Supporting Information

Synthesis and Antiviral Activity of 5-Substituted Cytidine Analogues: Identification of a Potent Inhibitor of Viral RNA-Dependent RNA Polymerases

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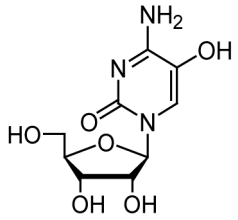
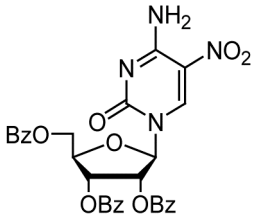
Chemical Synthesis Information

General. All reactions were performed under an anhydrous nitrogen atmosphere unless otherwise noted. 5-hydroxy-2'-deoxycytidine (**2**) and 5-bromo-2'-deoxycytidine (**3**) were acquired from Berry & Associates (Dexter, MI). 5-Bromocytidine (**5**) was purchased from Sigma-Aldrich (although no longer commercially available). Alternatively, it is readily synthesized from cytidine as previously described.[1] 5-bromocytidine triphosphate **8** was obtained from TriLink BioTechnologies (San Diego, CA). Commercial grade reagents (Aldrich, Acros) were used without further purification unless specifically noted. Tetrahydrofuran, acetonitrile, and *N*,*N*-dimethylformamide were rendered anhydrous by passing through the resin column of a solvent purification system (GlassContour; Laguna Beach, CA). Column chromatography employed ICN SiliTech silica gel (32-63 μm). HPLC purification was performed an Agilent 1100 series instrument (preparative scale) equipped with an Aquasil C18 preparative column (21.2 x 250 mm, 5 μm; Thermo Electron Corporation). Analysis of nucleotide purity was conducted on a Hewlett Packard 1100 series instrument (analytical scale) equipped with an Aquasil C18 analytical column (4.6 x 250 mm, 5 μm; Keystone Scientific Inc., [Thermo Electron Corp]). Nuclear magnetic resonance (NMR) spectroscopy employed Bruker CDPX-300, DPX-300, AMX-360, DRX-400, or AMX-2-500 MHz spectrometers. Internal solvent peaks were referenced in each case. Chemical shifts for ^{13}C NMR and ^{31}P NMR analyses performed in D_2O were indirectly referenced to 10% acetone in D_2O (CH_3 set to 30.89 ppm)[2] and 85% H_3PO_4 (0 ppm), respectively. ^{13}C chemical shifts for nucleoside diphosphates and triphosphates denoted with a (*) fail to resolve into clean singlets due to apparent conformational restrictions. Mass spectral data was obtained from either The University of Texas at Austin Mass Spectrometry Facility (FAB, ESI and CI) or The Pennsylvania State University Mass Spectrometry Facility (ESI and APCI). Elemental analyses were performed by Midwest Microlab, LLC (Indianapolis, IN).

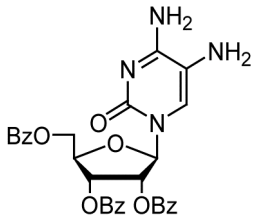


5-hydroxycytidine hydrate (4). This known compound was prepared by the method of Fukuhara and Visser.[3] ^1H NMR (DMSO-d_6, 400.1 MHz): δ 8.91 (br s, 1H), 7.36 (br s, 1H), 7.23 (s, 1H), 6.77 (s, 1H), 5.78 (d, J = 4.5 Hz, 1H), 5.23 (br s, 1H), 5.00 (m, 2H), 3.90 (m, 2H), 3.78 (m, 1H), 3.61-3.51 (m, 2H). ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 160.9, 154.2, 126.6, 122.3, 88.8, 84.2, 73.8, 70.0, 61.2. MS (APCI$^+$) calcd. for $C_9H_{14}N_3O_6$ $[M+H]^+$ 260.1, found 260.1. Anal. calcd. for $C_9H_{15}N_3O_7$: C 38.99; H 5.45; N 15.16. Found: C 38.79; H 5.45; N 14.77.

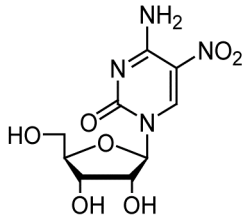


1-(2',3',5'-Tri-O-benzoyl-β-D-ribofuranosyl)-5-nitrocytidine (13).[4] 5-nitrocytosine[5] (**10**, 3.009 g, 19.28 mmole) was suspended in HMDS (40 mL, 191.8 mmole) containing TMSCl (1.0 mL, 7.82 mmole).[6-8] The mixture was refluxed for 36 h then concentrated *in vacuo* under high vacuum (ca. 0.4 Torr). To this crude material was added β-D-ribofuranose-1-acetate-2,3,5-tribenzoate (**12**, 9.737 g, 19.30 mmole) and the material was suspended in MeCN (125 mL). The suspension was degassed with N_2, then $SnCl_4$ (23 mL, 1.0 M solution in CH_2Cl_2) was added.[6-8] The suspension quickly clarified and the solution was stirred at 23 °C for 3 h. Distilled water (dH_2O, 100 mL) was added with vigorous stirring to hydrolyze the remaining $SnCl_4$, which facilitated the precipitation of the product. The solid was collected, suspended in EtOAc (500 mL) and washed with saturated aq. K_2CO_3 (100 mL, 4x). The organic layer was diluted with EtOAc (to 3.2 L) and washed with saturated aq. NaCl (800 mL, 1x) and dH_2O (800 mL, 2x). The organic layer was dried

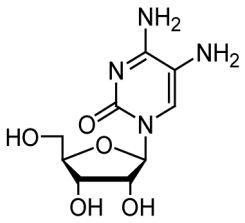
over anhydrous $MgSO_4$ and concentrated *in vacuo*. The resulting solid was triturated with warm $CHCl_3$ (100 mL, 2x) and dried *in vacuo* yielding **13** (9.008 g, 78% yield over 2 steps) as a cream colored solid. mp 211-213 °C (blackened) ^{1}H NMR (360.1 MHz, $CDCl_3$): δ 9.07 (s, 1H), 8.44 (br s, 1H), 8.07 (m, 2H), 7.91 (m, 5H), 7.52 (m, 3H), 7.42 (m, 2H), 7.33 (m, 4H), 6.34 (d, *J* = 3.6 Hz, 1H), 5.94 (m, 2H), 4.87-4.74 (m, 3H). ^{13}C NMR (90.6 MHz, $CDCl_3$): δ 166.2, 165.21, 165.19, 157.8, 152.0, 146.4, 133.73, 133.68, 133.5, 129.9, 129.8, 129.7, 129.1, 128.6, 128.54, 128.49, 128.42, 120.2, 90.5, 81.1, 74.9, 70.9, 63.4. IR (film): 1727, 1646, 1267 cm^{-1}. HRMS (ESI$^+$) calcd. for $C_{30}H_{24}N_4O_{10}Na$ $[M+Na]^+$ 623.1390, found 623.1387.



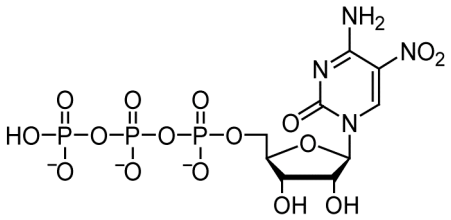
1-(2',3',5'-Tri-O-benzoyl-β-D-ribofuranosyl)-5-aminocytidine (14). To a degassed (N_2) solution of **13** (1.035 g, 1.72 mmole) in THF (65 mL) and glacial AcOH (5 mL) was added Pd/C (10%, 372 mg).[4] The solution was degassed again, charged with H_2 (1 atm), and stirred for 7 h at 23 ºC. The reaction was filtered through a pad of Celite, washed with excess MeOH, and concentrated *in vacuo*. The crude material was purified by column chromatography (10% MeOH in EtOAc) yielding **14** (624 mg, 63% yield) as a beige, glassy solid. mp 153-157 °C ^{1}H NMR (299.9 MHz, $CDCl_3$): δ 8.10 (m, 2H), 7.91 (m, 4H), 7.53-7.29 (m, 9H), 6.98 (s, 1H), 6.35 (d, *J* = 4.5 Hz, 1H), 5.91 (m, 1H), 5.81 (m, 1H), 4.87-4.58 (m, 3H), 2.81 (br s, 2H, D_2O exchangeable). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 166.1, 165.44, 165.35, 163.0, 155.0, 133.6, 133.5, 129.9, 129.8, 129.7, 129.4, 128.8, 128.68, 128.67, 128.4, 126.5, 115.3, 89.0, 79.7, 74.2, 71.2, 63.8. IR (film): 3342, 3196, 3068, 1726, 1268 cm^{-1}. HRMS (ESI$^+$) calcd. for $C_{30}H_{27}N_4O_8$ $[M+H]^+$ 571.1829, found 571.1833.



5-Nitrocytidine (6). Protected ribonucleoside **13** (1.27 g, 2.12 mmole) was suspended in aq. EtOH (50 mL, 4:1 absolute EtOH:distilled H_2O) at 23 °C. Three portions of NaOH (2.1 mL each, 1 N solution, 6.3 mmole total) was added at 20 min intervals and the solution was stirred for 3 h.[4] The reaction was then acidified to pH ca. 2 with aqueous HCl (1 N) and the EtOH was removed *in vacuo*. The resulting material was diluted to a total volume of ca. 75 mL by addition of distilled H_2O and warmed to resolubilize the material. The aqueous layer was extracted with $CHCl_3$ (50 mL, 3x) then neutralized to pH ca. 8 with aq. NH_4OH (10%). The material was concentrated *in vacuo* until a small amount of precipitate was observed, then cooled to 4 °C. The white solid was collected, washed with a minimal amount of water (ca. 10 mL), then redissolved in hot distilled H_2O (50 mL). The solution was quickly frozen and lyophilized to dryness. This step was repeated one additional time. The lyophilized material was dried *in vacuo* at 23 °C over P_2O_5 yielding **6** (318 mg, 52% yield) as a white solid. mp 129-131 °C (shrank at ca. 100 °C) ^{1}H NMR (360.1 MHz, DMSO-d_6): δ 9.72 (s, 1H), 8.48 (s, 1H), 8.03 (s, 1H), 5.69 (s, 1H), 3.97 (m, 3H), 3.82-3.59 (m, 2H). ^{13}C NMR (90.6 MHz, DMSO-d_6): δ 157.2, 151.8, 147.4, 119.4, 91.1, 83.8, 74.7, 67.6, 58.8. IR (KBr): 3323, 1648 cm^{-1}. UV (H_2O) 227 nm (λ_{max}, ε = 21,900 M^{-1} cm^{-1}), 321 nm (ε = 10,900 M^{-1} cm^{-1}). HRMS (ESI$^+$) calcd. for $C_9H_{13}N_4O_7$ $[M+H]^+$ 289.0784, found 289.0778.



5-Aminocytidine (7). Protected ribonucleoside **14** (1.12 g, 1.97 mmole) was suspended in aq. EtOH (50 mL, 4:1 absolute EtOH:distilled H_2O) at 23 °C. Three portions of NaOH (2.0 mL each, 1 N solution, 6.0 mmole total) was added at 20 min intervals and the solution was stirred for 3 h.[4] The reaction was then acidified to pH ca. 2 with HCl (1 N) and the EtOH was removed *in vacuo*. The residual water layer was warmed to resolubilize the material then extracted with $CHCl_3$ (40 mL, 3x). The aqueous layer was neutralized to pH ca. 8 with aq. NH_4OH (10%) then concentrated *in vacuo*. The crude material was dissolved in distilled H_2O (5 mL) followed by addition of absolute EtOH (50 mL) which yielded a cloudy solution. The solution was cooled to -20 °C and the material was allowed to precipitate. The resulting solid was collected, washed with excess Et_2O and set aside. Recrystallization of the mother liquor (2 additional crops) was achieved by redissolving the concentrated mother liquor in distilled H_2O (3 mL) followed by addition of absolute EtOH (50 mL), then cooling to -20 ºC. The three recrystallization batches were pooled together, dissolved in distilled H_2O (15 mL), and lyophilized to dryness. This step was repeated one additional time. The lyophilized material was dried *in vacuo* at 23 °C over P_2O_5 yielding (242 mg, 48% yield) of **7** as a light yellow solid. mp 191-192 °C ^{1}H NMR (360.1 MHz, DMSO-d_6): δ 8.43 (br s, 2H, D_2O exchangeable), 7.39 (s, 1H), 6.22 (br s, 2H, D_2O exchangeable), 5.78 (d, J = 4.9 Hz, 1H), 5.27 (m, 3H, D_2O exchangeable), 3.94 (m, 2H), 3.82 (m, 1H), 3.63-3.49 (m, 2H). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 158.1, 150.7, 122.8, 116.8, 88.5, 84.8, 73.8, 70.0, 61.1. IR (KBr): 3336 cm^{-1}. HRMS (ESI$^+$) calcd. for $C_9H_{15}N_4O_5$ $[M+H]^+$ 259.1042, found 259.1037.



5-Nitrocytidine-5'-triphosphate triethylammonium salt (9). This compound was prepared by the widely-utilized "one-pot, three-step" methodology for nucleotide synthesis.[9-20] 5-nitrocytidine (**6**, 66.3 mg, 0.23 mmole) was suspended in anhydrous pyridine (5 mL) and concentrated *in vacuo* (3x) to render the nucleoside anhydrous. To the dried material was added Proton-Sponge (100 mg, 0.47 mmole) and the material was dissolved in trimethyl phosphate (2.2 mL). The solution was cooled to 0 °C and $POCl_3$ (43 μL, 0.46 moles) was added dropwise. The resultant dark purple solution was stirred for 2 h at 0 °C, after which time Bu_3N (270 μL, 1.13 mmole) was added followed by a solution of tributylammonium pyrophosphate (560 mg) in DMF (2 mL). The solution was stirred for 2 min, then quenched by the addition of triethylammonium bicarbonate (TEAB, 5 mL, 1.0 M solution). The reaction components were frozen and lyophilized to dryness. The crude material was purified by stepwise preparative-scale HPLC. The initial purification of **9** employed the following linear gradient (flow rate = 20 mL/min): the mobile phase comprised 1% to 25% CH_3CN in triethylammonium acetate (TEAA) buffer (0 to 30 min, 20 mM TEAA, pH = 6) followed by 25% to 90% CH_3CN in TEAA buffer (30 to 35 min) and isocratic 90% MeCN in TEAA buffer (35 to 40 min). The material eluting broadly from 11–13 minutes was collected and concentrated *in vacuo*. This crude material was purified an additional time by preparative-scale HPLC utilizing the following linear gradient (flow rate = 20 mL/min): the mobile phase comprised isocratic 1% MeCN in TEAA buffer (0 to 5 min, 20 mM TEAA, pH = 6), 1% to 10% CH_3CN in TEAA buffer (5 to 20 min), 10% to 90% CH_3CN in TEAA buffer (20 to 25 min), isocratic 90% MeCN in TEAA buffer (25 to 30 min). The material eluting broadly from 17.5–19 minutes was collected and concentrated *in vacuo*. The material was redissolved in double distilled water (ddH_2O, 10 mL), frozen, then lyophilized to dryness. This step was repeated one additional time using ddH_2O (5 mL), providing 5-nitrocytidine triphosphate **9** (triethylammonium salt) as an oily solid (27.2 mg, 12% yield). ^{1}H NMR (300.1 MHz, D_2O): δ 9.13 (s, 1H),

5.66 (m, 1H), 4.19 (m, 5), 2.99 (q, *J* = 7.3 Hz, ca. 29 H, TEAA salt), 1.07 (t, *J* = 7.3 Hz, ca. 44 H, TEAA salt). ^{13}C NMR (75.5 MHz, D_2O): δ 163.4, 155.2, 147.6, 118.9, 92.1, 83.7*, 75.4, 68.7, 64.7*, 47.3 (TEAA salt), 8.9 (TEAA salt). ^{31}P NMR (145.8 MHz, D_2O): δ -8.99 (m), -10.92 (d, *J* = 21.8 Hz), -22.42 (br s). HRMS (FAB$^-$) calcd. for $C_9H_{14}N_4O_{16}P_3^-$ [M–TEAA+3H]$^-$ 526.9618, found 526.9613.

The chemical purity of **9** was further analyzed by analytical HPLC running the following linear gradient (flow rate = 1 mL/min). The mobile phase comprised 1% CH_3CN in KH_2PO_4 (0 to 5 min, 100 mM KH_2PO_4, pH = 6), 1% to 15% CH_3CN in KH_2PO_4 (5 to 20 min), and 15% to 80% CH_3CN in KH_2PO_4 (20 to 25 min). As shown in Figure S1, triphosphate **9** eluted at 4.3 min in 90% purity.

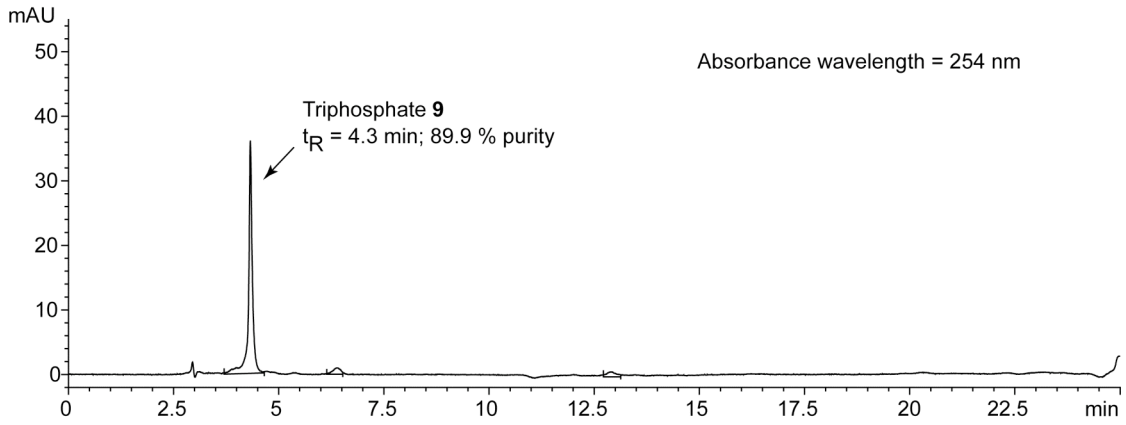


Figure S1. Analytical HPLC of synthetic 5-nitrocytidine triphosphate (**9**).

Cell Culture

HeLa S3 cells were maintained in DMEM/F-12 supplemented with 2% dialyzed fetal bovine serum and penicillin/streptomycin (1X, Invitrogen). Nucleosides were freshly suspended in 100% DMSO (200 mM) immediately prior to use. Ribavirin (**1**), a gift of Zhi Hong (Valeant Pharmaceuticals), was suspended in deionized water. For cytotoxicity studies, HeLa S3 cells (1 X 10^5) were plated the day before in 24-well plates. Cells were incubated with ribonucleosides at various concentrations for 7 hours at 37 °C. All wells were adjusted to a final concentration of 1% DMSO. Media was removed and cells were washed with PBS (0.5 mL). Cells were allowed to grow for an additional 24 h in the absence of compound. Cell monolayers were washed in PBS (0.5 mL), dissociated by treatment with trypsin (1X, Invitrogen), and viable cells were counted by trypan blue exclusion using a hemacytometer.

Antiviral Evaluation

Infection with poliovirus (PV) and coxsackievirus B3 (CVB3/0) employed HeLa S3 host cells (1 X 10^5) plated 1 day prior to treatment in 24-well plates. Cells were pretreated by addition of nucleoside at the specified concentration in fresh media adjusted to a final concentration of 1% DMSO. After a 1-hour incubation at 37 °C, media was removed and cells were infected with PV or CVB3/0 (1 X 10^6 PFU) in phosphate-buffered saline (PBS, total volume = 0.1 mL). Plates were incubated for 15 min at 23 °C, PBS was removed by aspiration, and fresh, prewarmed (37 °C) media containing the specified amount of nucleoside was added. The infection was allowed to proceed at 37 °C for 6 hours. Cells were washed with PBS and collected after treatment with trypsin. Cells were pelleted by centrifugation, resuspended in PBS (0.5 mL), and subjected to 3 freeze-thaw cycles. Cell debris was removed by centrifugation and the supernatant containing the cell-associated virus was saved. Titer was determined by applying serial dilutions of supernatant to HeLa S3 monolayers (plated in 6-well plates 1 day before at 5 X 10^5 cells/well) and overlaying with growth media containing low melting point agarose (1% for PV, 0.5% for CVB3/0). Plates were incubated for 2 (PV) or 3 (CVB3/0) days at 37 °C, at which time the agar was removed and plaques were visualized by staining with crystal violet (1%) in aqueous ethanol (20%).

Nucleotide Incorporation by Poliovirus RNA-Dependent RNA Polymerase in vitro

PV RNA-dependent RNA polymerase ($3D^{pol}$) was expressed and purified as previously described.[21] Extension assays utilizing symmetrical primer-template substrates (S/S) were performed as described.[22] S/S RNAs were synthesized by Dharmacon, Inc. In brief, PV $3D^{pol}$ was incubated with the appropriate S/S duplex for 90 s at 30 °C to allow formation of pre-initiation enzyme-RNA complexes. Extension reactions were initiated by the addition of nucleotide and reactions were incubated at 30 °C. For the experiment shown in Figure 2, the initiated reaction contained $3D^{pol}$ (5 μM), S/S RNA (1 μM), HEPES (50 mM, pH 7.5), 2-mercaptoethanol (10 mM), $MgCl_2$ (5 mM) and NTP (100 μM). This reaction also contained unlabeled S/S "trap" (100 μM) that was added along with initiating nucleotide to prevent re-initiation of dissociated enzyme. For the experiment shown in Figure 5, the initiated reaction contained $3D^{pol}$ (1 μM), S/S RNA (1 μM), HEPES (50 mM, pH 7.5), 2-mercaptoethanol (10 mM), $MgCl_2$ (5 mM), and NTP (10 μM each). Reactions were quenched by addition of EDTA (final conc. = 50 mM, pH 8.0). Product was added to an equal volume of loading buffer (90% formamide, 0.025% bromphenol blue, and 0.025% xylene cyanol) and heated to 65 °C prior to loading on a denaturing polyacrylamide gel containing 23% acrylamide, 1.5% bisacrylamide, 40% formamide, TBE buffer (1X, 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA), and urea (4 M). Electrophoresis was performed in TBE buffer (1X) at 30 mA for ca. 2 h. Products were visualized using a PhosphorImager (Molecular Dynamics). Quantitation was performed using ImageQuant software (Molecular Dynamics) and fit by non-linear regression using KaleidaGraph 3.5 software (Synergy Software, Reading, PA).

Cell Treatment and Preparation of Cell Extracts

This procedure is a modification of the method described by Pogolotti and Santi.[23] HeLa S3 cells were maintained in DMEM/F-12 supplemented with 2% dialyzed fetal bovine serum and penicillin/streptomycin (1X, Invitrogen). Prior to initiation of the experiment, HeLa cells (7.5×10^6) were plated in 100 mm dishes (50% confluency) and grown for 24 hours at 37 °C. The media was removed, replaced with fresh media (5 mL) containing actinomycin D (2.5 μg/mL, inhibits cellular transcription), and incubated for 15 min at 37 °C. The cells were then treated with either 5-nitrocytidine (**6**, 2 mM in DMSO) or DMSO (control) and incubated for an additional 3 h at 37 °C. The final concentration of DMSO in the media following treatment was 1%. The media was again removed, and the cells were washed with PBS (1 mL) and dissociated by treatment with trypsin (1X, Invitrogen). The dissociated cells were centrifuged to a pellet (ca. 5,000 g for 2 min), then resuspended in aqueous trichloroacetic acid (0.6 M, 200 μL), and incubated on ice for 10 minutes. *Important: the following steps must be performed on ice or in a cold room.* Cellular material was pelleted by centrifugation at 12,000 g for 2 minutes at 4 °C. The resulting supernatant was collected and 1,1,2-trichlorotrifluoroethane (200 μL) containing tri-n-octylamine (0.5 M) was added. The solution was mixed by vortexing, then centrifuged at 12,000 g for 30 sec. The aqueous upper phase of this extraction was collected, frozen, and stored at -80 °C until immediately prior to analysis.

Analysis of Cell Extracts by Reverse-Phase HPLC

HeLa cell extracts were analyzed on a Hewlett Packard 1100 series instrument equipped with an Aquasil C18 analytical column (4.6 x 250 mm, 5 μm; Keystone Scientific Inc., [Thermo Electron Corp]) running the following mobile phase (flow rate = 1 mL/min): isocratic 1% CH_3CN in KH_2PO_4 buffer (0 to 5 min, 100 mM KH_2PO_4, pH = 6), gradient 1% - 15% CH_3CN in KH_2PO_4 buffer (5 to 20 min), 15% - 80% CH_3CN in KH_2PO_4 buffer (20 to 25 min). The phosphate buffer was prepared by dissolving KH_2PO_4 in double distilled water at 100 mM and adjusting the pH to 6.0 by addition of aqueous KOH (10% solution). The integration of peak areas and generation of 3D UV plots were obtained from the ChemStation for LC 3D software (Rev.A.09.03, Agilent Technologies).

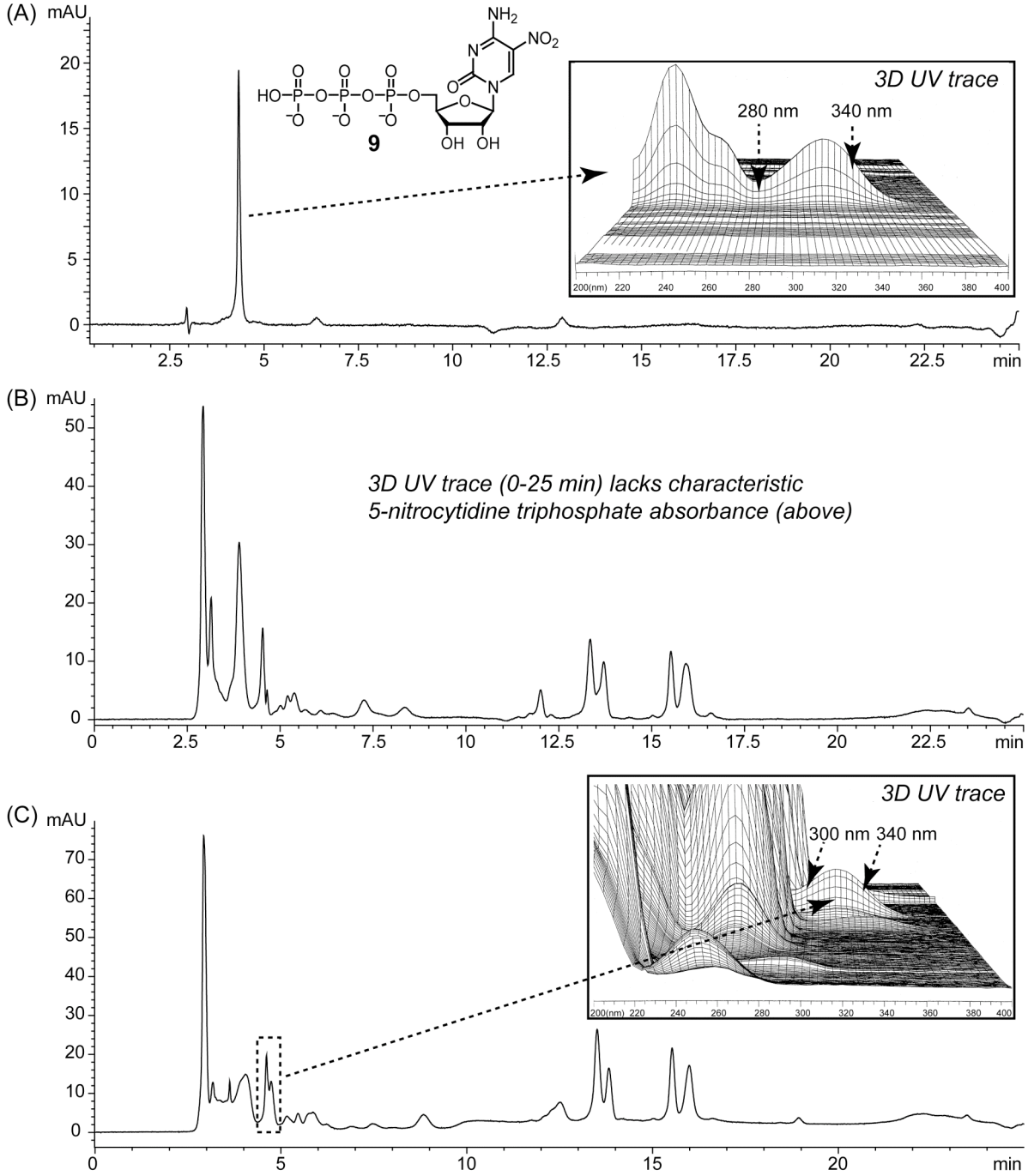


Figure S2. Analysis of HeLa cell extracts by reverse-phase HPLC. Panel A: Trace of 5-nitrocytidine triphosphate (**9**, 0.56 nmol) with characteristic UV trace (inlay). Panel B: Separation of untreated (DMSO only) HeLa cell extracts. Panel C. Separation of HeLa cell extracts treated with 5-nitrocytidine (**6**, 2 mM, 3 hr). The characteristic UV absorbance is shown in the inlay. The absorbance wavelength for all HPLC traces is 295 nm.

Luciferase-Based Reporter Assay for Poliovirus Replication

RNA transcripts carrying a luciferase reporter gene were generated as described for viral genomes from ApaI-linearized plasmids encoding pRLucRA.[24-26] HeLa cell cultures were propagated in DMEM/F-12 (Invitrogen) supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen), and maintained between 20% and 80% confluence. Subconfluent HeLa monolayers were detached from culture flasks by trypsin treatment, washed with 1X phosphate-buffered saline (PBS), adjusted to 2.4 x 10^6 cells/mL suspended in DMEM/F-12 containing ribavirin (**1**, 2 mM), 5-nitrocytidine (**6**, 1 mM), or no nucleotide as a control. All samples were adjusted to a final concentration of 1% DMSO. After 1 h incubation in a 37 °C water bath with agitation, HeLa cells were again pelleted, then resuspended in PBS, mixed with pRLucRA RNA (9 μg) in a microcentrifuge tube, transferred to an electroporation cuvette (0.2-cm gap width; Bio-Rad), and subjected to an electric pulse (500 microfarads, 0.13 V) with a Gene Pulser system (Bio-Rad). Electroporated cells were immediately transferred to pre-warmed (37 °C) DMEM/F-12 containing ribavirin (**1**, 2 mM), 5-nitrocytidine (**6**, 1 mM), or no nucleotide, with 1% DMSO and plus or minus guanidine hydrochloride (3 mM). The volume of electroporated cells added was calculated by multiplying 33 μL by n + 1 (where n equals the number of time points to be measured). The volume of DMEM/F-12 added to electroporated cells was calculated by multiplying 500 μL by n + 1. After mixing the appropriate volume of electroporated cells with the appropriate volume of medium, 500 μL aliquots were prepared in microcentrifuge tubes for each time point to be measured. These aliquots were incubated in a 37 °C water bath with agitation. At fixed time points, cells were pelleted by centrifugation (14,000 rpm, 2 min), lysed by addition of 1X cell culture lysis reagent (Promega, 100 μL), and vortexed. Cell lysates were maintained on ice for 2 min, then pelleted by centrifugation (14,000 rpm, 2 min) to remove cellular debris and nuclei. Cell lysates were maintained on ice at 4 °C until all time points were collected. Assays of cell lysates for luciferase activity were conducted by mixing of lysate (10 μL) with the luciferase assay substrate (10 μL, Promega) followed by quantifying in a Lumat LB 9501 luminometer (Berthold). Protein assays were performed to quantify the relative lights units (RLU) per milligram of protein. In this regard, cell lysate (5 μL) was added to Bio-Rad protein assay dye reagent (1 mL, Bio-Rad) and vortexed. Protein concentrations were obtained by measuring the UV absorbance at 595 nm.

References

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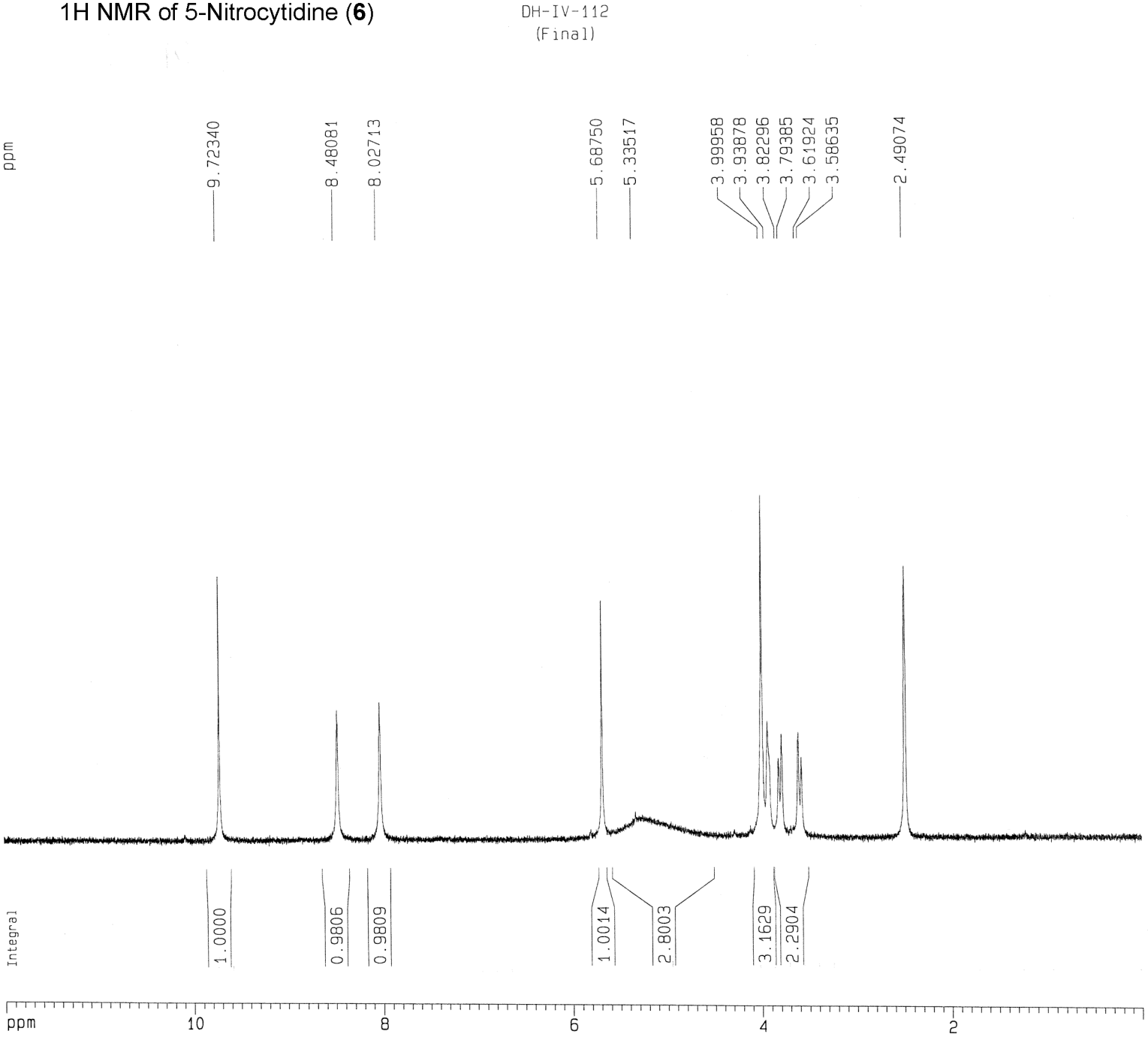
1H NMR of 5-Nitrocytidine (**6**)



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PROCNO	1

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DS	2
SWH	7462.687 Hz
FIDRES	0.113872 Hz
AQ	4.3909621 sec
RG	2048
DW	67.000 usec
DE	95.71 usec
TE	300.0 K
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D1	1.00000000 sec
P1	10.00 usec
SFO1	360.1378726 MHz
NUCLEUS	1H

F2 - Processing parameters

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WDW	no
SSB	0
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GB	0
PC	1.00

1D NMR plot parameters

CX	20.00 cm
F1P	12.000 ppm
F1	4321.63 Hz
F2P	0.000 ppm
F2	0.00 Hz
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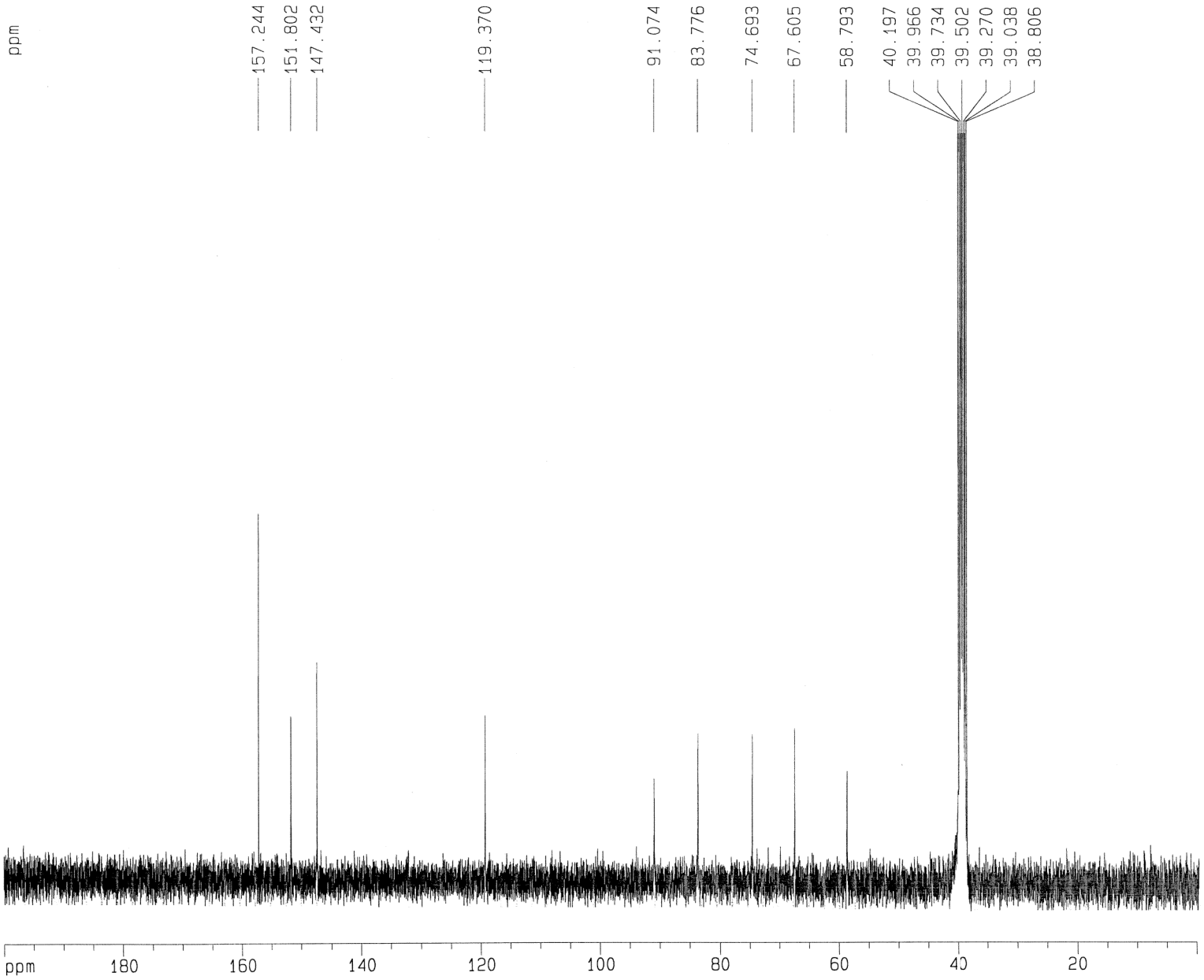
13C NMR of 5-Nitrocytidine (**6**)

DH-IV-112
(Final)



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TD	65536
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NS	9216
DS	4
SWH	22727.273 Hz
FIDRES	0.346791 Hz
AQ	1.4418420 sec
RG	32768
DW	22.000 usec
DE	31.43 usec
TE	300.0 K
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CPDPRG	waltz16
P31	110.00 usec
S4	31 dB
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S2	31 dB
P1	8.00 usec
SFO1	90.5663271 MHz
NUCLEUS	13C

F2 - Processing parameters

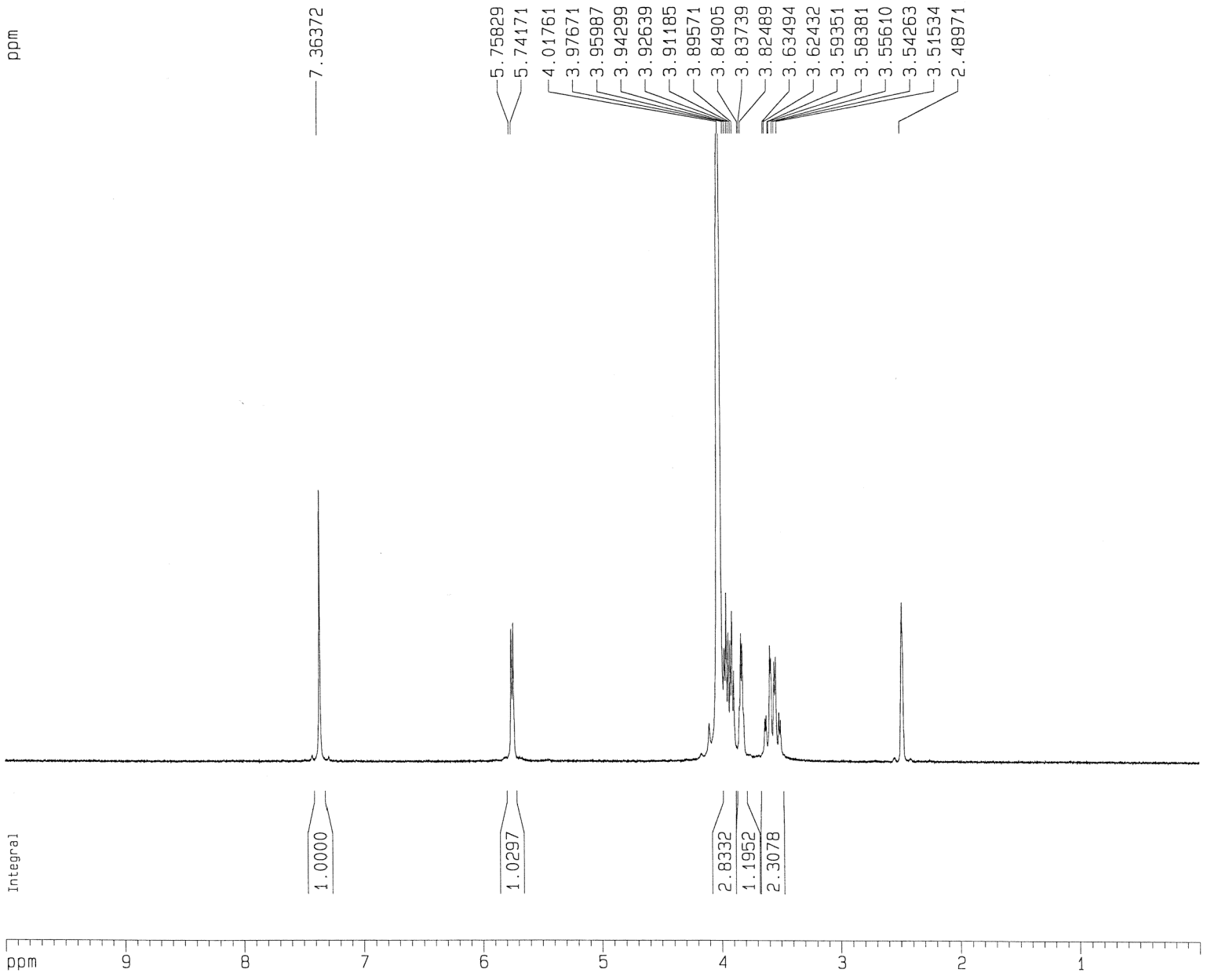
SI	32768
SF	90.5562332 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	200.000 ppm
F1	18111.25 Hz
F2P	0.000 ppm
F2	0.00 Hz
PPMCM	10.00000 ppm/cm

1H NMR of 5-Aminocytidine (**7**)

DH-IV-110 (all)
DMSO (+ D2O added)

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PROCNO                 1

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PULPROG            zg30
TD                65536
SOLVENT            DMSO
NS                    16
DS                     2
SWH           6172.839 Hz
FIDRES        0.094190 Hz
AQ            5.3084660 sec
RG                574.7
DW               81.000 usec
DE                6.00 usec
TE               300.0 K
D1            1.00000000 sec

============ CHANNEL f1 ========
NUC1                  1H
P1                 9.60 usec
PL1               -6.00 dB
SFO1        300.1318534 MHz

F2 - Processing parameters
SI                 32768
SF          300.1300045 MHz
WDW                   no
SSB                    0
LB                0.00 Hz
GB                     0
PC                 1.00

1D NMR plot parameters
CX                20.00 cm
F1P              10.000 ppm
F1               3001.30 Hz
F2P               0.000 ppm
F2                0.00 Hz
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HZCM         150.06500 Hz/cm
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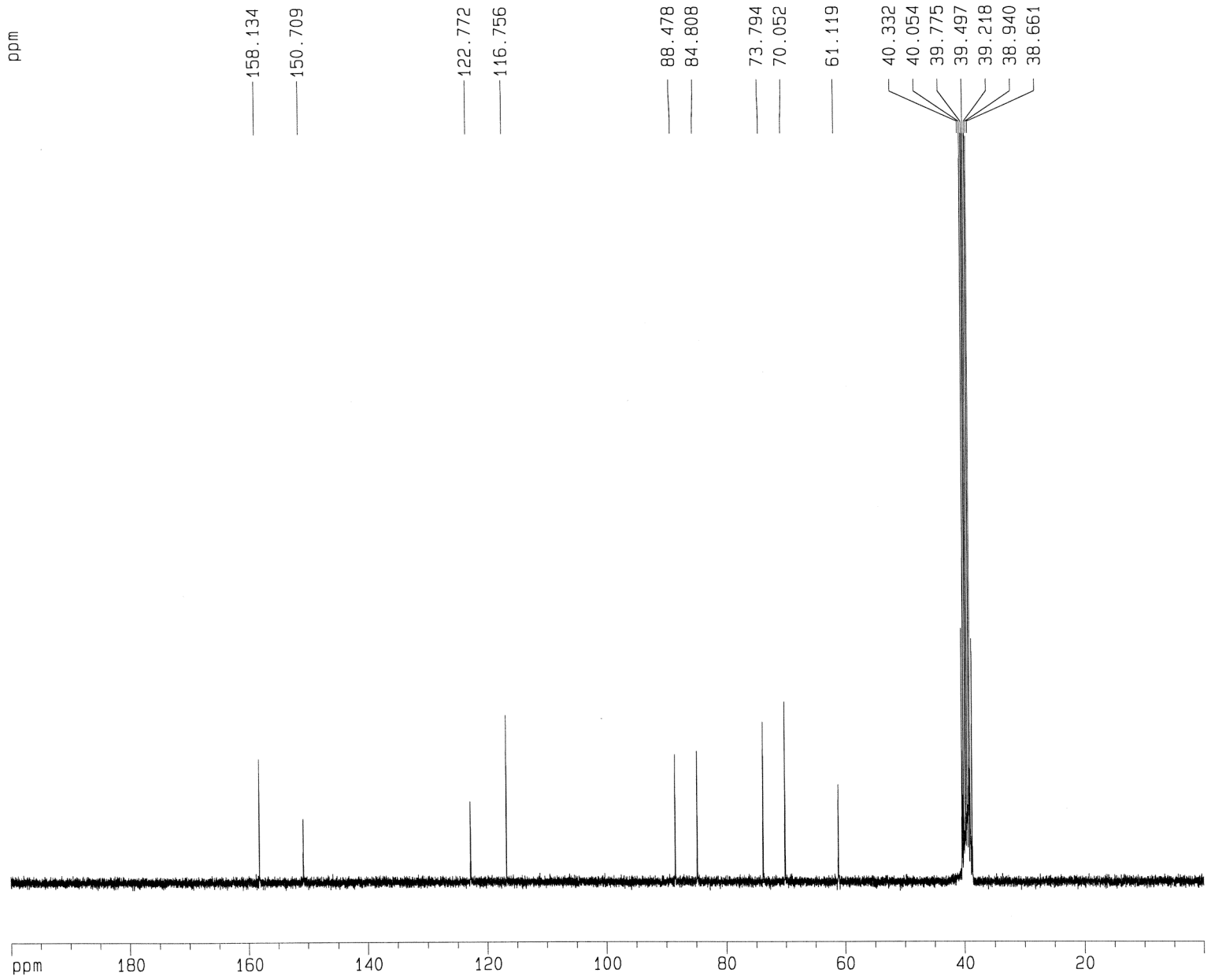
13C NMR of 5-Aminocytidine (**7**)

DH-IV-110 (all)

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PROCNO                 1

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PULPROG          zgpg30
TD                 65536
SOLVENT             DMSO
NS                 10240
DS                     4
SWH          18796.992 Hz
FIDRES        0.286819 Hz
AQ            1.7433076 sec
RG                   512
DW              26.600 usec
DE               6.00 usec
TE               300.0 K
D1          2.00000000 sec
D11         0.03000000 sec
D12         0.00002000 sec

============ CHANNEL f1 ============
NUC1                13C
P1              5.40 usec
PL1             -6.00 dB
SFO1        75.4106357 MHz

============ CHANNEL f2 ============
CPDPRG2        waltz16
NUC2                1H
PCPD2          115.00 usec
PL2             0.00 dB
PL12            20.00 dB
PL13            20.00 dB
SFO2       299.8711995 MHz

F2 - Processing parameters
SI                 32768
SF          75.4024057 MHz
WDW                   no
SSB                    0
LB              0.00 Hz
GB                     0
PC                  1.40

1D NMR plot parameters
CX                 20.00 cm
F1P               200.000 ppm
F1              15080.48 Hz
F2P               0.000 ppm
F2                 0.00 Hz
PPMCM          10.00000 ppm/cm
HZCM          754.02405 Hz/cm
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