NMR}$ (400 MHz, DMSO- d_6): δ 2.12-2.20 (ddd, $J = 3.4, 6.0, 13.3$ Hz, 1H, H-2'), 2.30-2.38 (m, 1H, H-2'), 3.47-3.53 (m, 2H, H-5', H-5''), 3.70-3.75 (m, 1H, H-4'), 3.82 (s, 3H, OCH₃), 4.20-4.28 (m, 1H, H-3'), 4.88 (t, $J = 5.4$ Hz, 1H, OH-5'), 5.18 (d, $J = 4.7$ Hz, 1H, OH-3'), 5.57 (dd, $J = 6.2, 7.9$ Hz, 1H, H-1'), 6.79 (d, $J = 0.9$ Hz, 1H, H-4 Im), 6.84 (br s, 2H, NH₂), 7.90 (s, 1H, H-6 Pyr), 8.00 (d, $J = 0.9$ Hz, 1H, H-2 Im); $^{13}\text{C NMR}$ (200 MHz, DMSO- d_6): δ 41.7, 53.7, 62.0, 71.1, 84.6, 87.9, 98.9, 125.8, 128.2, 136.4, 159.9, 164.2, 167.5; HRMS (ESI-TOF) m/z calcd for [HRMS (ESI-TOF) m/z calcd for [C₁₃H₁₇N₅O₄ + H]: 308.1359, found: 308.1355.

2,6-Dimethoxy-4-[1-(2'-deoxy- β -D-ribofuranosyl)-1H-imidazol-4-yl]-pyrimidine (3a).

Compound **3a** was obtained starting from **3** (8.0 mg, 0.039 mmol) and thymidine (40.0 mg, 0.17 mmol) in the presence of NDT (100 μL) in citrate buffer (4.0 mL, 5% v/v DMSO) at 37°C overnight (100% conversion). Purification by reverse phase HPLC gave **3a** (8.2 mg, 66% yield); $t_R = 13.33$ min (G2); $^1\text{H NMR}$ (400 MHz, DMSO- d_6): δ 2.24-2.32 (ddd, $J = 3.3, 6.2, 13.2$ Hz, 1H, H-2'), 2.37-2.46 (ddd, $J = 6.0, 7.3, 13.3$ Hz, 1H, H-2''), 3.48-3.61 (m, 2H, H-5', H-5''), 3.62-3.87 (m, 1H, H-4'), 3.91 (s, 3H, OCH₃), 3.94 (s, 3H, OCH₃), 4.30-4.37 (m, 1H, H-3'), 4.95 (br s, 1H, OH-5'), 5.28 (br s, 1H, OH-3'), 6.11 (t, $J = 6.1$ Hz, 1H, H-1'), 6.83 (s, 1H, H-6 Pyr), 8.00 (d, $J = 1.3$ Hz, 1H, H-5 Im), 8.04 (d, $J = 1.3$ Hz, 1H, H-2 Im); $^{13}\text{C NMR}$ (100 MHz, DMSO- d_6): δ 41.5, 54.1, 54.7, 62.1, 71.1, 86.4, 88.3, 94.5, 119.1, 138.0, 139.8, 162.6, 165.5, 172.3; HRMS (ESI-TOF) m/z calcd for [C₁₄H₁₈N₄O₅ + H]: 323.1356, found: 323.1352.

2-Amino-6-methoxy-4-[1-(2'-deoxy- β -D-ribofuranosyl)-1H-imidazol-4-yl]-pyrimidine

(4a). Compound **4a** was obtained starting from **4** (5.5 mg, 0.029 mmol) and thymidine (27.5 mg, 0.11 mmol) in the presence of NDT (35 μL) in citrate buffer (2.5 mL, 5% v/v DMSO) at 37°C overnight (100% conversion). Purification by reverse phase HPLC gave **4a** (3.2 mg, 36% yield); $t_R = 15.42$ min (G1); $^1\text{H NMR}$ (400 MHz, DMSO- d_6): δ 2.11-2.41 (ddd, $J = 3.1, 6.1, 13.3$ Hz, 1H, H-2'), 2.32-2.43 (ddd, $J = 5.9, 7.4, 13.3$ Hz, 1H, H-2''), 3.51 (br s, 2H, H-5', H-5''), 3.81-3.85 (m, 4H, H-4', OCH₃), 4.29-4.34 (m, 1H, H-3'), 4.92 (br s, 1H, OH-5'), 5.27 (br s, 1H, OH-3'), 6.08 (t, $J = 6.4$ Hz, 1H, H-1'), 6.40 (br s, 2H, NH₂), 6.45 (s, 1H, H-6 Pyr), 7.76 (d, $J = 1.2$ Hz, 1H, H-5 Im), 7.94 (d, $J = 1.2$ Hz, 1H, H-2 Im); $^{13}\text{C NMR}$ (100 MHz, DMSO- d_6): δ 41.5, 53.3, 62.2, 71.2, 86.2, 88.2, 90.3, 117.5, 137.6, 140.8, 161.9, 163.9, 171.1; HRMS (ESI-TOF) m/z calcd for [C₁₃H₁₇N₅O₄ + H]: 308.1359, found: 308.1363.

NDT-catalyzed transglycosylation of imidazo[4',5':4,5]-thieno[3,2- d]pyrimidin-5(6H)-one (5).

Compound **5** (3.25 mg, 0.016 mmol) and thymidine (16.3 mg, 0.07 mmol) in citrate buffer (2.0 mL, 5% v/v DMSO) were incubated in the presence of NDT (20 μL) at 37°C overnight (75% conversion). After purification by reverse phase HPLC, three compounds were isolated.

1'-(2'-deoxy- β -D-ribofuranosyl)-1H-imidazo[4',5':4,5]-thieno[3,2- d]pyrimidin-5(6H)-one

(5a): 0.8 mg (15% yield); $t_R = 13.33$ min (G1); $^1\text{H NMR}$ (800 MHz, DMSO- d_6): δ 2.29-2.37 (m, 1H, H-2'), 2.74-2.83 (m, 1H, H-2''), 3.50-3.69 (m, 2H, H-5', H-5''), 3.88-3.95 (m, 1H, H-4'), 4.37-4.45 (m, 1H, H-3'), 4.25 (br s, 1H, OH-5'), 5.31 (d, $J = 3.4$ Hz, 1H, OH-3'), 6.52 (dd,

$J = 5.8, 8.0$ Hz, 1H, H-1'), 8.22 (s, 1H, H-2 Pyr), 8.49 (s, 1H, H-2' Im), 12.62 (br s, 1H, NH); ^{13}C NMR (200 MHz, DMSO- d_6): δ 41.1, 62.2, 71.1, 86.3, 88.7, 121.4, 121.3, 128.3, 143.4, 144.7, 148.7, 160.1; HRMS (ESI-TOF) m/z calcd for $[\text{C}_{12}\text{H}_{12}\text{N}_4\text{O}_4\text{S} + \text{H}]$: 309.0658, found: 309.0646.

1-(2'-deoxy- β -D-ribofuranosyl)-6-(2'-deoxy- β -D-ribofuranosyl)-1H-

imidazo[4',5':4,5]thieno[3,2-d]pyrimidin-5(6H)-one (5b): 1.2 mg (17% yield); $t_R = 16.75$ min (G1); ^1H NMR (800 MHz, DMSO- d_6): δ 2.23-2.43 (m, 3H, H-2'a, H-2'b, H-2"b), 2.72-2.79 (m, 1H, H-2"a), 3.53-3.72 (m, 4H, H-5', H-5"), 3.89-3.97 (m, 2H, H-4'), 4.31-4.37 (m, 1H, H-3'b), 4.38-4.43 (m, 1H, H-3'a), 4.96 (br s, 1H, OH-5'), 5.13 (br s, 1H, OH-5'), 5.30-5.36 (m, 2H, OH-3'), 6.48 (t, $J = 6.1$ Hz, 1H, H-1'b), 6.55 (t, $J = 7.2$ Hz, 1H, H-1'a), 8.56 (s, 1H, H-2' Im), 8.83 (s, 1H, H-2 Pyr); ^{13}C NMR (200 MHz, DMSO- d_6): δ 40.5, 42.1, 61.7, 62.1, 70.4, 71.5, 85.2, 86.3, 88.6, 88.7, 120.3, 128.2, 142.3, 145.0, 146.6, 149.5, 157.0; HRMS (ESI-TOF) m/z calcd for $[\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_7\text{S} + \text{Na}]$: 447.0950, found: 447.0958.

3-(2'-deoxy- β -D-ribofuranosyl)-3H-imidazo[4',5':4,5]-thieno[3,2-d]pyrimidin-5(6H)-one (5c): 0.6 mg (11% yield); $t_R = 11.25$ min (G1); ^1H NMR (800 MHz, DMSO- d_6): δ 2.32-2.37 (ddd, $J = 3.5, 5.8, 13.3$ Hz, 1H, H-2'), 2.74-2.83 (ddd, $J = 5.8, 7.2, 13.3$ Hz 1H, H-2"), 3.56 (br s, 2H, H-5', H-5"), 3.89-3.92 (m, 1H, H-4'), 4.36 (br s, 1H, H-3'), 4.92 (br s, 1H, OH-5'), 5.41 (br s, 1H, OH-3'), 6.30 (dd, $J = 6.2, 7.1$ Hz, 1H, H-1'), 8.21 (s, 1H, H-2 Pyr), 8.32 (s, 1H, H-2' Im), 12.80 (br s, 1H, NH); ^{13}C NMR (200 MHz, DMSO- d_6): δ 39.3, 62.3, 71.2, 86.5, 88.4, 112.3, 120.3, 133.9, 142.3, 143.8, 148.2, 159.6; HRMS (ESI-TOF) m/z calcd for $[\text{C}_{12}\text{H}_{12}\text{N}_4\text{O}_4\text{S} + \text{H}]$: 309.0658, found: 309.0660.

PNP-catalyzed transglycosylation reaction.

Bacterial purine nucleoside phosphorylase (N2415, 10 U/mg) was purchased from Sigma-Aldrich. Enzymatic reaction mixtures (0.1 mL) contained a flex-base (1 μmol) and adenosine as donor (2 μmol). A 0.2 M stock solution of flex-base in DMSO was added slowly to 10 mM phosphate buffer (pH 7.4) containing adenosine to reach a final volume of 0.1 mL. The reaction was started by adding PNP (2 μL at 0.1 U/ μL) and run at 37°C or 50°C. Conversion was monitored by analytical reverse phase HPLC. Products were purified by reverse phase HPLC and characterized by NMR. Due to the limited quantities available, some ^{13}C NMR and heteronuclear correlation spectra are missing.

2,6-Dimethoxy-4-(1- β -D-ribofuranosyl-1H-imidazol-4-yl)-pyrimidine (3c). Compound **3c** was obtained starting from **3** (3.0 mg, 0.015 mmol) and adenine (0.03 mmol) in the presence of PNP (30 μL , 3 U) for 16 h at 50°C (80% conversion). Purification by reverse phase HPLC gave 3.45 mg (68% yield) of **3c**. $t_R = 12.6$ min (G2). ^1H NMR (400 MHz, DMSO- d_6): δ 3.53-3.60 (m, 1H, H-5'), 3.60-3.67 (m, 1H, H-5"), 3.91 (s, 3H, OCH₃), 3.91 (br s, 1H, H-4'), 3.94 (s, 3H, OCH₃), 4.05-4.09 (m, 1H, H-3'), 4.21 (t, $J = 5.4$ Hz, 1H, H-2'), 5.06 (br s, 1H, OH-5'), 5.20 (br s, 1H, OH-3'), 5.46 (br s, OH-2'), 5.64(d, $J = 6.02$ Hz, 1H, H-1'), 6.84 (s, 1H, H-6 Pyr), 8.01 (d, $J = 1.3$ Hz, 1H, H-5 Im), 8.06 (d, $J = 1.3$ Hz, 1H, H-2 Im); ^{13}C NMR (100 MHz, DMSO- d_6): 54.1, 54.7, 61.8, 71.0, 76.1, 86.2, 90.2, 94.5, 119.2, 138.3, 139.9, 162.5, 165.5, 172.3; HRMS (ESI-TOF) m/z calcd for $[\text{C}_{14}\text{H}_{18}\text{N}_4\text{O}_6 + \text{H}]$: 339.1305, found: 339.1303

PNP-catalyzed transglycosylation of imidazo[4',5':4,5]-thieno[3,2-*d*]pyrimidin-5(6H)-one (5). Compound **5** (1.0 mg, 4.8 μ mol) and adenine (2 equiv) were incubated in the presence of PNP (10 μ L, 1 U) for 16 h at 37°C (80% conversion). After purification by reverse phase HPLC, two compounds were isolated.

1-(β -D-ribofuranosyl)-1*H*-imidazo[4',5':4,5]thieno[3,2-*d*]pyrimidin-5(6*H*)-one (5d): 0.4 mg (26% yield). t_R = 16.5 min (G1). ^1H NMR (600 MHz, DMSO- d_6): δ 3.53-3.65 (m, 1H, H-5'), 3.64-3.76 (m, 1H, H-5''), 4.00 (br s, 1H, H-4'), 4.16 (br s, 1H, H-3'), 4.59 (br s, 1H, H-2'), 5.20 (br s, 1H, OH-3'), 5.48 (br s, 1H, OH-2'), 5.90 (br s, 1H, OH-5'), 5.95 (d, J = 7.2 Hz, 1H, H-1'), 8.15 (s, 1H, H-2), 8.44 (s, 1H, H-2' Im); ^{13}C NMR (150 MHz, DMSO- d_6): 62.2, 70.8, 74.7, 86.9, 89.7, 121.6, 128.4, 143.4, 145.8, 150.6, 162.6 (^{13}C missing); HRMS (ESI-TOF) m/z calcd for [$\text{C}_{12}\text{H}_{11}\text{N}_4\text{O}_5\text{S} + \text{H}$]: 325.0607, found: 325.0609.

3-(β -D-ribofuranosyl)-3*H*-imidazo[4',5':4,5]thieno[3,2-*d*]pyrimidin-5(6*H*)-one (5e): 0.2 mg (13% yield). t_R = 13.8 min (G1). ^1H NMR (600 MHz, DMSO- d_6): δ 3.55-3.71 (m, 2H, H-5', H-5''), 3.96 (br s, 1H, H-4'), 4.08 (br s, 1H, H-3'), 4.31 (br s, 1H, H-2'), 5.04 (br s, 1H, OH-5'), 5.32 (br s, 1H, OH-3'), 5.61 (br s, OH-2'), 5.74 (t, J = 6.9 Hz, 1H, H-1'), 8.06 (br s, 1H, H-2 Pyr), 8.12 (s, 1H, H-2' Im); ^{13}C NMR (150 MHz, DMSO- d_6): 62.5, 71.2, 73.6, 86.2, 89.8, 121.6, 128.4, 143.4, 145.8, 150.6, 162.6 (^{13}C missing); HRMS (ESI-TOF) m/z calcd for [$\text{C}_{12}\text{H}_{11}\text{N}_4\text{O}_5\text{S} + \text{H}$]: 325.0607, found: 325.0607.

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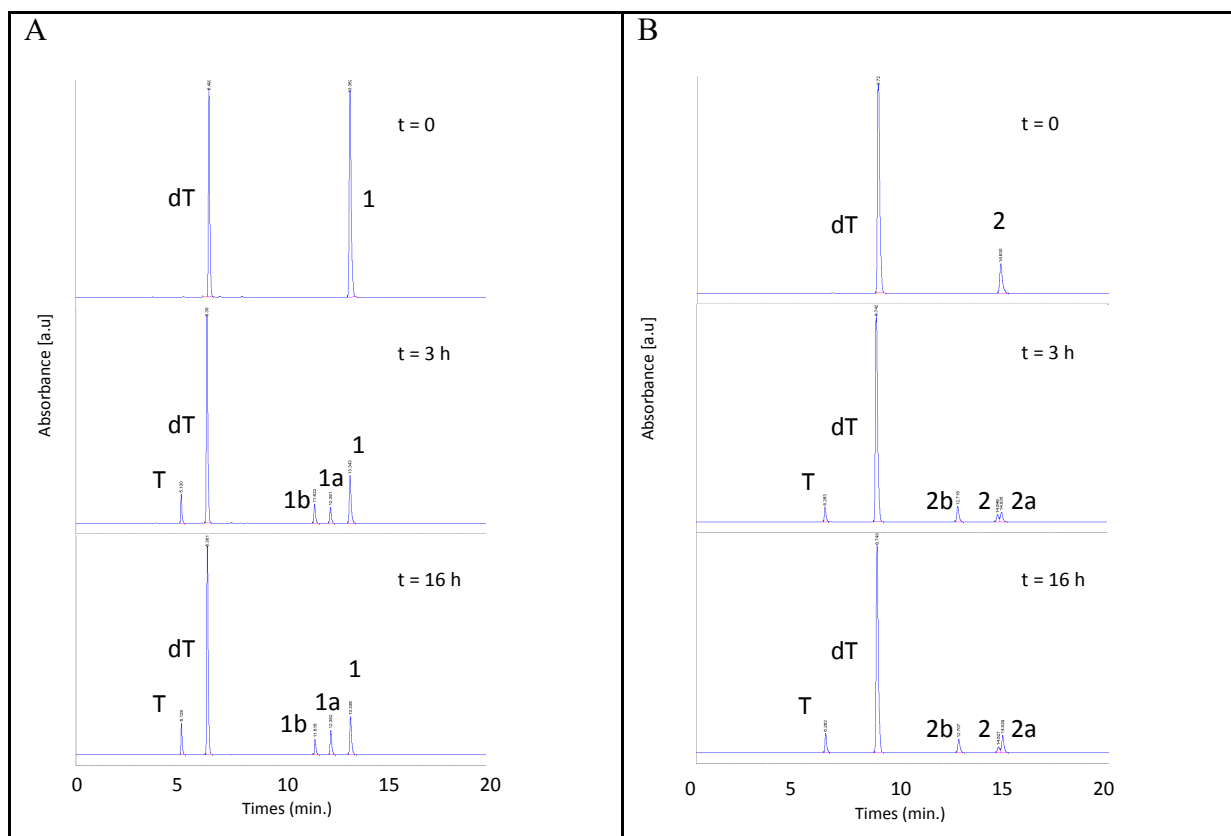


Figure S1. HPLC monitoring of the transglycosylation reaction of **1** (panel A) or **2** (panel B) in the presence of thymidine (4 equiv) and NDT ($1.25 \mu\text{L}/\mu\text{mol}$ acceptor) at $t = 0, 3 \text{ h}$ and 16 h at 37°C . HPLC conditions: 0–40% (panel A) or 0–20% (panel B) linear gradient of acetonitrile in 10 mM TEAA buffer pH 6.0 over 20 min at a flow rate of 1mL min^{-1} . Detection at 254 nm.

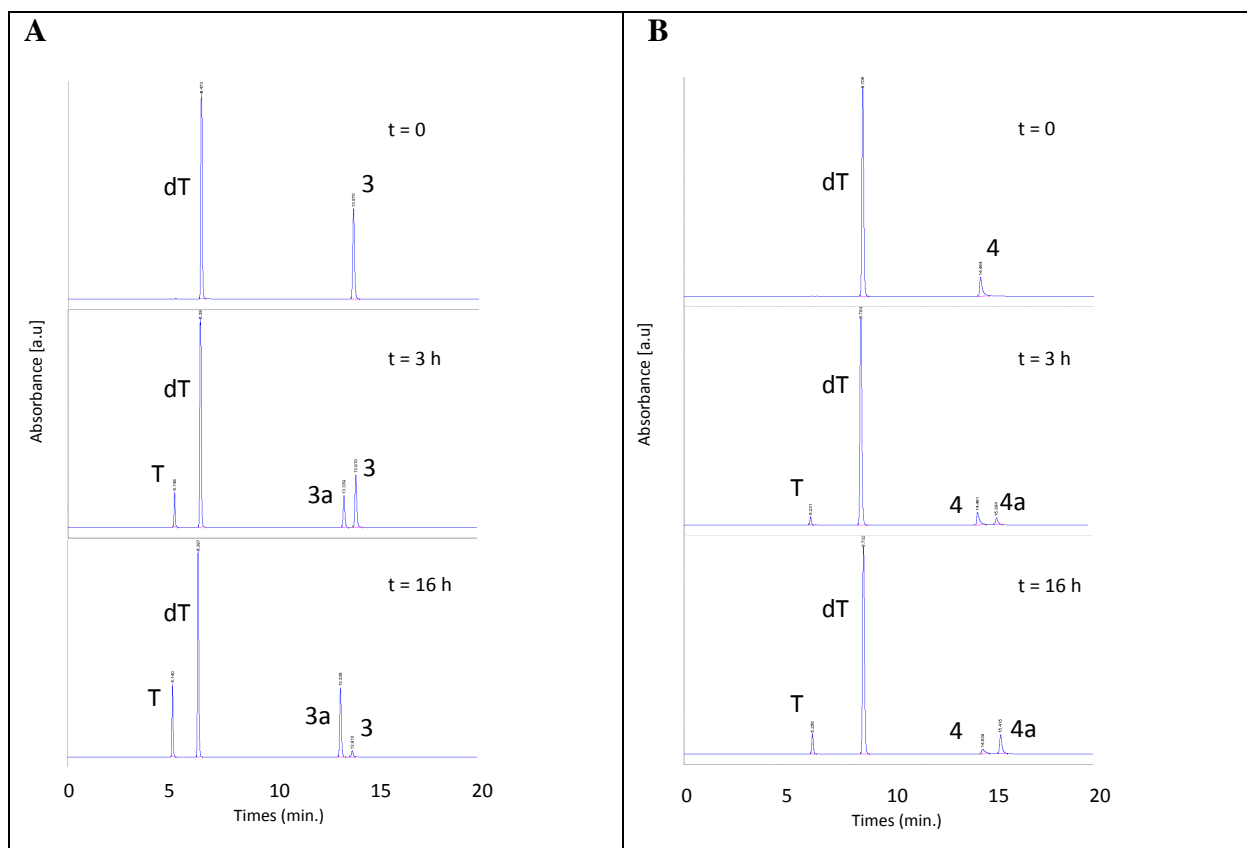


Figure S2. HPLC monitoring of the transglycosylation reaction of **3** (panel A) or **4** (panel B) in the presence of thymidine (4 equiv) and NDT ($1.25 \mu\text{L}/\mu\text{mol}$ acceptor) at $t = 0, 3 \text{ h}$ and 16 h at 37°C . HPLC conditions: 0–40% (panel A) or 0–20% (panel B) linear gradient of acetonitrile in 10 mM TEAA buffer pH 6.0 over 20 min at a flow rate of 1 mL min^{-1} . Detection at 254 nm.

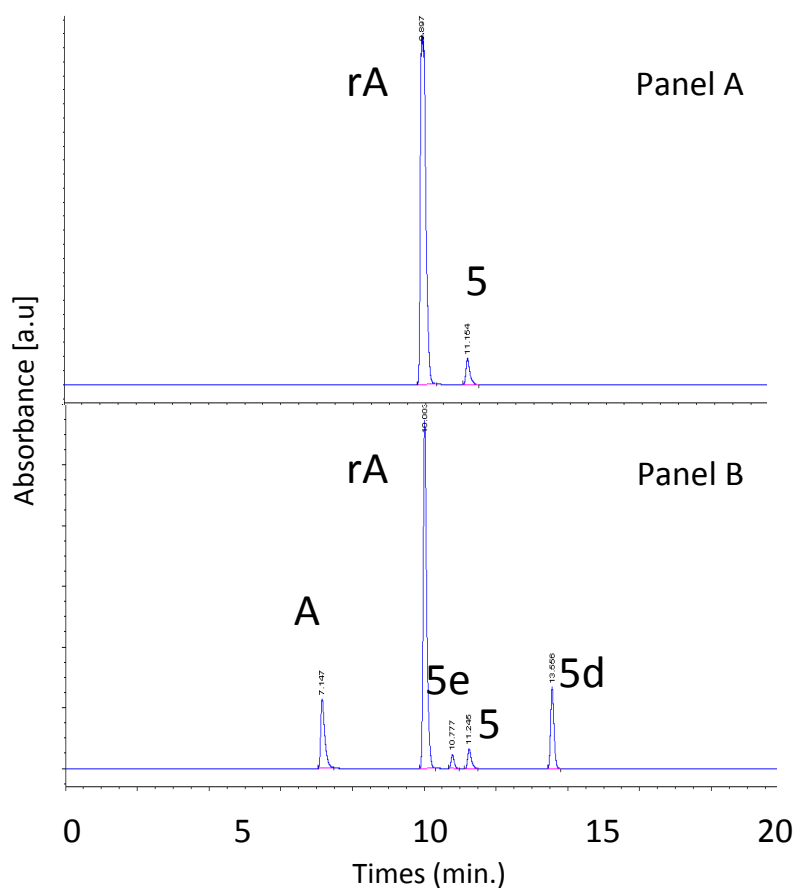
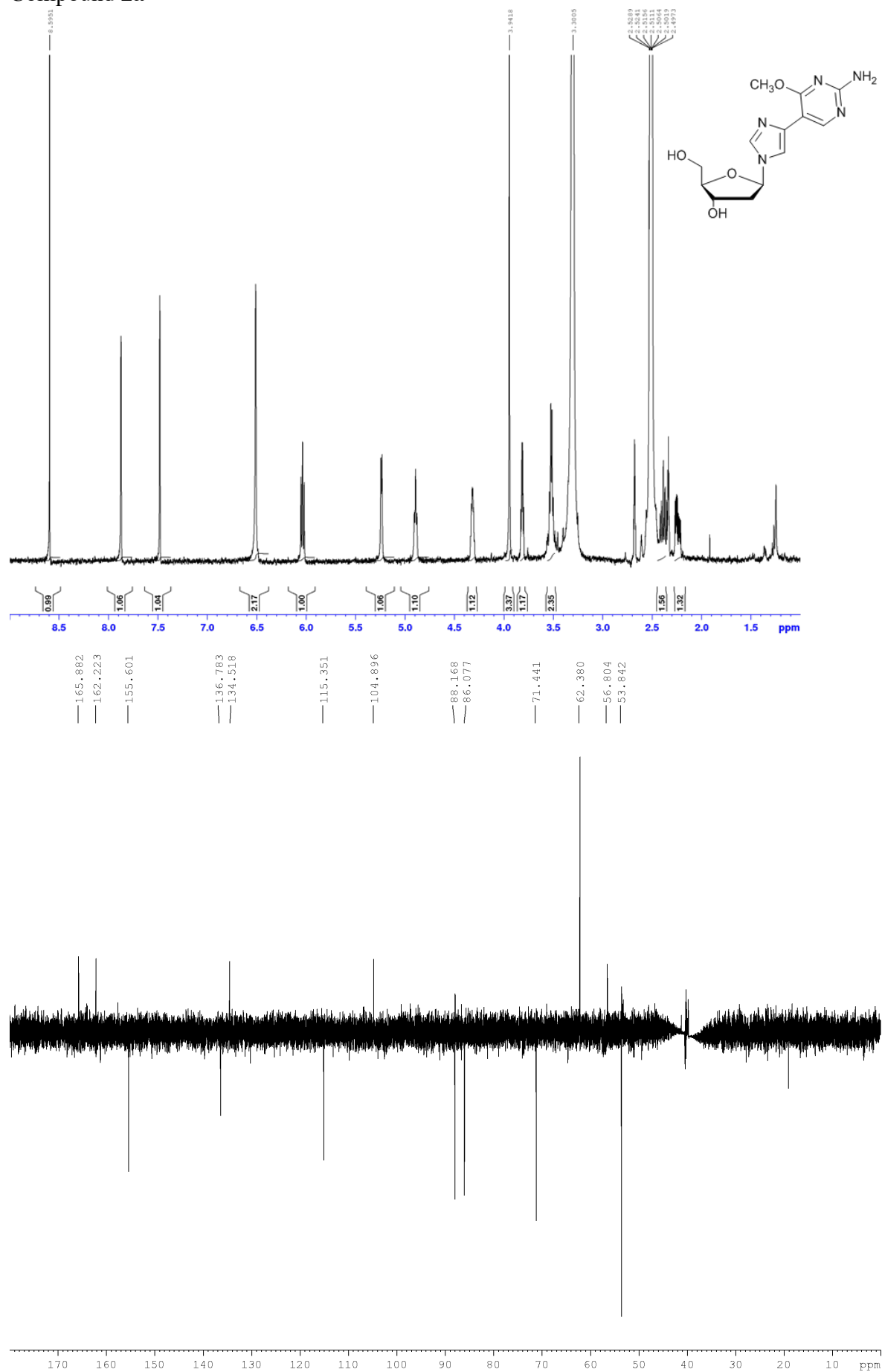
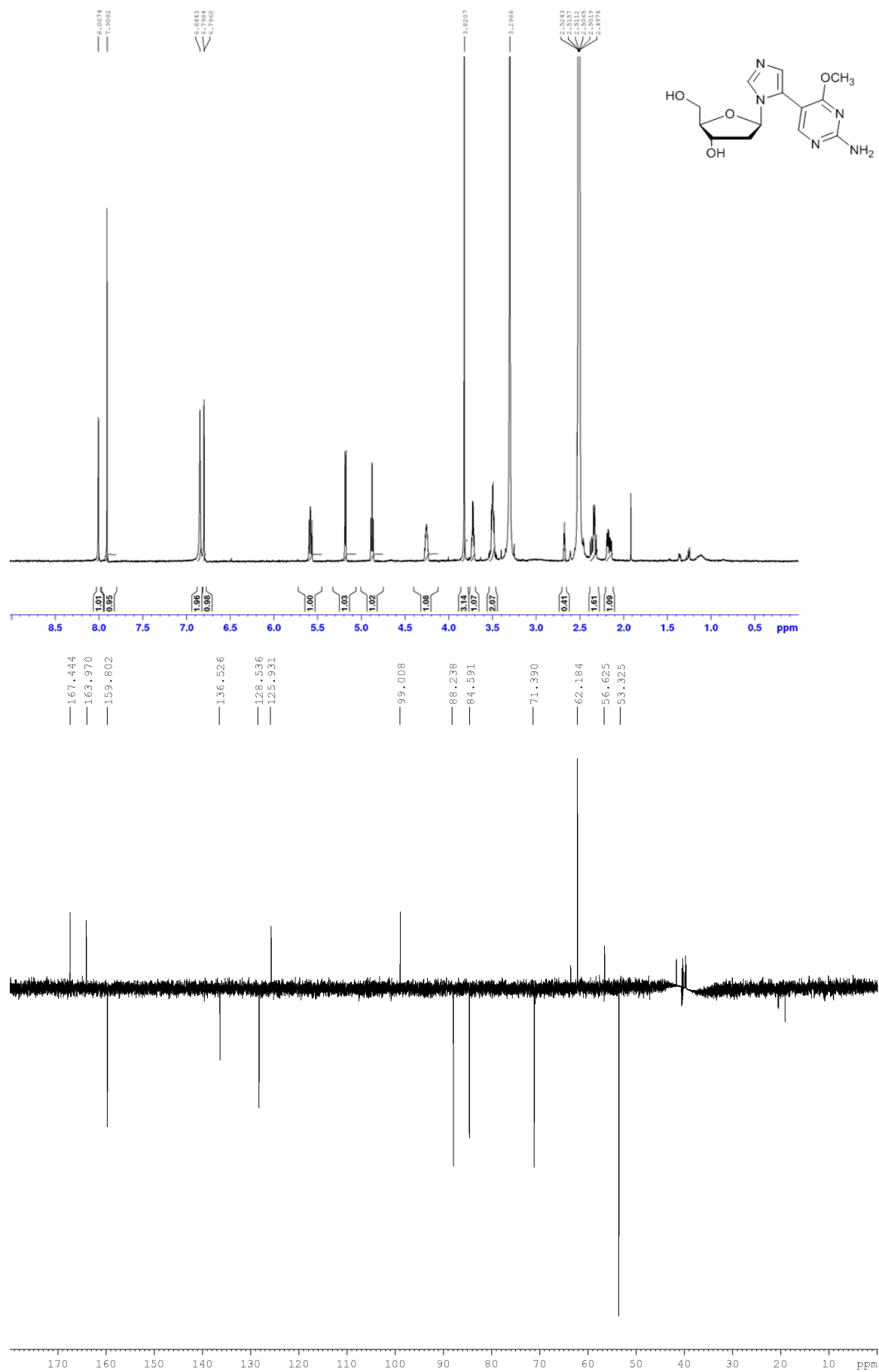


Figure S3. HPLC monitoring of the transglycosylation reaction of **5** in the presence of adenosine (2 equiv) and PNP (6.25 $\mu\text{L}/\mu\text{mol}$ acceptor) at $t = 0$ (panel A) and 12 h (panel B) at 37°C. HPLC conditions: 0–20% linear gradient of acetonitrile in 10 mM TEAA buffer pH 6.0 over 20 min at a flow rate of 1 mL min^{-1} . Detection at 254.

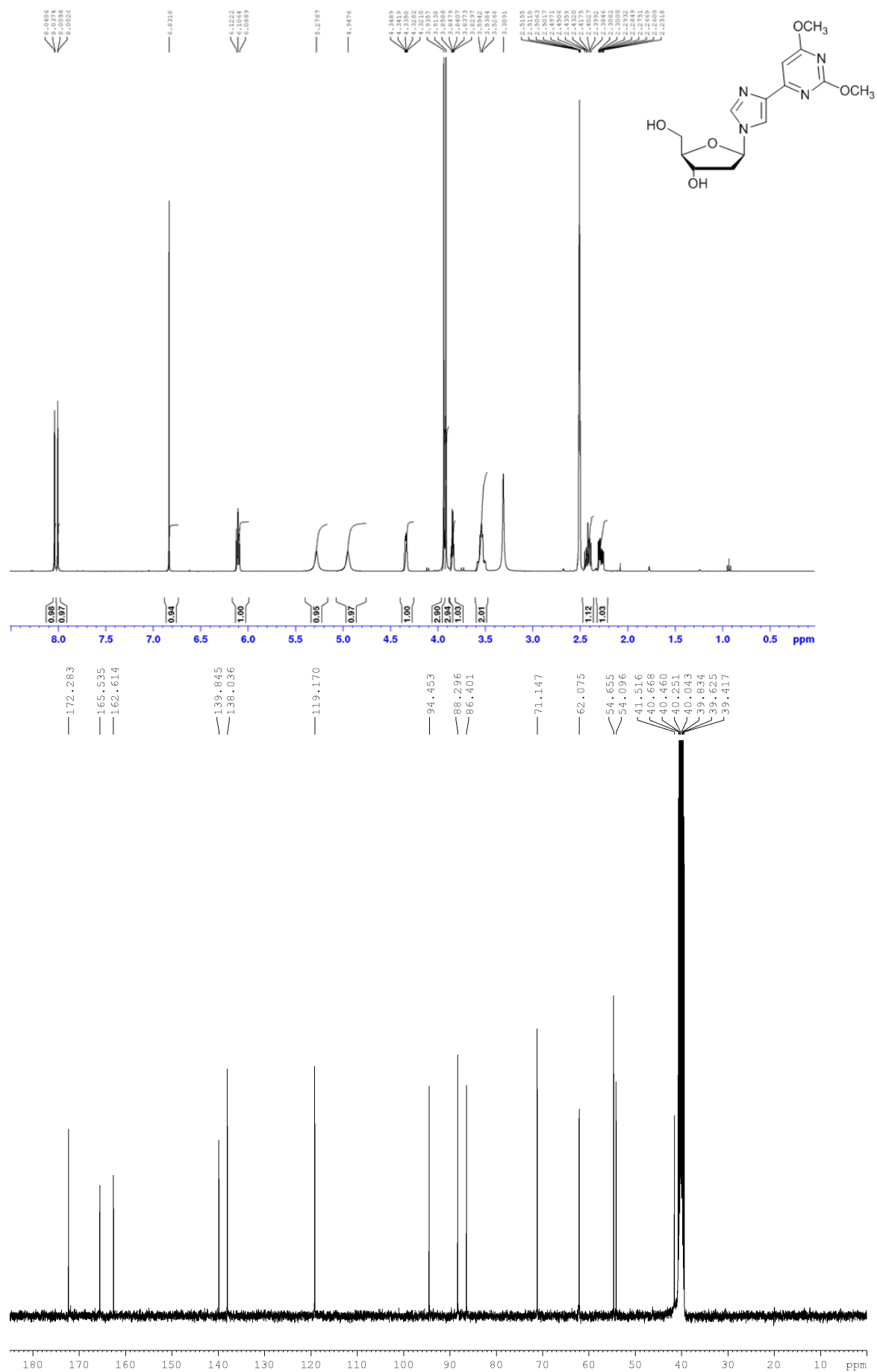
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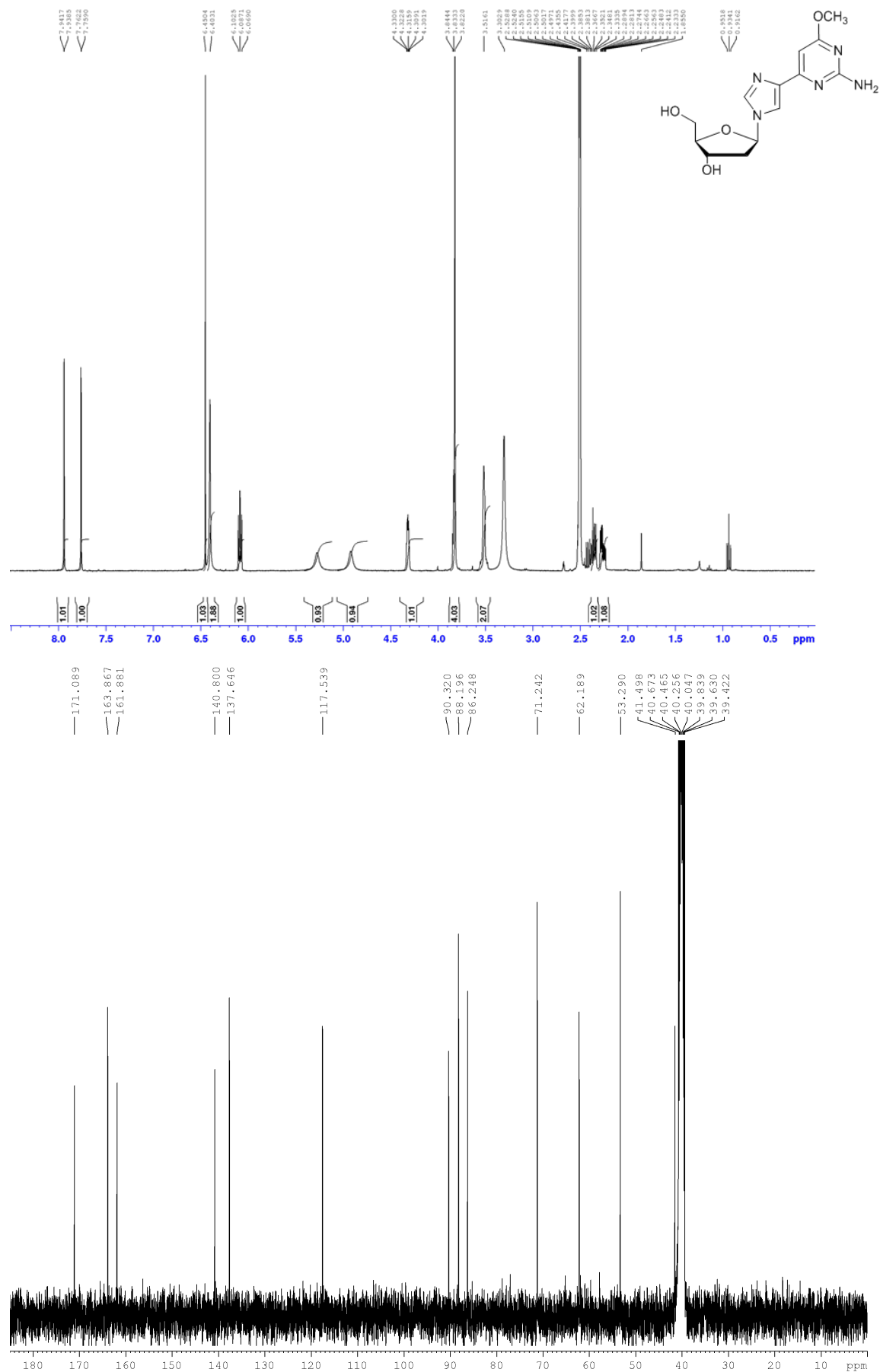
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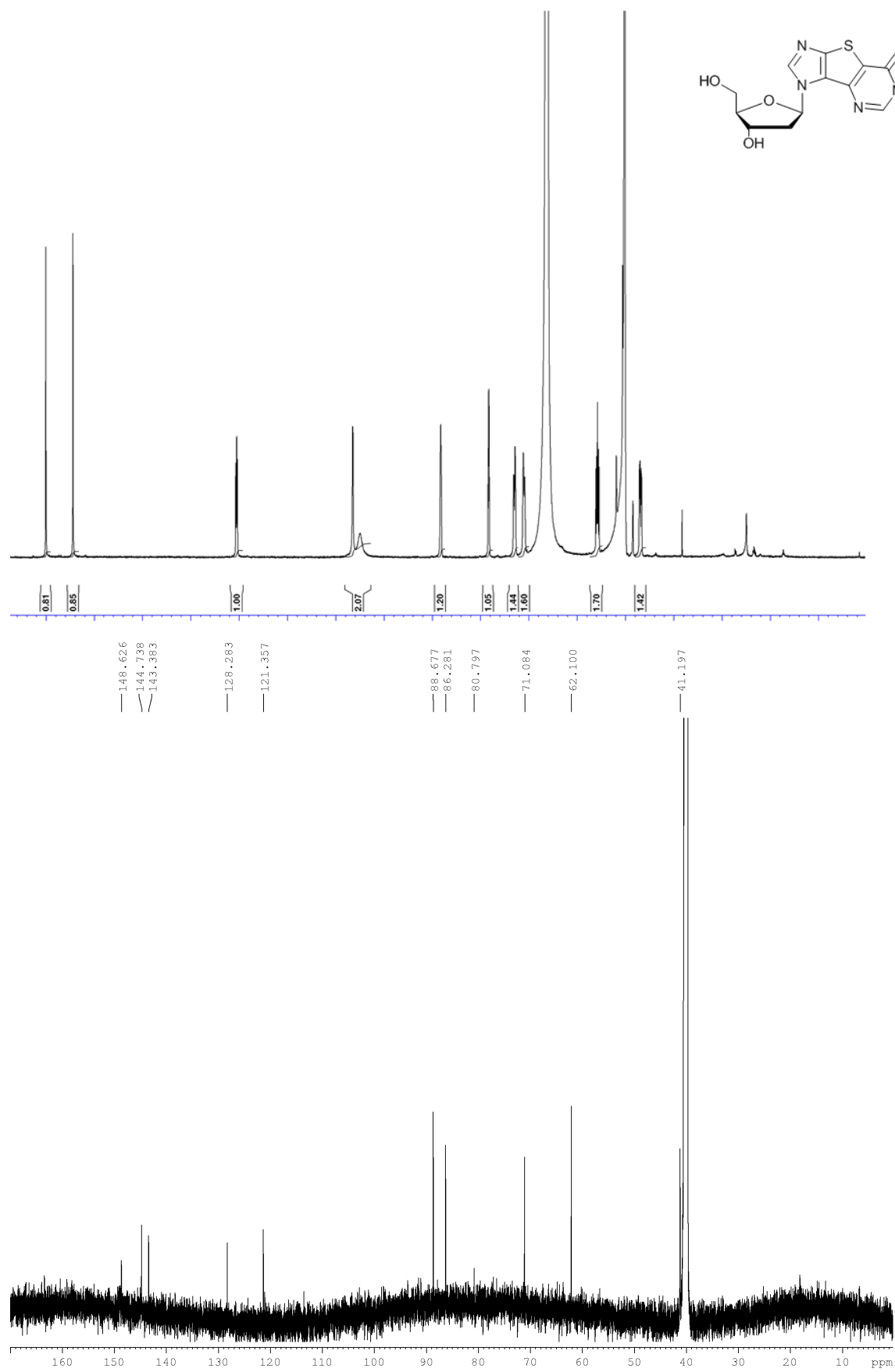
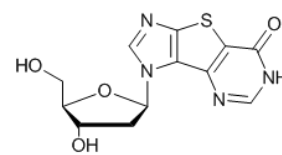
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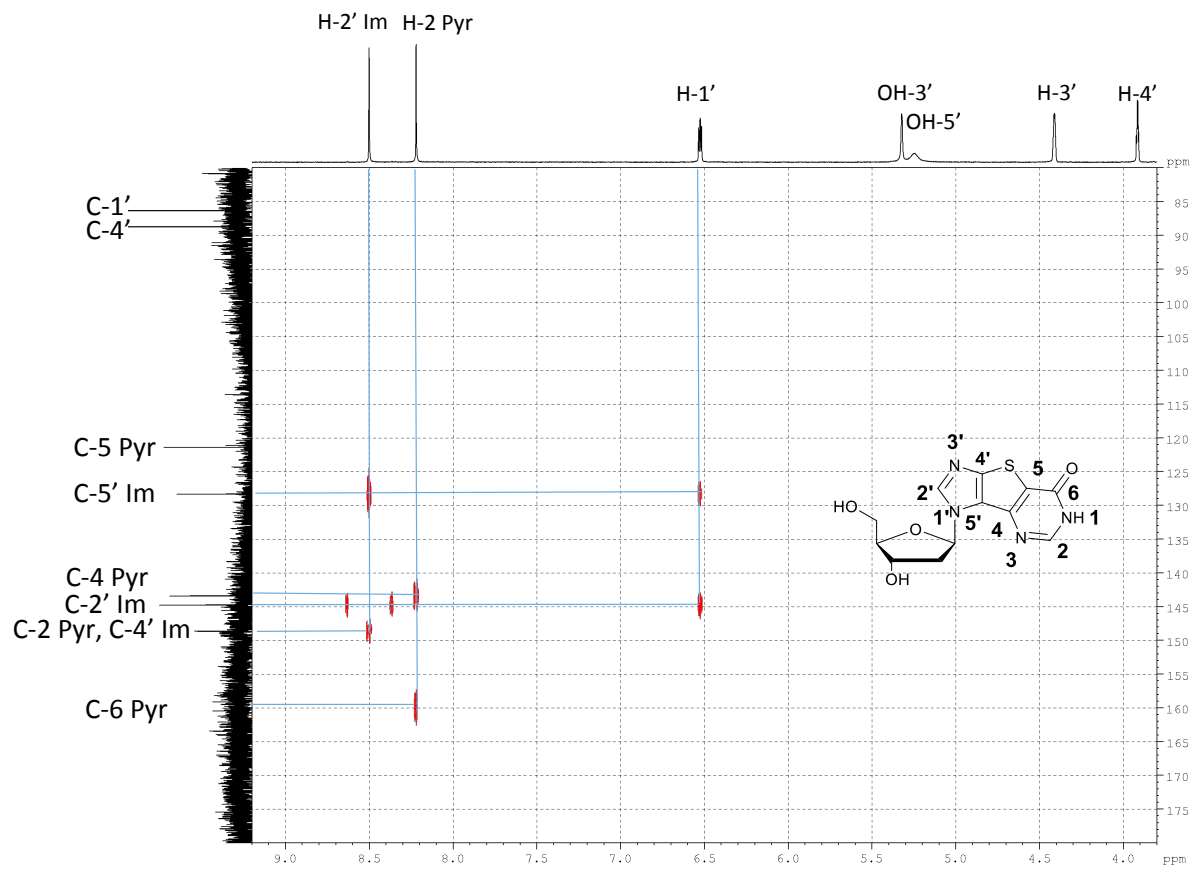


Compound 4a

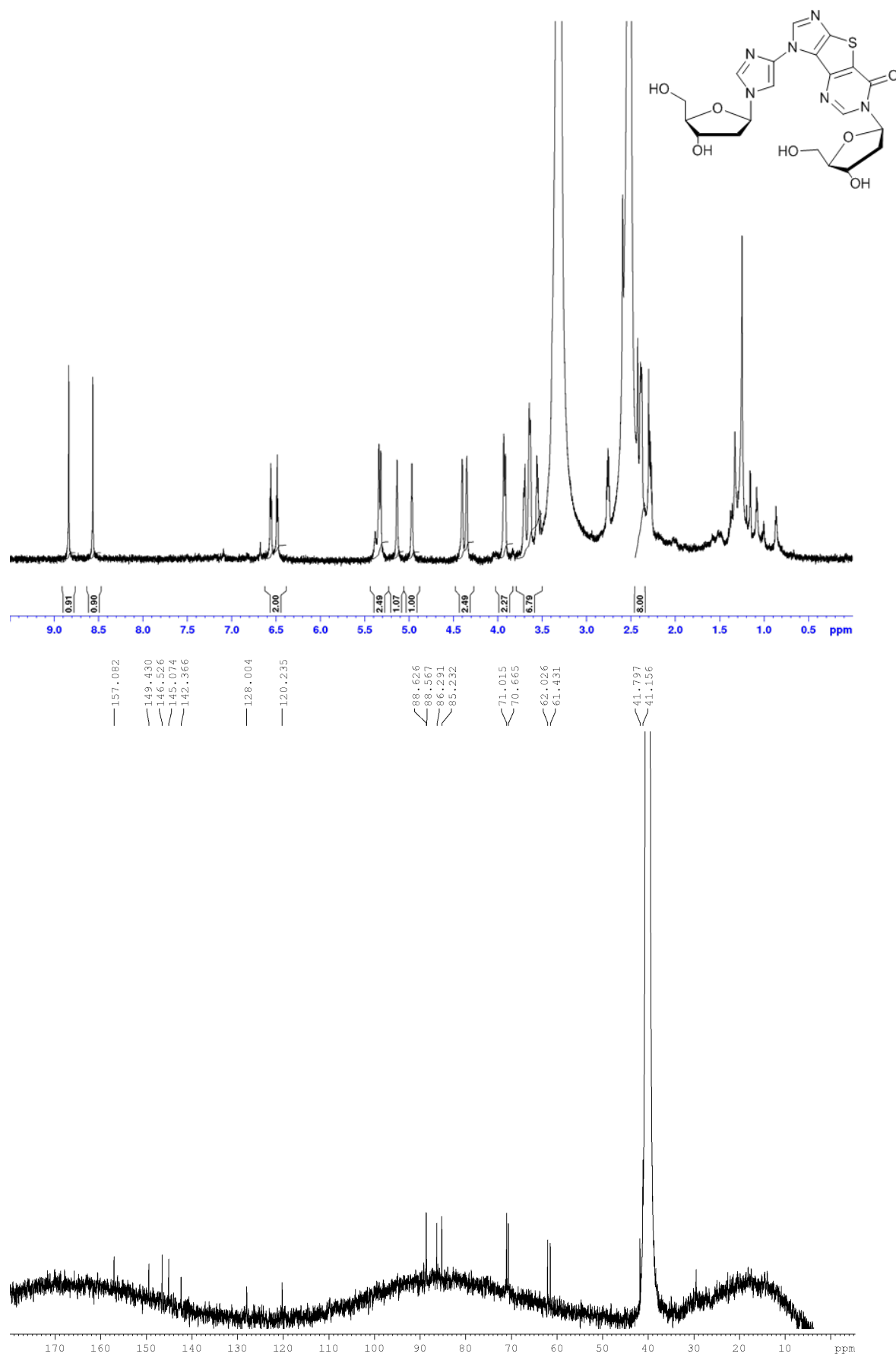


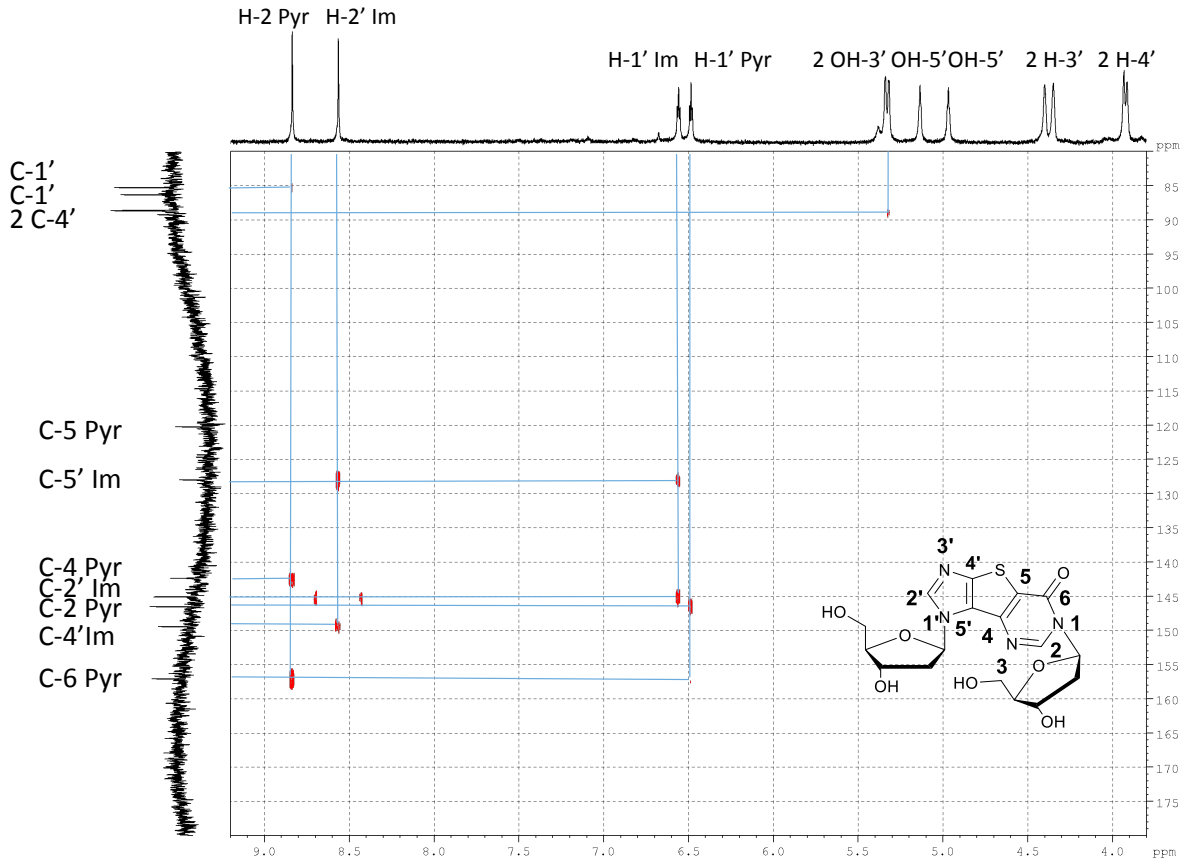
Compound 5a



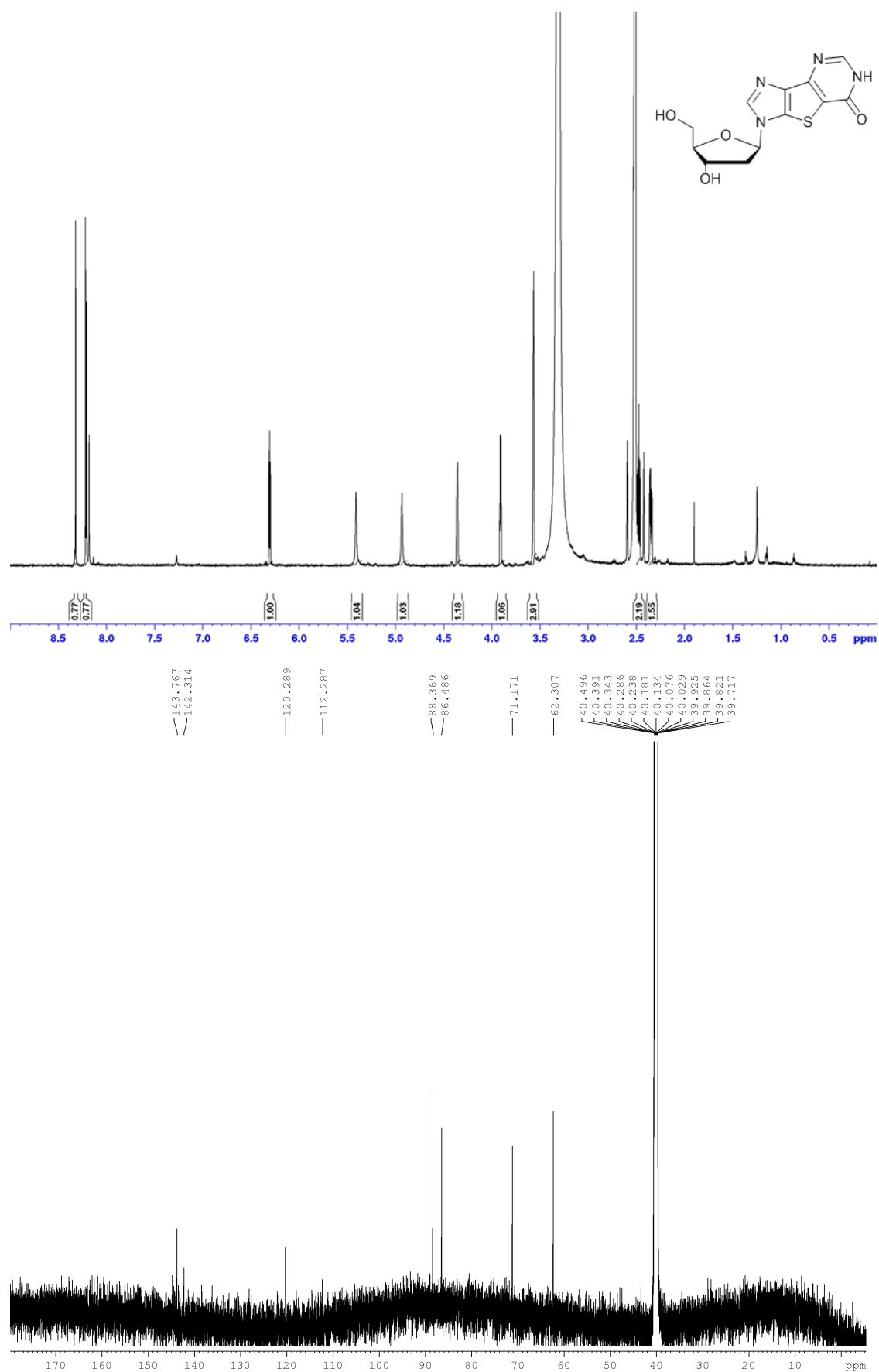


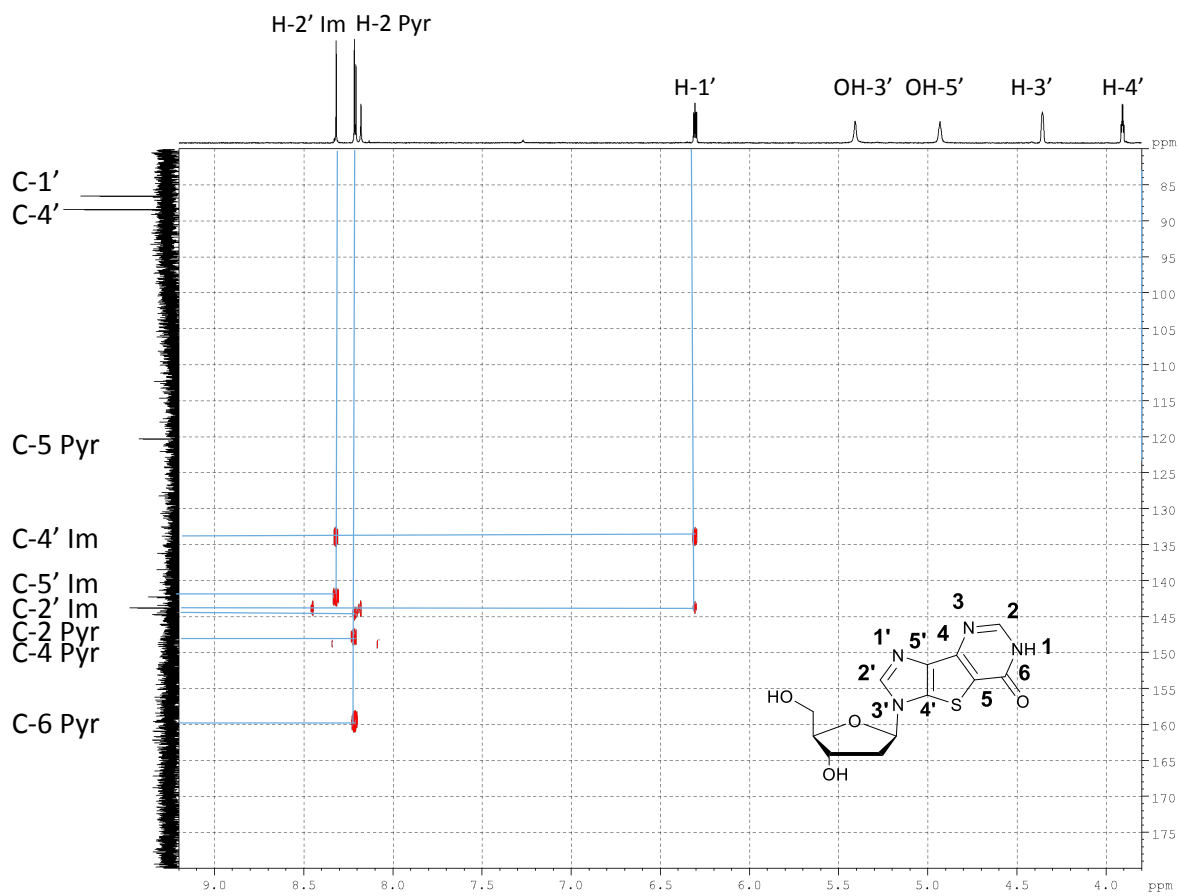
Compound 5b



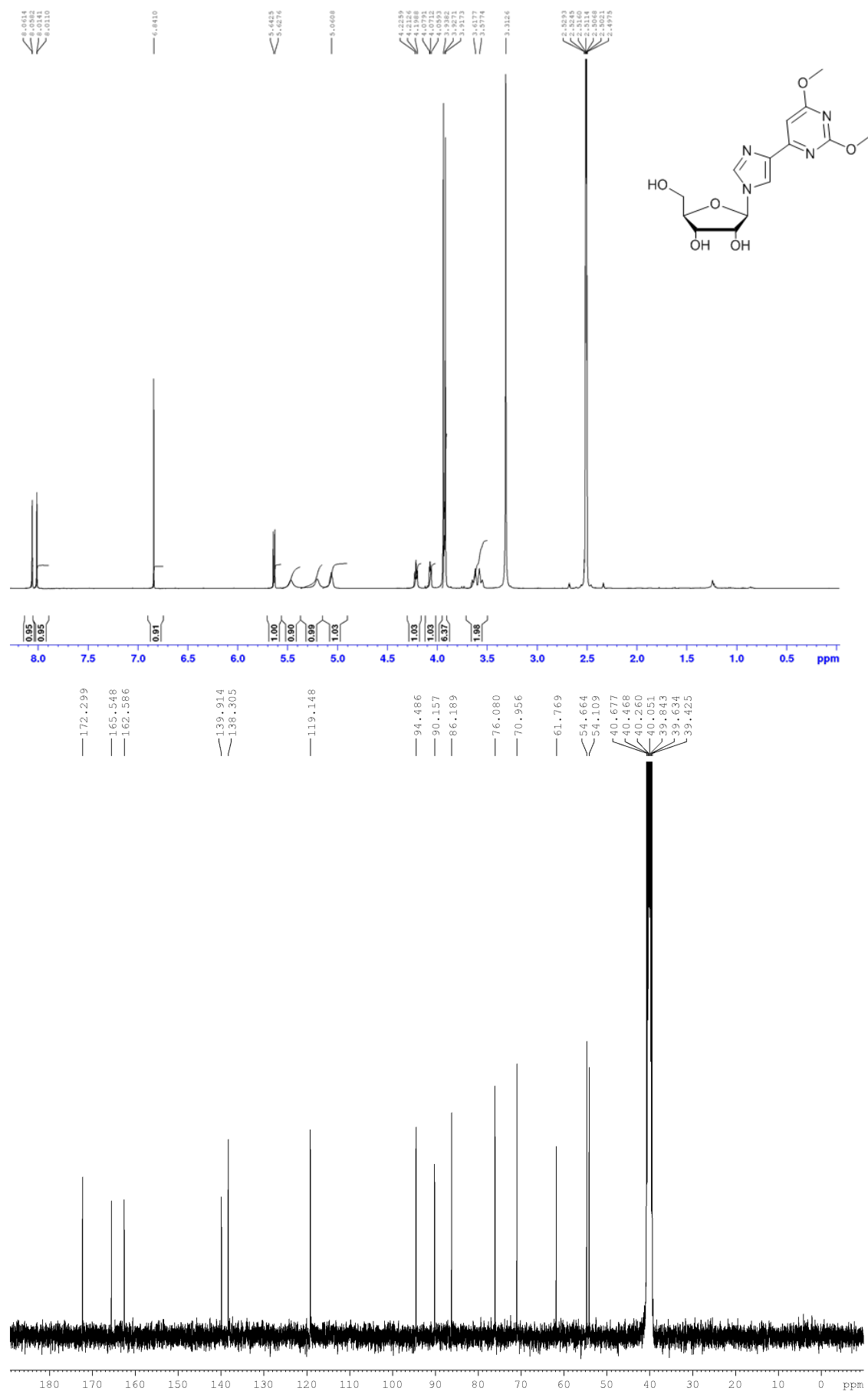


Compound 5c

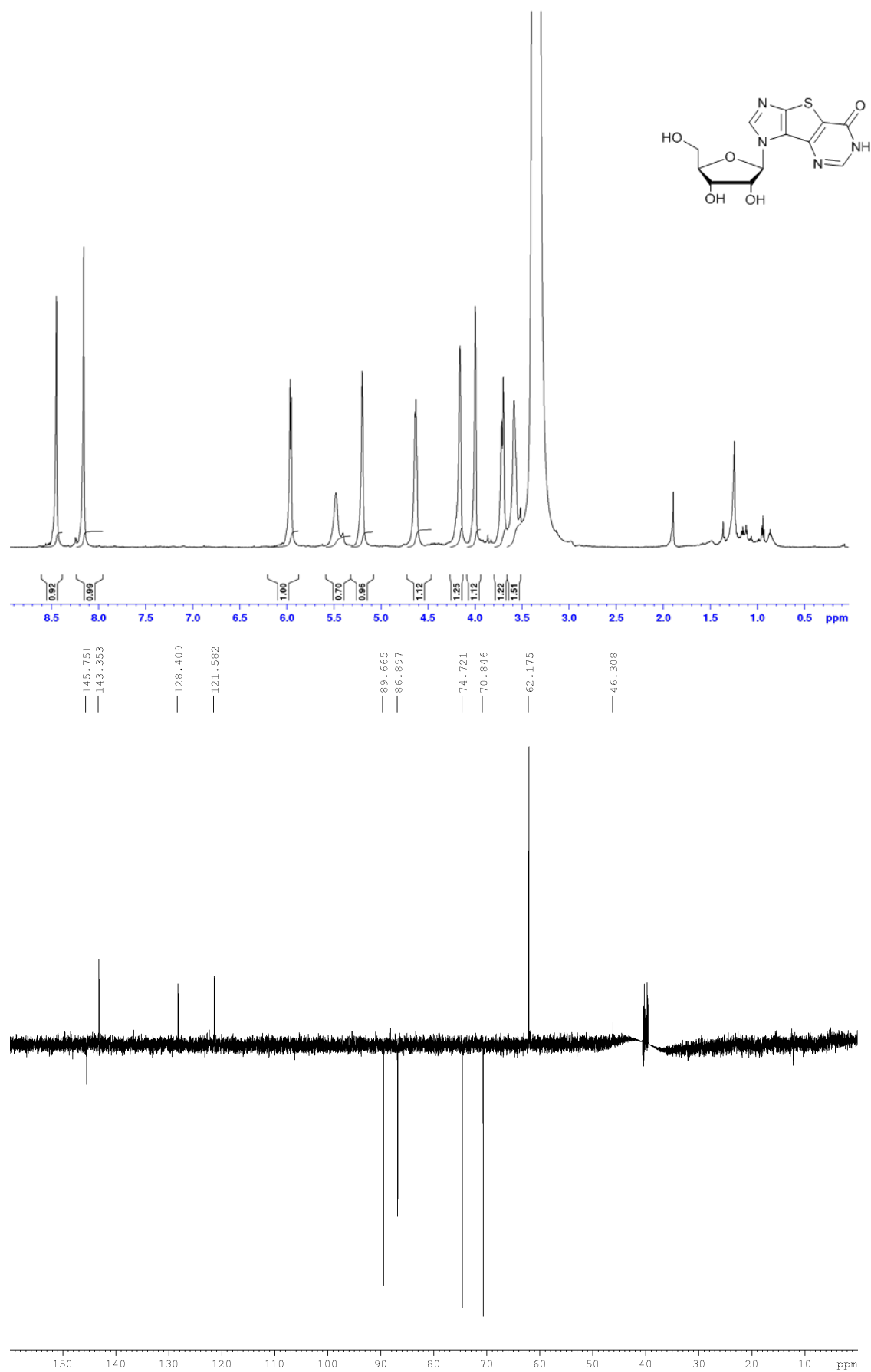


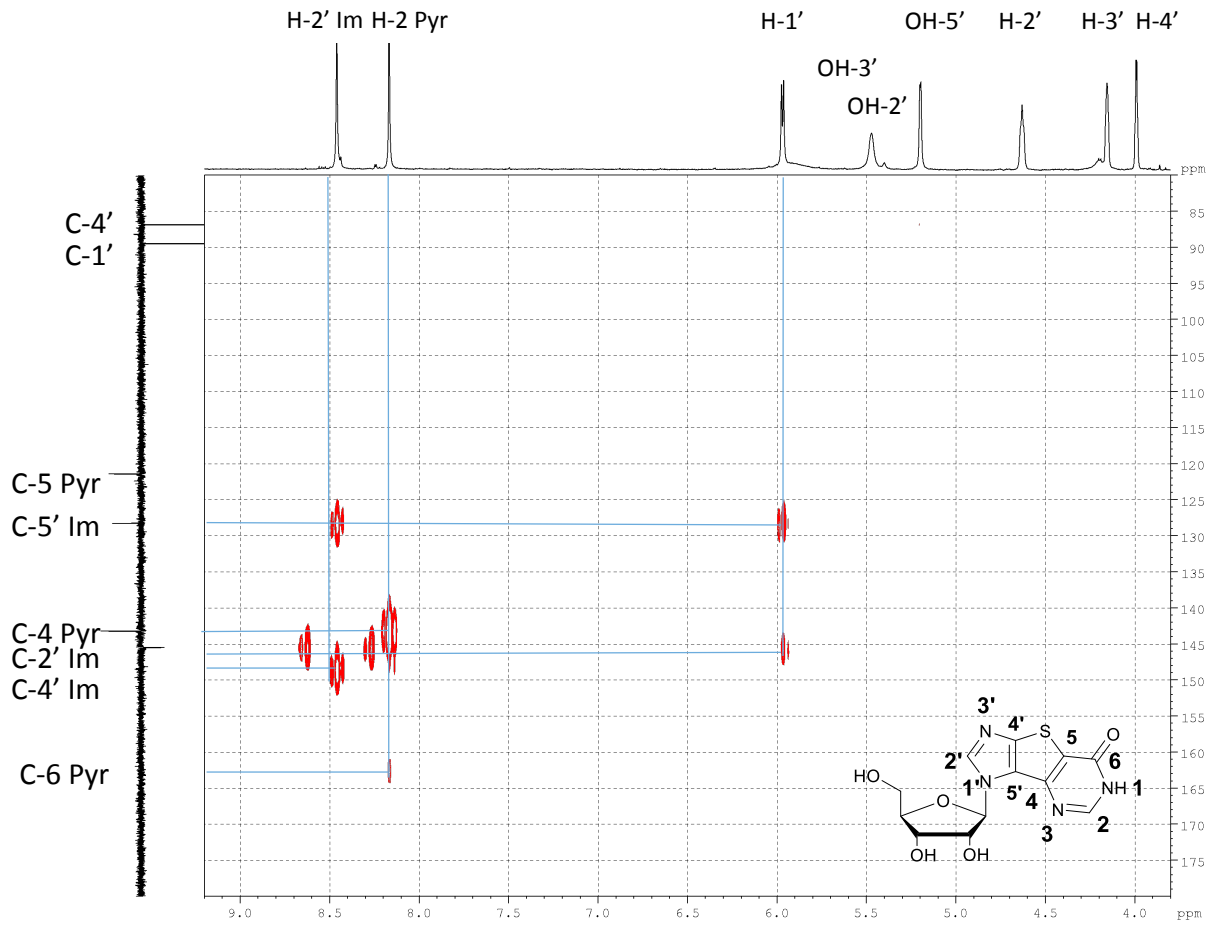


Compound 3c



Compound 5d





Compound 5e

