Synthesis and Characterization of π-Stacked Phenothiazine Labeled Oligodeoxynucleotides

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General procedures. All solvents were dried and freshly distilled prior to use (CH_2Cl_2 with CaH_2; THF with Na; pyridine with CaH_2). All chemicals were purchased from Aldrich or Acros as highest purity grade and used without further purification. All reactions were performed under nitrogen atmosphere. NMR spectra were recorded on a Varian INOVA spectrometer (for ^1H at 400 MHz and for ^1H NOE at 500 MHz). Chemical ionization mass spectra were obtained on a Hewlett Packard HP 5988A spectrometer using NH_3. Fast atom bombardment mass spectra (FABMS) were obtained on a JOEL JMS-SX102A spectrometer using a 3-nitrobenzyl alcohol matrix. MALDI-TOF mass spectra were obtained using a PerSpective Biosystems Voyager-DE Biospectrometry Workstation operating in the positive ion mode using 2-(4-hydroxyphenylazo)-benzoic acid (HABA).

Syntheses

1-(α,β)-O-methyl-2-deoxy-D-ribose

1-(α,β)-O-methyl-2-deoxy-D-ribosewas purchased from Aldrich or prepared as described below. 2-deoxy-D-ribose (1.34 g, 10 mmol) was dissolved in 50 mL of dry methanol. Hydrogen chloride gas generated in situ, via reacting concentrated sulfuric acid with sodium chloride, and was then bubbled through the reaction flask. The reaction was complete within 2 hours as indicated by TLC. The resulting reaction solution was then neutralized with an Amberlyst A-21 ion exchange resin to pH = 7. After the resin was filtered from the solution, the filtrate was concentrated to dryness using a rotary evaporator. The final residue was pure by TLC (1.43 g, 96%). ^1H-NMR ($CDCl_3$), δ 2.00-2.22 (m, 2H, C2), 3.31-3.33 (d, 3H, methyl), 3.57-3.63 (m, 2H, C5), 3.91-4.03 (m, 1H, C3), 5.02-5.06 (m, 1H, C1).

1-(α,β)-O-methyl-3, 5-di-(O-p-toluoyl)-2-deoxy-D-ribose

1-O-methyl-2-deoxy-D-ribose (1.40 g, 9.22 mmol) was dissolved in 30 mL of dry pyridine, and the flask was cooled to 0 ^0C (ice-water). p-Toluoyl chloride (3.19 mL, 24.1 mmol) was then added to the reaction solution dropwise, and the resulting mixture was stirred at room temperature overnight under nitrogen. The reaction was stopped, the

solvent was removed, and the residue was dissolved in dichloromethane (20 mL) and washed with H_20 (20 mL) and saturated $NaHCO_3$ (20 mL). The organic phase was then concentrated to dryness and the crude mixture was further purified by column chromatography (10-15% ethylacetate in hexanes) to afford 1-(α, β)-O-methyl-3, 5-di-(O- p-toluoyl)-2-deoxy-D-ribose **3** (3.02 g, 85% yield). ^{1}H-NMR ($CDCl_3$), δ 2.39-2.41 (d, 6H, toluoyl-methyl α+β), 2.18-2.60 (m, 2H, C2 α+β), 3.36-4.42 (d, 3H, methoxy α+β), 4.45-4.66 (m, 3H, C5+C3 α+β), 5.18-5.23 (m, 1H, C4 α+β), 5.39-5.43 (m, 0.45H, C1α), 5.58-5.62 (m, 0.55H, C1 β), 7.20-7.24 (t, 4H, benzoyl), 7.91-7.99 (m, 4H, benzoyl). FABMS calculated MW= 384, found M+H=385

1-(α)-chloro-3, 5-di-(O- p-toluoyl)-2-deoxy-D-ribose 3

Compound **3** was prepared following a modified literature procedure[1-3] as described below. 1-O-methyl-3, 5-di- (O- p-toluoyl)-2-deoxy-D-ribose, (3.00 g, 7.81 mmol), was dried and mixed with 25 mL of 1N HCl in acetic acid. Five minutes later, a white precipitate was formed, and the reaction mixture was stirred at 10 ^{o}C for an additional hour. The white solid was filtered and washed with 50 mL of cold ethyl ether to afford 1-(α)-chloro-3, 5-di-(O-p-toluoyl)-2-deoxy-D-ribose **4** (2.19 g, 72% yield). ^{1}H-NMR ($CDCl_3$), δ 2.41-2.42 (d, 6H, toluoyl-methyl), 2.83-2.91 (m, 2H, C2), 4.57-4.70 (m, 2H, C5), 4.85-4.86 (q, 1H, C4), 5.55-5.58 (m, 1H, C3), 6.46-6.48 (d, 1H, C1), 7.23-7.28 (m, 4H, benzoyl), 7.89-8.00 (m, 4H, benzoyl). FABMS calculated MW = 388.11, found M-Cl = 354.14

3-Bromo-10-methyl-phenothiazine, 2

Compound **2** was prepared following a literature procedure.[4] First, a sodium acetate buffer was prepared by dissolving sodium hydroxide (0.61 g) in acetic acid (37 mL). Next, 10-methyl-phenothiazine, **5**, (1.07 g, 5 mmol) was added to this buffer, followed by 10 mL of CH_2Cl_2. Bromine (0.26 mL, 5 mmol) in 5 mL of glacial acetic acid was added to the reaction mixture slowly over a one hour period. After an additional hour, the reaction was stopped and all the solvents were removed. The dark red residue was dissolved in 50 mL of CH_2Cl_2 and this solution was washed with 50 mL of 1 % $NaHCO_3$ solution. Column chromatography (5% ethylacetate in hexanes) afforded 3-bromo-10-methyl-phenothiazine, **2** (0.96 g, 66% yield). ^{1}H-NMR ($CDCl_3$), δ3.34 (s, 3H, 10-methyl), 6.63-6.66 (m, 1H, C1), 6.80-6.82 (d, 1H, C9), 6.93-6.96 (t, 1H, C7), 7.11-7.20 (m, 2H, C6+C8), 7.23-7.27 (m, 2H, C2+C4). FABMS calculated MW=292, found M+H=293.

1-(β)-(10-methyl-phenothiazin-3-yl)-3, 5-di- (O-p-toluoyl)-2-deoxy-D-ribose, 4

A solution of 3-bromo-10-methyl-phenothiazine, **2**, (1.26 g, 4.35 mmol) in 10 mL of dry THF was added slowly to a reaction flask containing an excess of magnesium turning and a chip of iodine in 5 mL of dry THF at 70 ^{o}C. After addition, this reaction mixture was refluxed for two hours before it was cooled down and the excess magnesium was removed. 1-chloro-3, 5-di-(O-p-toluoyl)-2-deoxy-D-ribose, **3**, (1.26 g, 3.25 mmol) was then added to this solution, and reaction proceeded under nitrogen at room temperature overnight. The solvent was removed and the resulting crude residue was purified by column chromatography (5% to 15% ethylacetate in hexane) to afford 1-(β)-(10-methyl-phenothiazin-3-yl)-3, 5-di-(O-p-toluoyl)-2-deoxy-D-ribose, **4** (0.24 g, 13% yield) and 1-

(α)-(10-methyl-phenothiazin-3-yl)-3, 5-di-(O-p-toluoyl)-2-deoxy-D-ribose, (0.8 g, 44%). The α anomer was also converted to the β anomer following a literature procedure by Kool (60-65% yield).[5] The reaction could be followed by TLC, since the lower spot was the α anomer and the upper spot was the β anomer (confirmed by ^{1}H NOE experiments).
1-(β)-(10-methyl-phenothiazin-3-yl)-3, 5-di-(O-p-toluoyl)-2-deoxy-D-ribose, **7**
^{1}H-NMR ($CDCl_3$), δ 2.16 - 2.22 (m, 1H, C-2 β), 2.39 - 2.49 (m, 1H, C-2 α), 2.4 (s, 3H, CH_3 toluoyl), 2.44 (s, 3H, CH_3 toluoyl), 3.36 (s, 3H, N-CH_3 phenothiazine), 4.5 - 4.53 (m, 1H, C-4'), 4.61 - 4.67 (m, 2H, C-5'), 5.15 - 5.18 (q, 1H, C-1'), 5.58 - 5.59 (d, 1H, C-3'), 6.74 - 6.81 (dd, 2H, C1 and C9 of phenothiazine), 6.91 - 6.94 (t, 1H, C7 of phenothiazine), 7.11 - 7.28 (m, 8H toluoyl, C2, C4, C6, and C8 of phenothiazine), 7.94 - 7.98 (m, 4H, aromatic toluoyl). FAB-HRMS calcd for $C_{34}H_{31}NO_5S$ 565.1923, found 565.1914. See Figure SI-1 and SI-2.

1-(α)-(10-methyl-phenothiazin-3-yl)-3, 5-di-(O-p-toluoyl)-2-deoxy-D-ribose
^{1}H-NMR ($CDCl_3$), δ 2.27 - 2.31 (m, 1H, C-2 α), 2.33 (s, 3H, CH3 toluoyl), 2.42 (s, 3H, CH3 toluoyl), 2.83 - 2.89 (m, 1H, C-2 β), 3.38 (s, 3H, N-CH3, phenothiazine), 4.51 - 4.58 (m, 2H, C-5'), 4.65 - 4.67 (m, 1H, C-4'), 5.3 - 5.32 (dd, 1H, C-1'), 5.57 - 5.6 (m, 1H, C-3'), 6.77 - 6.84 (dd, 2H, C1 and C9 of phenothiazine), 6.94 - 6.97 (t, 1H, C7 of phenothiazine), 7.01 - 7.03 (d, 2H, aromatic toluoyl), 7.14 - 7.29 (m, 6H, toluoyl, C2, C4, C6, and C8 of phenothiazine), 7.64 - 7.65 (d, 2H, aromatic toluoyl), 7.95 - 7.97 (d, 2H, aromatic toluoyl). See Figure SI-3 and SI-4.

1-(β)-(10-methyl-phenothiazin-3-yl)-5-(O-p-toluoyl)-2-deoxy-D-ribose, 5
1-(β)-(10-methyl-phenothiazin-3-yl)-3, 5-di-(O-p-toluoyl)-2-deoxy-D-ribose, **4** (0.74 g, 1.31 mmol) and sodium methoxide (0.054 g, 1 mmol) were mixed in 10 mL of methanol. The reaction mixture was monitored by TLC. After stirring for 1.5 hours at room temperature, the reaction was stopped and the solvent was removed. Column chromatography purification (30% ethylacetate in hexanes) afforded 1-(β)- (10-methyl-phenothiazin-3-yl)-5-(O-p-toluoyl)-2-deoxy-D-ribose, **5** (0.14 g, 24% yield). The other product isolated was 1-(β)-(10-methyl-phenothiazin-3-yl)-3-(O-p-toluoyl)-2-deoxy-D-ribose. Alternatively, **5** can be obtained by first removing both p-toluoyl groups (3 equivalents of NaOMe and a 3 hour reaction time) followed by re-protection of the 5'-hydroxyl group with p-toluoyl chloride. The product 1-(β)- (10-methyl-phenothiazin-3-yl)- 5-(O- p-toluoyl)-2-deoxy-D-ribose was obtained in 27% yield. ^{1}H-NMR ($CDCl_3$), δ 2.0 - 2.09 (m, 1H, C-2 β), 2.23 - 2.27 (m, 2H, C-2 a), 2.4 (s, 3H, CH_3 toluoyl), 3.33 (s, 3H, N-CH3 phenothiazine), 4.24 - 4.26 (q, 1H, C-4), 4.32 - 4.58 (m, 3H, C-3' and C-5'), 5.13 - 5.16 (q, 1H, C-1'), 6.74 - 6.81 (dd, 2H, C1and C9 of phenothiazine), 6.91 - 6.94 (t, 1H, C7 of phenothiazine), 7.12 - 7.25 (m, 6H, phenothiazine, aromatic toluoyl), 7.93 - 7.97 (m, 2H, aromatic toluoyl). FAB-HRMS calcd for $C_{26}H_{25}NO_4S$ 447.1504, found 447.1498. See Figure SI-5 and Figure SI-6.

Phosphoramidite synthesis
2-Cyanoethyl-N, N'-diisopropylchlorophosphoramidite (1.2 equiv) was added to a solution of the PTZ-2'-deoxynucleoside in dry CH_3CN containing diisopropylethylamine

(3 equiv). The reaction mixture was stirred under nitrogen for 1 hour. Next, the reaction was quenched with methanol. The solvent was then removed and the product was washed with hexane and dried under high vacuum (^{31}P-NMR ($CDCl_3$): δ 150 ppm).

Oligodeoxynucleotide Syntheses

Oligodeoxynucleotide syntheses were performed on an ABI 395 DNA synthesizer from the 3' to 5' end using standard automated DNA synthesis protocols (1.0 μmol scale). A 0.1 M solution of **6** in dry acetonitrile was prepared and installed on the DNA synthesizer in a standard reagent bottle. Normal solid-phase oligodeoxynucleotide synthesis was performed. In the last step, the PTZ-nucleobase analog phosphoramidite was introduced and allowed to react with the oligodeoxynucleotide for 15 minutes. Use of the toluoyl protecting groups instead of the DMT group prevents monitoring the coupling efficiency. After syntheses, the PTZ-labeled oligodeoxynucleotides were cleaved from the column and the collected oligodeoxynucleotides were incubated at 55° C for 16 hours in a 30% NH_4OH solutions for the deprotection of base and phosphate moieties.

Purification and Characterization

HPLC purification of the PTZ-labeled oligodeoxynucleotides was accomplished using a Rainin HPLC instrument. Reverse phase chromatography was performed on a C18 column (25 cm x 4.6 mm) with acetonitrile (ACN) and 0.1 M triethylamine acetate (TEAA) as eluting solvents. A flow rate of 3 mL/min was used and the concentration of ACN was increased from 5% to 50% over 40 minutes. The retention times of the phenothiazine labeled oligodeoxynucleotides **7** and **8** (R_t = 29 min) were well separated from the unlabeled oligodeoxynucleotide (R_t = 15 min). Besides purification, HPLC also provides a way to determine the yield. The overall yield for incorporating a phenothiazine-nucleoside in oligodeoxynucleotides was approximately 28%.

MALDI mass spectrometry (positive ions) was used to characterize the phenothiazine labeled oligodeoxynucleotides.

Sequence	Calculated MW	Found by MALDI
7	5268.9	5272
8	5268.9	5272.3

Melting Curves

The stability of the duplex formed between two complementary oligodeoxynucleotides was determined from the melting curve profiles as a function of temperature. The T_m value was determined from the first derivative. The concentrations of stock solutions of oligodeoxynucleotide single strands were determined from the UV-vis absorbance spectra. Enzyme digestion of the oligdeoxynucleotides to yield the individual nucleotides with Nuclease P1 *penicillium citrinum* was performed by the addition of 1 μL of a 1 mg/mL solution of enzyme to 5.0 μL of oligodeoxynucleotide single strand solution and 44.0 μL of 20 mM sodium acetate buffer at pH 5.5. The solutions were incubated at 55 ^{o}C for 30 min. A sample of 20.0 μL of digest solution was diluted to 2.00 mL with water, and the absorption spectrum measured. The total molar absorptivity was determined from the sum of all the individual contributions of each nucleoside and PTZ. The concentrations of the stock solutions were then calculated. Equimolar amounts of each single strand were measured out of the stock solutions, combined, and diluted with phosphate buffer (5 mM NaH_2PO_4, 50 mM NaCl, pH = 7) to the appropriate concentration for UV-vis measurements. The solution was heated to 90 ^{o}C for 3 min and allowed to slowly cool to room temperature. After cooling, the thermal denaturation experiment was performed using the following parameters on a HP UV-vis: a) monitoring wavelength, 260 nm, b) temperature range, 25 - 85 ^{o}C, c) temperature step, 0.2 ^{o}C, d) averaging time constant, 15 s, e) 0.2 ^{o}C rate of change.

Fluorescence experiments

Emission spectra were measured on a Jobin-Yvon-Horiba Fluorolog-3 luminescence spectrophotometer. The PTZ-puplex (**8·9)** was dissolved in an aqueous buffer (50 mM sodium phosphate, 50 mM sodium chloride at pH = 7.0) at a concentration of 2 x 10^{-5} . Measurements were performed in deoxygenated solutions at room temperature. Excitation of **5** at 315 nm produces a broad emission centered at approximately 451 nm in CH_3CN/H_2O (4:1), and agrees with the reported literature value.[6] The phenothiazine labeled DNA duplex, **8·9**, possess an emission blue-shifted at 397 nm (50 mM NaCl, 5 mM NaH_2PO_4, pH = 7). The phenothiazine labeled DNA duplex, **7·8**, possess an emission at 397 nm The emission quantum yields were calculated from the integrated spectra relative to Me-PTZ. The quantum yield of Me-PTZ in ACN:H_2O (4:1) is 0.0081.[6] The error in our analysis is 0.0003. Anisotropy measurements were performed in deoxygenated solutions at room temperature with 20% v/v of PEG (350 MW) added to increase the viscosity of the buffer solution. Addition of PEG did not alter the T_m of duplex **7·8**. No anisotropy was observed for Me-PTZ in methanol.

References:

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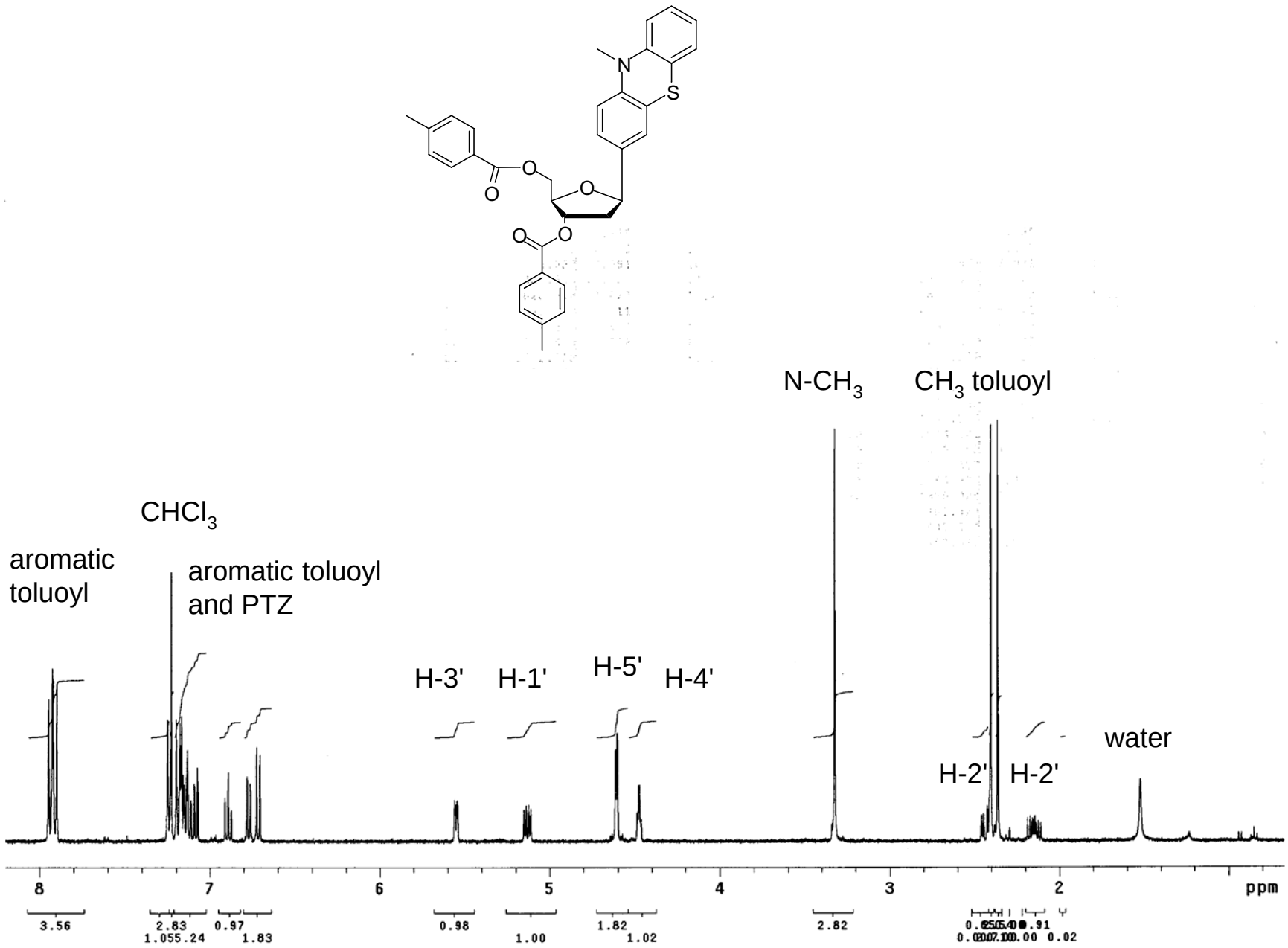
(5) Ren, R. X.-F.; Chaudhuri, N. C.; Paris, P. L.; Rumney, S.; Kool, E. T. *J. Am. Chem. Soc.* **1996**, *118*, 7671-7678.
(6) Kawanishi, Y.; Tazuke, S. *J. Phys. Chem.* **1986**, *90*, 2469-2475.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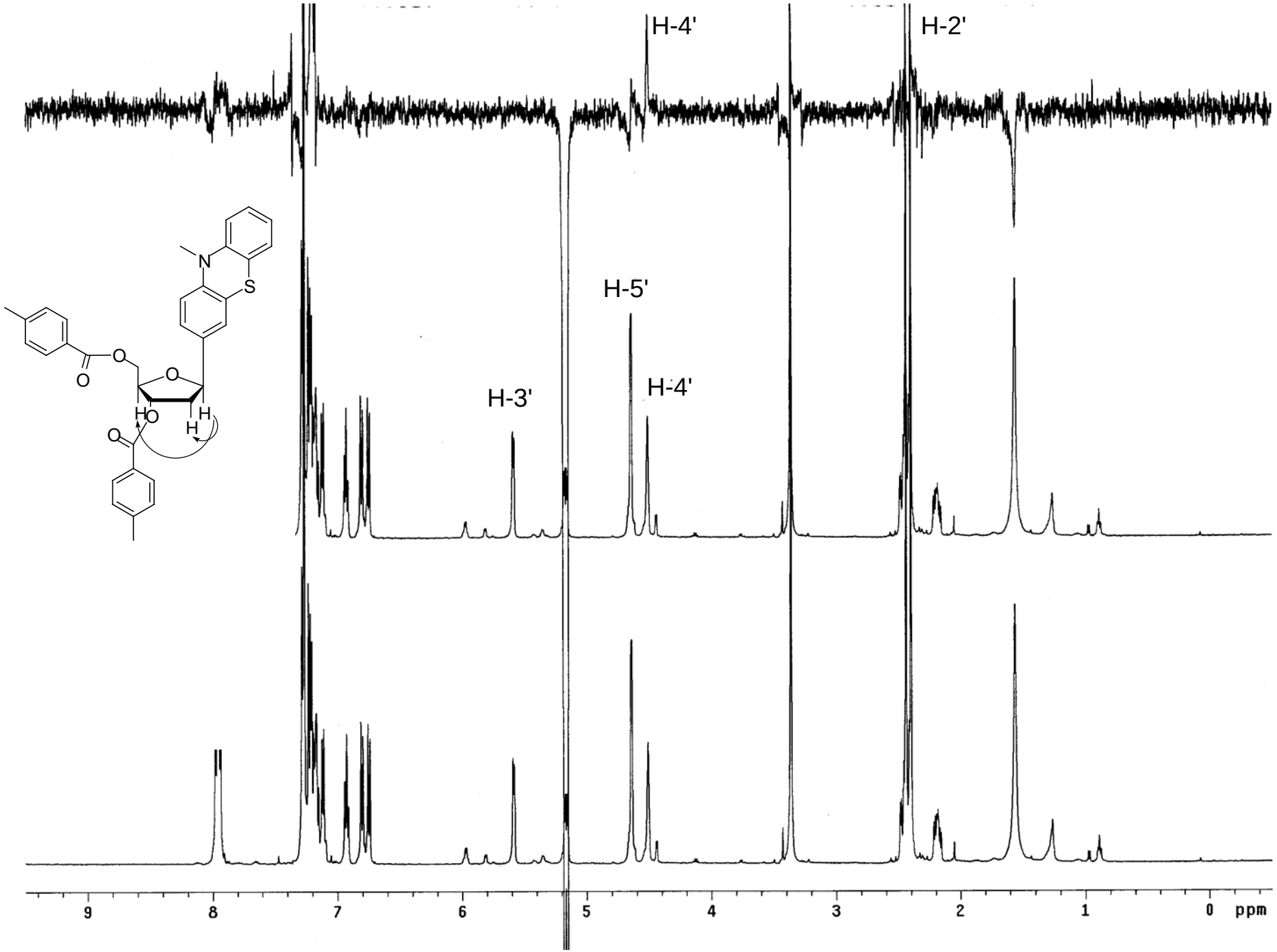
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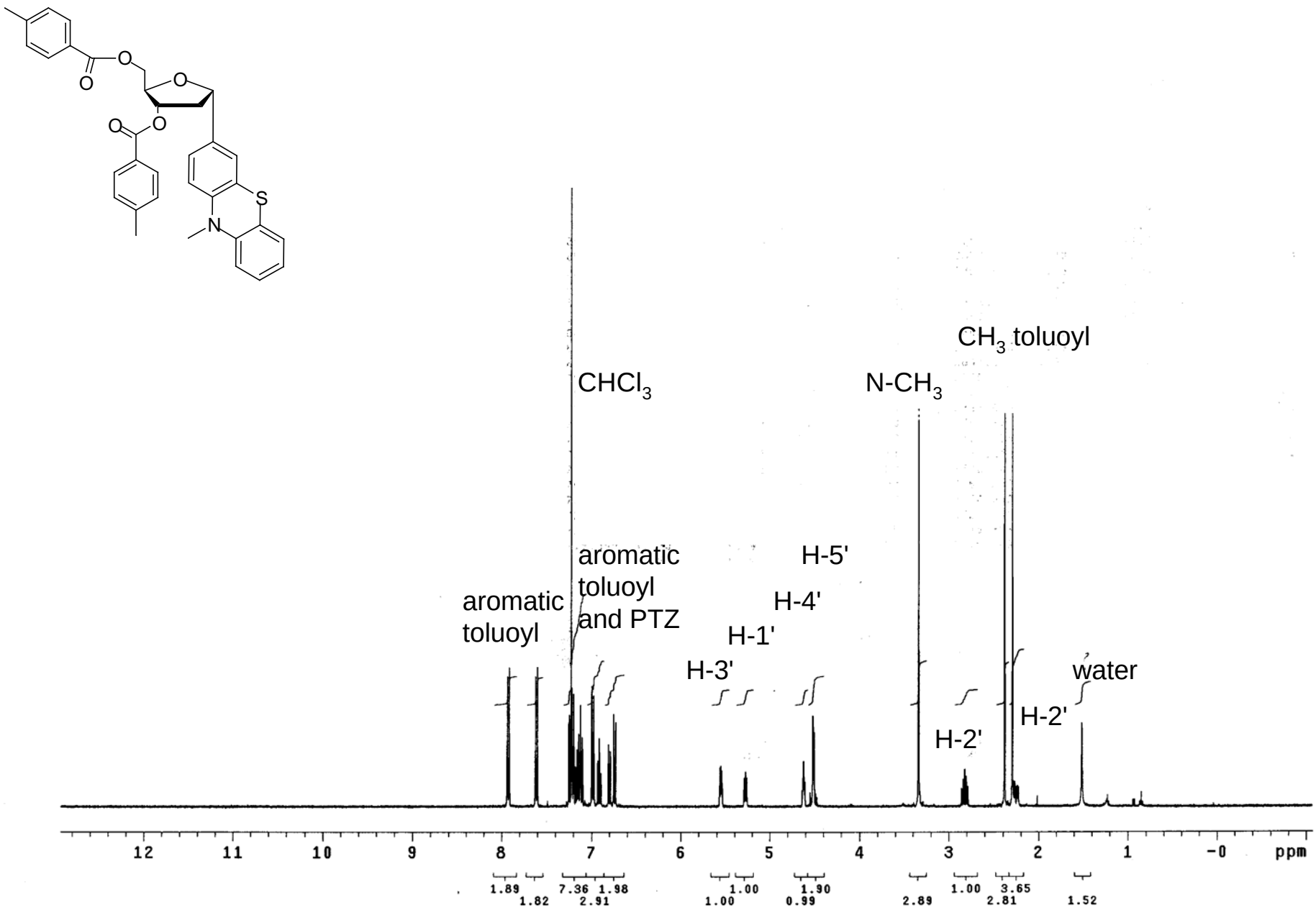
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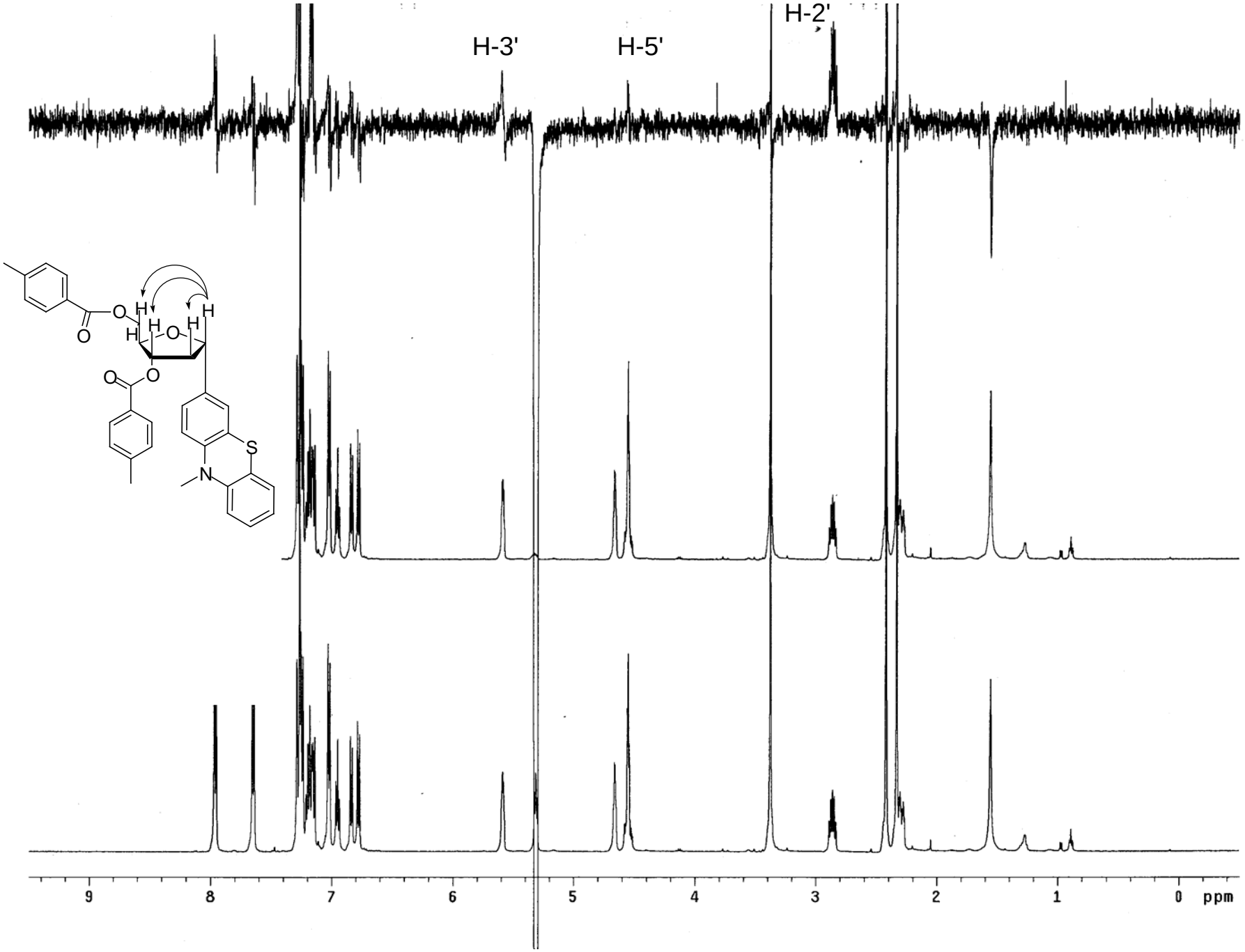
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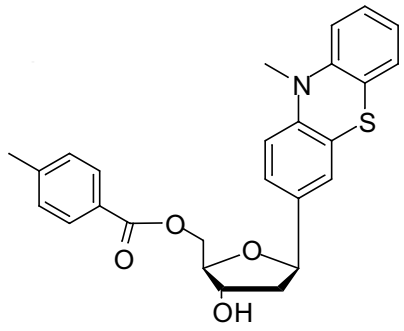
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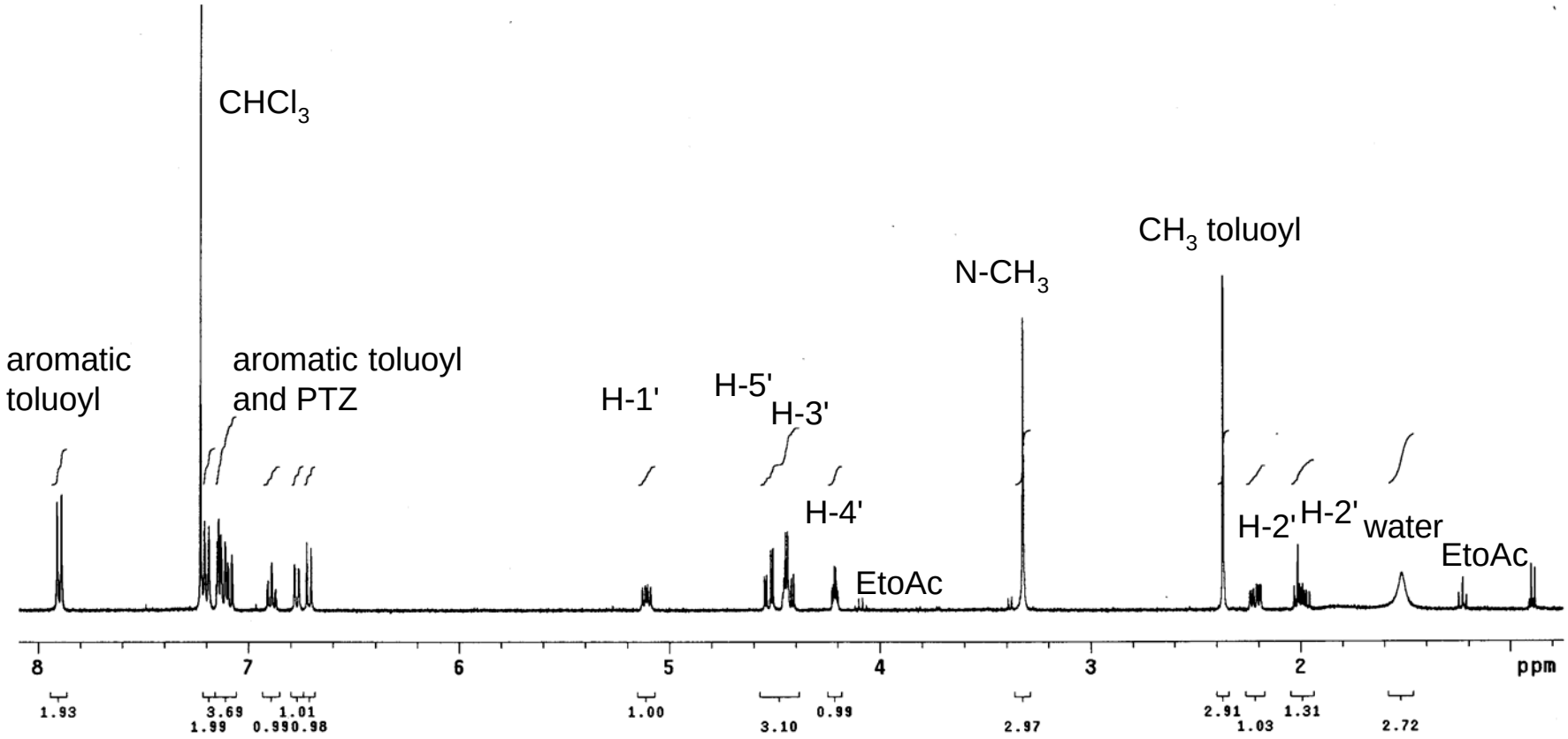
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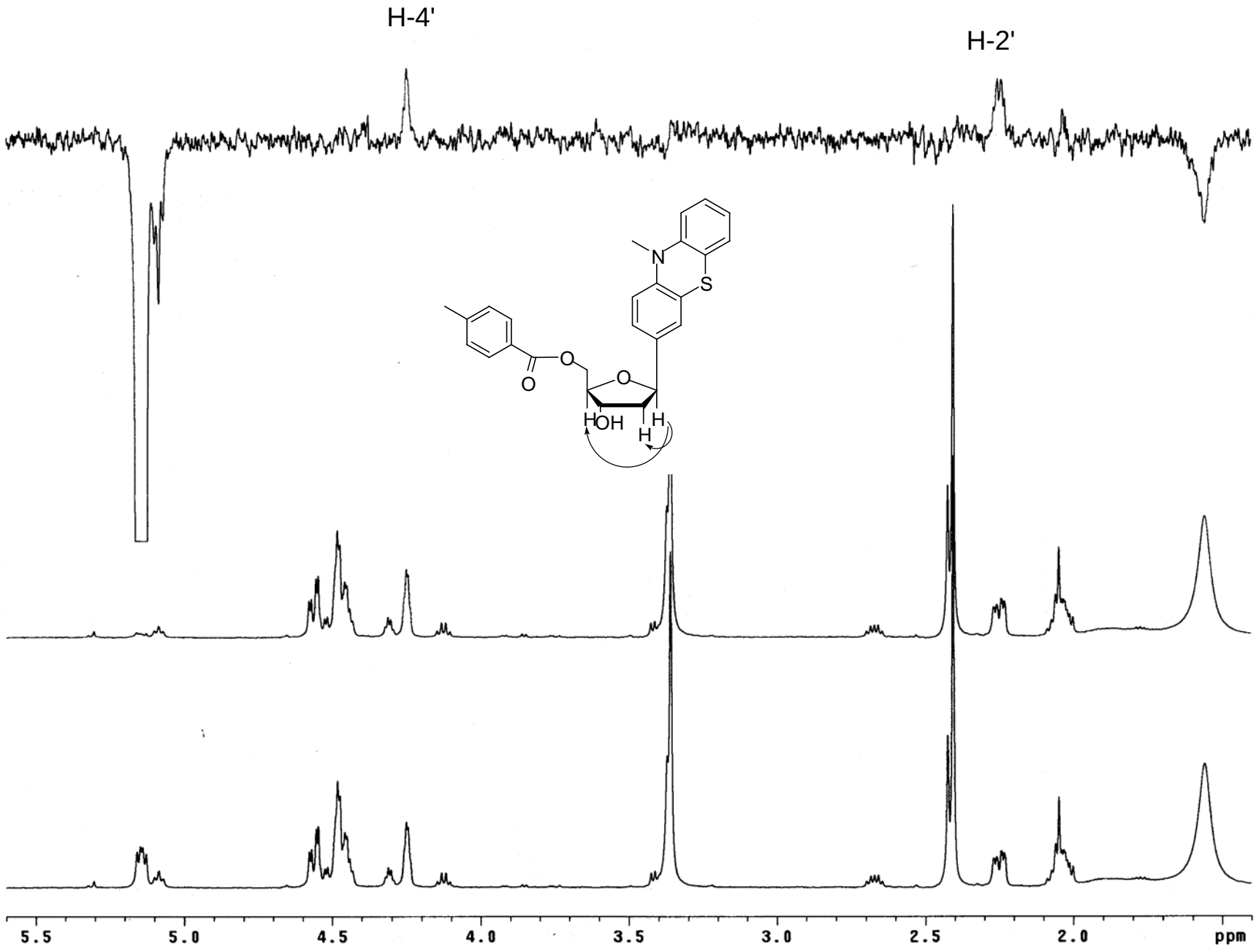
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