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Remote Interactions Explain the Unusual Regioselectivity of Lipase from Pseudomonas cepacia toward the Secondary Hydroxyl of 2'-Deoxynucleosides

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General Spectroscopic and Experimental Data

Immobilized *Pseudomonas cepacia* lipase (PCL-C, also known as PSL-C, 783 U/g) was obtained from Amano Pharmaceutical Co. Melting points were measured in open capillary tubes and are uncorrected. IR spectra were recorded on a Fourier transform infrared spectrophotometer using KBr pellets. Flash chromatography was performed using silica gel 60 (230-400 mesh). ^{1}H-, ^{13}C-NMR, and DEPT were obtained using AC-200 (^{1}H, 200.13 MHz and ^{13}C, 50.3 MHz), and AC-300 (^{1}H, 300.13 MHz and ^{13}C, 75.5 MHz), or DPX-300 (^{1}H, 300.13 MHz and ^{13}C, 75.5 MHz) spectrometers for routine experiments. An AMX-400 spectrometer operating at 400.13 and 100.61 MHz for ^{1}H and ^{13}C, respectively, was used for the acquisition of ^{1}H-^{1}H and ^{1}H-^{13}C correlation experiments. The chemical shifts are given in delta (d) values and the coupling constants (J) in Hertz (Hz). ESI^{+} was used to record mass spectra (MS). Microanalyses were performed on a Perkin-Elmer model 2400 instrument.

Molecular Modeling

The crystal structure of the PCL was relaxed before addition of the substrate to the experimental structure. The PDB file 3LIP was opened with all atoms of the crystal structure, including water molecules. Hydrogen atoms were added to the structure to correspond to pH 7, tested for partial charge balance and corrected for atom types within the AMBER force field. The pH of the catalytic histidine was adjusted to pH 4 to protonate the residue and the structure was then relaxed. Geometry optimizations were carried out in two steps. First, geometry optimization using the steepest descent algorithm to a local minima with a RMS deviation of about 0.02 Å mol^{-1}corrected major structural problems. Second, conjugate gradient algorithm gave a faster minimization near the local minima, obtaining a RMS value of 0.005 Å mol^{-1} or lower. The entire structure was relaxed by a systematic manner that avoided large structural changes. First only the water molecules were geometry optimized; next , both the water molecules and the side chains of amino acids were geometry optimized. Finally, all atoms of enzyme and solvent were geometry optimized. For all further geometry optimizations in this study the solvent molecules were never fixed. This was to ensure that water molecules were mobile and that their position did not hinder the nucleoside or the enzyme. Energy refers to the potential energy of the enzyme-tetrahedral intermediate complex.

Geometry Optimization of Phosphonate Core. A phosphonate core of the tetrahedral intermediate mimicking butanoylation of ethanol (X= O; R= Pr) was assembled in the crystal structure. The partial charges on the intermediate atoms were set to those of the tetrahedral intermediate (carbon, not phosphorus), the values of which were obtained by semi-empirical calculation performed using Chem3D using the AM1 parameters. The formal charge of the phosphonate moiety was -1.

For the optimization of the phosphonate core a systematic approach of releasing different motifs was applied. Initially, the intermediate was allowed to adjust to the enzyme active site by keeping the entire enzyme fixed during the geometry optimization. The side chains of the lipase were then released and allowed to adjust and finally the entire complex was allowed to adjust. This approach avoided drastic changes in the lipase structure caused by nonoptimal conformations of the intermediate. A hydrogen bond calculation was performed after each minimization in order to assure the presence of the critical hydrogen bonds around the catalytic site.[1]

Phosphonates including thymidinyl group. After obtaining a catalytically productive intermediate core, the remainder of the nucleoside was added and different conformations were produced by manual adjustment of dihedral angles identified in Figure S1. Catalytic requirements for a covalent link to the catalytic serine and hydrogen bonds to the catalytic histidine and oxyanion restrict how the nucleoside can orient in the active site. For this reason, we did not use automated docking procedures, but relied on a systematic conformational search. Geometry optimizations were again performed using the same approach used to optimize the phosphonate core with systematic releasing of the substrate, amino acid side chains and the entire enzyme complex. Again, minimizations were carried out with both the steepest descent and conjugate gradients until an RMS value of at least 0.005 $Å.mol^{-1}$ was obtained.

For reaction at the 3' position, the conformational search involved 120° rotations of two dihedral angles identified in Figure S1 to find all possible staggered conformations. The geometry of each conformation was then optimized. For at the 5' position, the conformational search involved an additional dihedral angle, Figure S1. Small adjustments of the dihedral angles caused large changes in the orientation of the thymine moiety and the interaction of substrate with the convoluted enzyme surface produced more local minima than for a nucleoside in solution. Large steps of 120° did

[1] To identify hydrogen bonds, a donor atom to acceptor atom distance of less than 3.20 Å and a donor atom - hydrogen - acceptor atom angle of 120° or greater are required.

not find all possible local minim. A more extensive search used manual adjustments of 10-20° were performed for both dihedral angles adjacent to the 5'-position. Structures with obvious steric problems were ignored. Additional manual adjustments oriented the substrate into visible pockets.

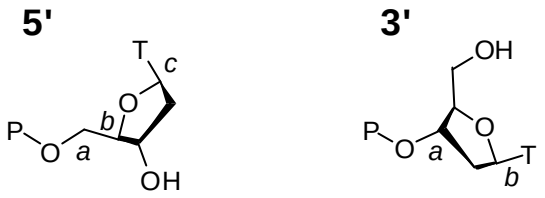

Figure S1. Manipulated dihedral angles for 5' and 3' structures. Dihedral angles corresponding to all the bonds labeled above were adjusted during manual conformational searching. For most angles, adjustments of 120° were made of each dihedral angle to produce possible conformations. Angle *a* of the 5' structure was manipulated using smaller adjustments to account for large changes in substrate conformations caused by these adjustments. In addition, different conformations of the ribose ring were explored.

Molecular dynamics. Starting from the transition state analogue derived from the proposed productive conformation for butanoylation at the 5'-hydroxyl group (PCL-5'-A), we performed molecular dynamics for 1 ps at 300 K for the equilibration period and 100 ps at 300 K for the simulation using a time step of 1 fs with a history file generated every 500 fs. Models with significantly different conformations were extracted and minimized as described above. Figure S2 and Table S1 contain details for the catalytically productive conformers. The linking to the catalytic serine and the shape of the active site dramatically limited the conformational range of the nucleoside and molecular dynamics did not identify significantly different conformations. For example, the seven conformations in Figure S2 all lead to the same conclusion – that the thymine ring remains in the solvent.

Table S1. Catalytically productive conformations of phosphonate analogues of tetrahedral intermediates for butanoylation of thymidine catalyzed by PCL obtained by molecular dynamics, followed by geometry optimization.[a]

Conformer	Key H-bonds	Thymine ring binding	Unfavorable intramolecular interactions
PCL-5'-A	5 of 5	unbound	none
PCL-5'-B	5 of 5	unbound	none
PCL-5'-C	5 of 5	unbound	none
PCL-5'-D	5 of 5	unbound	none
PCL-5'-E	5 of 5	unbound	none
PCL-5'-F	5 of 5	unbound	none
PCL-5'-G	5 of 5	unbound	none

[a] Productive conformations are those with catalytically relevant hydrogen bonds N–O distances of <3.2 Å and N–H–O angles >120° and no severe steric interactions with enzyme residues.

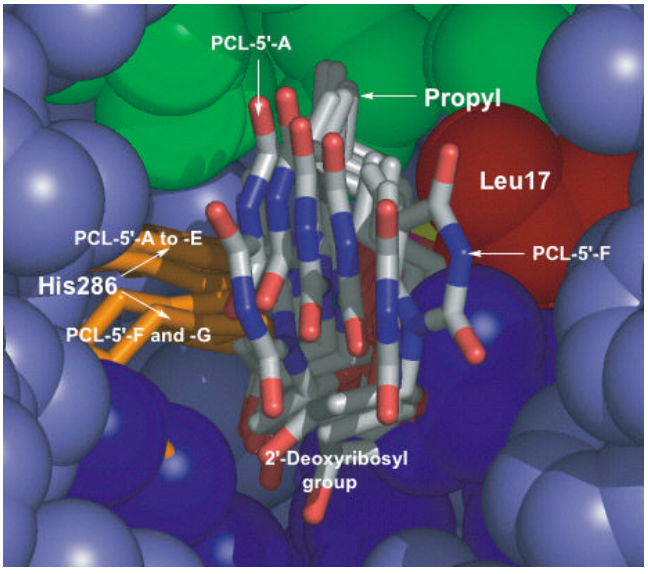

Figure S2. Overlay of seven catalytically productive conformations for butanoylation of the 5'-hydroxyl generated using molecular dynamics, followed by geometry optimization. Residues surrounding the large hydrophobic pocket (Val266, Val267, Leu167, Phe119 and Pro113) are colored green and residues surrounding the alternate hydrophobic pocket (Ile290, Leu287, Thr18 and Tyr29) are colored dark blue. Oxyanion hole residues Leu17 and Gln88 are colored red and yellow (barely visible), respectively. Conformers PCL-5'-A to PCL-5'-E have similar conformations as the proposed productive conformer PCL-5'-A as shown by a similar catalytic histidine 286 (orange). PCL-5'-F and PCL-5'-G have significantly different conformations than PCL-5'-A to PCL-5'-E as shown by a different His286 orientation. However, all conformers place the thymine ring in the solvent and not bound in an enzyme pocket.

Experimental Procedures and Spectroscopical Data

Oxime acetyl,[2] butanoyl,[3] decanoyl,[2] benzoyl,[4] and phenylacetyl[5] have been previously described.

*Enzymatic Acylation of Thymidine (**1**).* Oxime ester (1.25 mmol) and THF (5 mL) were added to a mixture of **1** (121 mg, 0.50 mmol), PCL-C (750 mg), and 3',5'-di-*O*-TMS-2'-deoxyuridine (25 mg, as internal standard) under nitrogen atmosphere in a 50-mL Erlenmeyer flask. The suspension was shaken at 250 rpm at 30 ºC or 60 °C until high or total conversions were achieved, or failing that, until no further reaction was apparent. Periodically, samples were taken from the reaction mixture, enzyme was filtered and the solvent was evaporated under reduced pressure and silylated as described by Sweeley *et al.*[6] The samples were analyzed by high performance liquid chromatography (HPLC) for the acetylation reaction on a Spherisorb W column (250 mm $\times$ 4.6 mm $\times$ 5 μm), with 3% MeOH/EtOAc as eluent, 0.9 mL/min at 40 ºC. 3',5'-Di-*O*-TMS-2'-deoxyuridine (as internal standard) appeared at 3.33 min; 3',5'-di-*O*-TMS-thymidine at 7.45 min; 3'-*O*-acetyl-5'-*O*-TMS-thymidine at 4.53 min; 5'-*O*-acetyl-3'-*O*-TMS-thymidine at 5.10 min; 3',5'-di-*O*-acetylthymidine at 3.96 min. The other acyl-ations were followed by gas chromatography (GC) on a TRACSIL TRB-5A capillary column (30 m $\times$ 0.32 mm $\times$ 0.50 μm), with nitrogen as carrier gas and a flame ionization detector. Injector and detector temperatures were set at 300 °C, head column pressure at 14 psi and split 90:1, column initial temperature 220 °C (3 min), rate 5 °C/min until 260 °C, then 15 min at 260 °C, followed by heating rate 5 °C/min until 300 °C, column final temperature was 300 °C (10 min). 3',5'-Di-*O*-TMS-2'-deoxyuridine (as internal standard) appeared at 11.8 min; 3',5'-di-*O*-TMS-thymidine at 11.0 min; 3'-*O*-butyryl-5'-*O*-TMS-thymidine at 16.3 min; 5'-*O*-butyryl-3'-*O*-TMS-thyidine at 15.9 min; 3',5'-di-*O*-butyrylthymidine at 23.3 min; 3'-*O*-decanoyl-5'-*O*-TMS-thymidine at 35.7 min; 3'-*O*-benzoyl-5'-*O*-TMS-thymidine at 32.7 min; 5'-*O*-benzoyl-3'-*O*-TMS-thymidine at 30.9 min; 3'-*O*-phenylacetyl-5'-*O*-TMS-thymidine at 33.3 min; 5'-*O*-phenylacetyl-3'-*O*-TMS-thymidine at 32.2 min. Conversion of the reaction was calculated by disappearance of the starting material with respect to internal standard.

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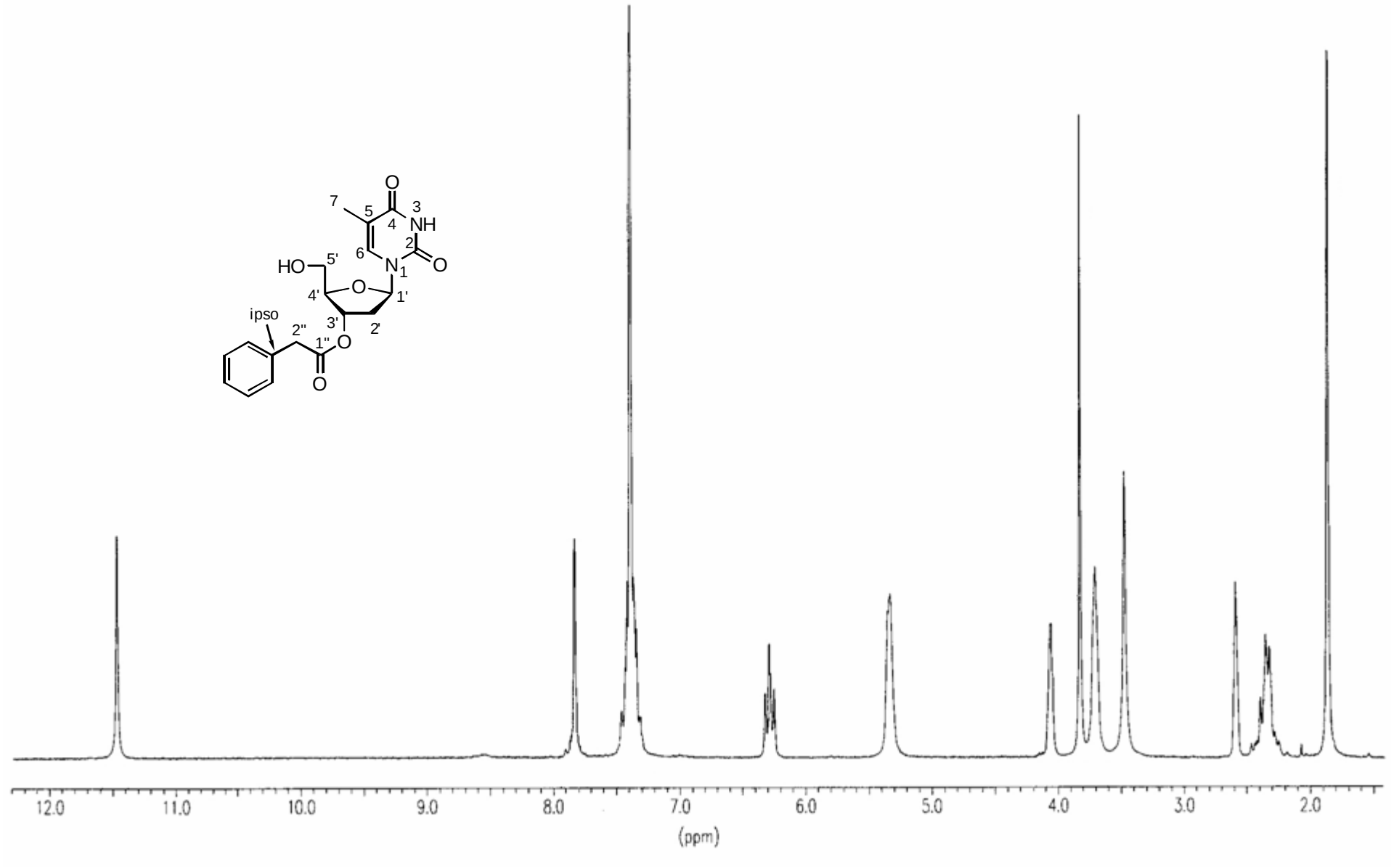
[2] Kirsch, J. F.; Jencks, W. P. *J. Am. Chem. Soc.* **1964**, *86*, 833-837.
[3] Gotor, V.; Pulido, R. *J. Chem. Soc., Perkin Trans. 1* **1991**, 491-492.
[4] Exner, O.; Horak, M.; Pliva, J. *Chem. Ind. (London)* **1958**, 1174-1175.
[5] Coelho, P. J.; Blanco, L. *Eur. J. Org. Chem.* **2000**, 3039-3046.
[6] Sweeley, C. C.; Bentley, R.; Makita, M.; Wells, W. W. *J. Am. Chem. Soc.* **1963**, *85*, 2497-2507.

3'-*O*-Acetylthymidine,[7] 5'-*O*-acetylthymidine,[8] 3',5'-di-*O*-acetylthymidine,[8] 3'-*O*-butyrylthymidine,[7] 5'-*O*-butyrylthymidine,[8] 3',5'-di-*O*-butyrylthymidine,[8] 3'-*O*-decanoylthymidine,[7] 3'-O-benzoylthymidine,[7] 5'-*O*-benzoylthymidine,[8] and 5'-*O*-phenylacetylthymidine,[9] were synthesized as has been previously reported.

3'-*O*-Phenylacetylthymidine. Oxime phenylacetyl (240 mg, 1.25 mmol) and THF (5 mL) were added to a mixture of **1** (121 mg, 0.50 mmol), and PCL-C (750 mg) under nitrogen atmosphere in a 50-mL Erlenmeyer flask. The suspension was shaken at 250 rpm at 60 °C during 22 h. Then, enzyme was filtered, washed with MeOH, and the solvent was evaporated under reduced pressure, and the crude products were purified by chromatography column (gradient eluent 60% EtOAc/hexane-EtOAc), obtaining a white solid. R_f (EtOAc) 0.4; mp 171-172 °C; IR (KBr) 1723 (C=O) cm^{-1}; ^{1}H NMR ([D_6]DMSO, 200 MHz) 1.83 (s, 3H, H_7), 2.42 (m, 2H, $H_{2'}$), 3.70 (m, 2H, $H_{5'}$), 3.83 (br s, 2H, $H_{2''}$), 4.08 (m, 1H, $H_{4'}$), 5.38 (m, 2H, $H_{3'}$+OH), 6.30 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 8.0 $^3J_{HH}$ 6.4 Hz), 7.48 (m, 5H, H_{ar}), 7.87 (s, 1H, H_6), and 11.50 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50.3 MHz) 12.4 (C_7), 36.5 ($C_{2'}$), 40.2 ($C_{2''}$), 61.4 ($C_{5'}$), 75.3 ($C_{3'}$), 83.7 ($C_{1'}$), 84.6 ($C_{4'}$), 109.8 (C_5), 126.9 (CH), 128.4 (CH), 129.4 (CH), 134.1 (C_{ipso}), 135.9 (C_6), 150.5 (C_2), 163.7 (C_4), and 170.9 (C=O); MS (ESI$^+$, *m/z*) 399 [*M*+K]$^+$, (100 %), 383 [*M*+Na]$^+$, (100), and 361 [*M*+H]$^+$, (2); elemental analysis calcd (%) for $C_{18}H_{20}N_2O_6$: C 59.99, H 5.59, N 7.77; found: C 60.2, H 5.3, N 8.0.

[7] Gotor, V.; Morís, F. *Synthesis* **1992**, 626-628.
[8] Morís, F.; Gotor, V. *J. Org. Chem.* **1993**, *58*, 653-660.
[9] Lavandera, I.; Fernández, S.; Magdalena, J.; Ferrero, M.; Kazlauskas, R. J.; Gotor, V. *ChemBioChem* **2005**, *6*, 1381-1390.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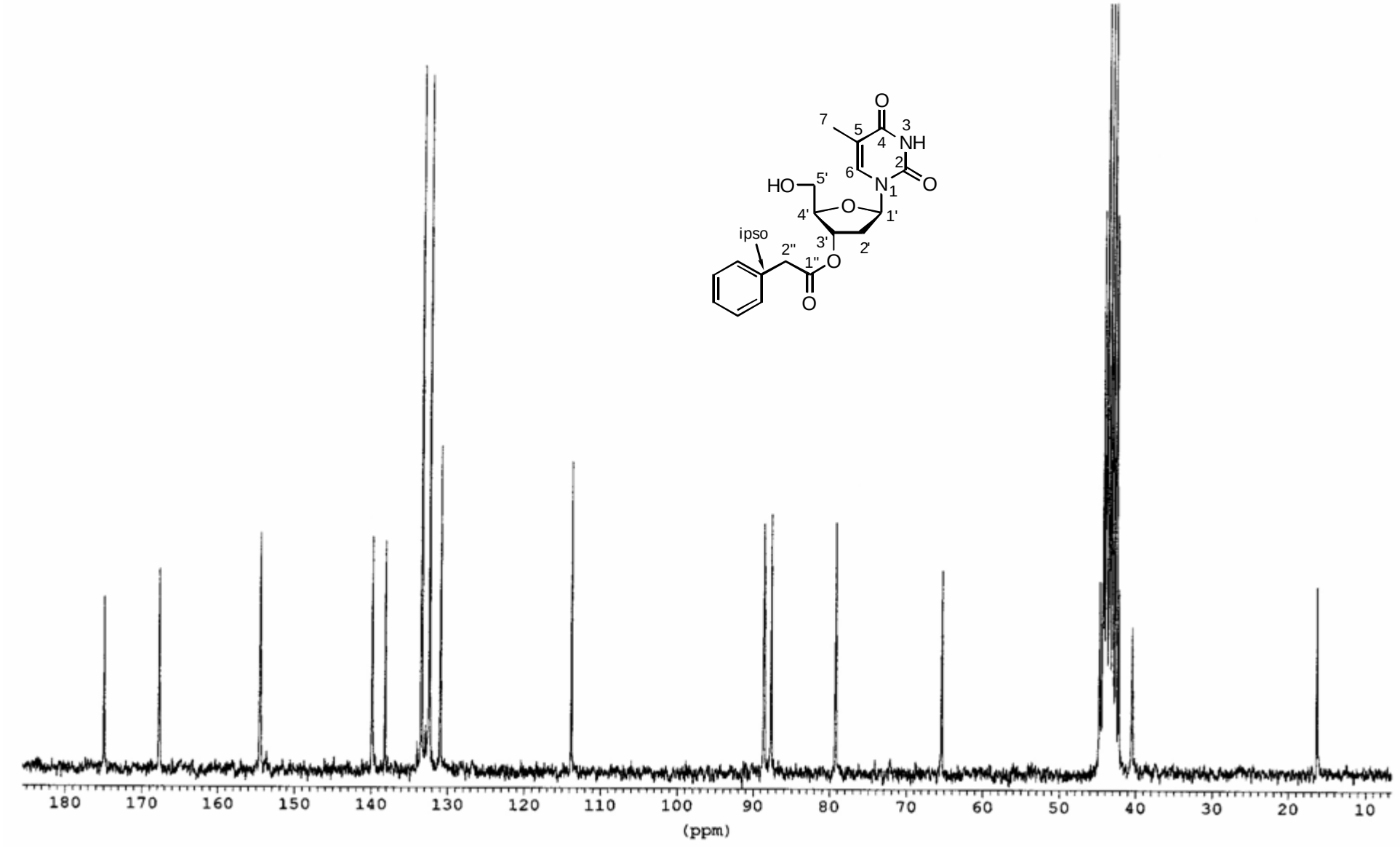
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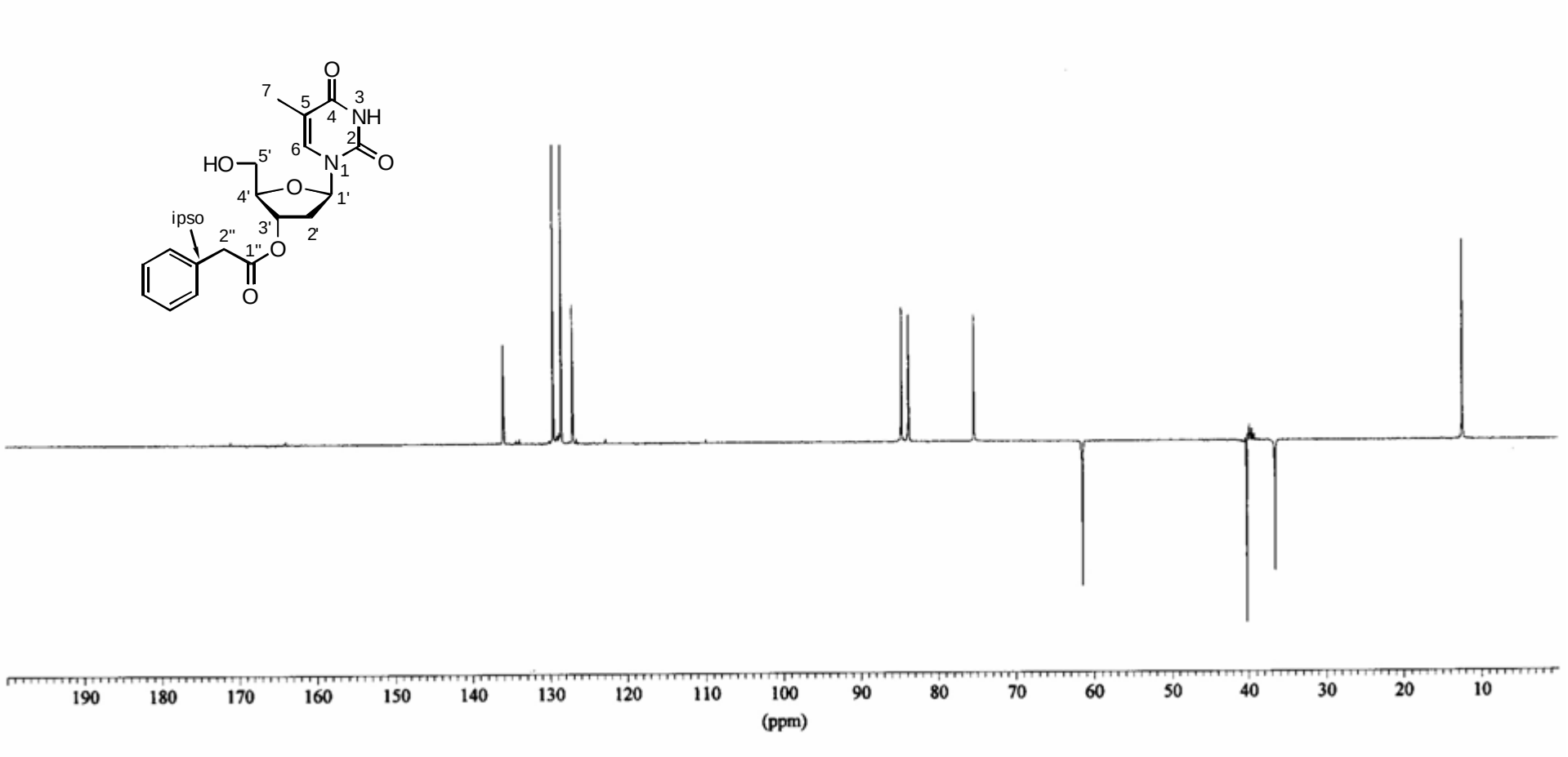
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