Synthesis and Antiviral Evaluation of a Mutagenic and Non−Hydrogen Bonding Ribonucleoside Analogue: 1−β− d − Ribofuranosyl−3−nitropyrrole †

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Synthesis and Antiviral Evaluation of a Mutagenic and Non-Hydrogen Bonding Ribonucleoside Analogue: 1-β-D-Ribofuranosyl-3-nitropyrrole†

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ABSTRACT: Synthetic small molecules that promote viral mutagenesis represent a promising new class of antiviral therapeutics. Ribavirin is a broad-spectrum antiviral nucleoside whose antiviral mechanism against RNA viruses likely reflects the ability of this compound to introduce mutations into the viral genome. The mutagenicity of ribavirin results from the incorporation of ribavirin triphosphate opposite both cytidine and uridine in viral RNA. In an effort to identify compounds with mutagenicity greater than that of ribavirin, we synthesized 1-β-D-ribofuranosyl-3-nitropyrrole (3-NPN) and the corresponding triphosphate (3-NPNTP). These compounds constitute RNA analogues of the known DNA nucleoside 1-(2′-deoxy-β-D-ribofuranosyl)-3-nitropyrrole. The 3-nitropyrrole pseudobase has been shown to maintain the integrity of DNA duplexes when placed opposite any of the four nucleobases without requiring hydrogen bonding. X-ray crystallography revealed that 3-NPN is structurally similar to ribavirin, and both compounds are substrates for adenosine kinase, an enzyme critical for conversion to the corresponding triphosphate in cells. Whereas ribavirin exhibits antiviral activity against poliovirus in cell culture, 3-NPN lacks this activity. Evaluation of 3-NPNTP utilization by poliovirus RNA-dependent RNA polymerase (RdRP) revealed that 3-NPNTP was not accepted universally. Rather, incorporation was only observed opposite A and U in the template and at a rate 100-fold slower than the rate of incorporation of ribavirin triphosphate. This diminished rate of incorporation into viral RNA likely precludes 3-NPN from functioning as an antiviral agent. These results indicate that hydrogen bonding substituents are critical for efficient incorporation of ribonucleotides into RNA by viral RdRPs, thus providing important considerations for the design of improved mutagenic antiviral nucleosides.

Recent advances in our understanding of the broad-spectrum antiviral nucleoside, ribavirin (**1**), have demonstrated that agents which promote viral mutagenesis represent a promising new class of antiviral therapeutics (*1*−*11*). Studies of poliovirus, a model RNA virus, have revealed that a modest 9.7-fold increase in the rate of viral mutagenesis is sufficient to confer a 99.3% loss in infectivity (*1*). Hence, mutagens such as error-prone substrates of viral RNA-dependent RNA polymerases (RdRPs)[1] have the potential to misincorporate into the replicating viral genome, increase the frequency of viral genomic mutations, exceed the error catastrophe threshold, and extinguish the virus population (*1*, *6*).

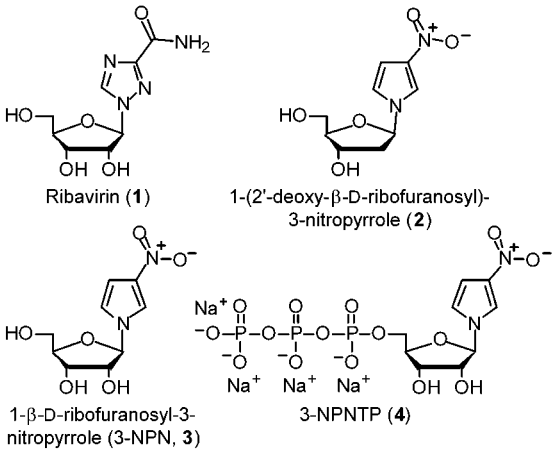

FIGURE 1: Structures of synthetic compounds.

Ribavirin (**1**, Figure 1) was recently shown to constitute an RNA virus mutagen. This drug is converted intracellularly into ribavirin triphosphate, which is a substrate of poliovirus RdRP. This enzyme recognizes ribavirin as a mimic of purine nucleosides (G and A) and incorporates ribavirin into the viral genome opposite C and U in the RNA template. Once in the genome, ribavirin templates incorporation of both C and U with equal efficiency, thus promoting A to G and G

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[1] Abbreviations: 3-NPN, 1-β-D-ribofuranosyl-3-nitropyrrole; 3-NPNTP, 1-β-D-ribofuranosyl-3-nitropyrrole triphosphate; RdRP, RNA-dependent RNA polymerase; A, adenosine; G, guanosine; C, cytidine; U, uridine; Et_2O, diethyl ether; DMF, *N*,*N*-dimethylformamide; PFU, plaque-forming units; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

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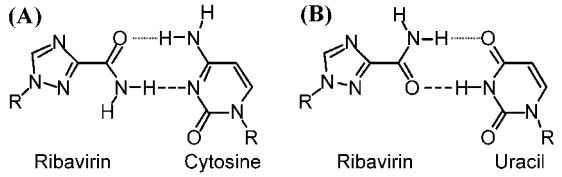


FIGURE 2: Base pairing between two conformations of the purine mimic ribavirin and the pyrimidine bases cytosine and uracil. R is ribofuranosyl phosphate or triphosphate.

to A transition mutations during multiple rounds of viral replication. Ribavirin mimics the purines by presenting two distinct isoenergetic hydrogen bonding conformations to complementary bases through rotation of the exocyclic carboxamide moiety (Figure 2) (*3*).

We hypothesize that ribonucleoside analogues more highly mutagenic than ribavirin have the potential to exhibit more potent antiviral activity. Ribonucleoside analogues that template all four bases ("universal bases") may provide useful lead compounds in this regard by enabling the introduction of more mutations per round of viral genome replication. Previous work on universal bases has focused almost exclusively on deoxyribonucleosides; few investigations have been conducted on analogous ribonucleosides even though RNA polymerases are generally more promiscuous in their ability to incorporate synthetic analogues (reviewed in ref *12*). The synthetic deoxyribonucleoside 1-(2′-deoxy-β-D-ribofuranosyl)-3-nitropyrrole (**2**) (*13*, *14*) is perhaps the best characterized universal DNA base analogue (*12*). This compound (**2**) was designed to circumvent the restrictive hydrogen bonding patterns controlling typical purine−pyrimidine hydrogen bonding motifs by incorporating the weakly hydrogen bonding nitro substituent. By reducing both favorable and unfavorable contributions from hydrogen bonding, compound **2** can hybridize with all four DNA nucleobases and function as a universal base (*13*). In addition, **2** confers stability within oligonucleotide duplexes by maximizing favorable aromatic π-stacking interactions between adjacent bases in the DNA duplex (*14*). The universality of **2** has been utilized for diverse applications in hybridization of duplex DNA (*15*−*22*), oligonucleotide probes and primers (*13*, *23*−*26*), peptide nucleic acids (*27*, *28*), and sequencing by MALDI mass spectrometry (*29*).

We report here the chemical synthesis, structural characterization, biochemical analysis, and antiviral evaluation of 1-β-D-ribofuranosyl-3-nitropyrrole (3-NPN, **3**), which is the RNA analogue of the corresponding universal DNA base (**2**). We further describe the synthesis of the triphosphate derivative of **3** (3-NPNTP, **4**) and analysis of the kinetics of incorporation of this compound into RNA by poliovirus RdRP.

MATERIALS AND METHODS

General Synthesis Information. All reactions were performed under an argon atmosphere unless otherwise noted. Commercial grade reagents (Aldrich) were used without further purification unless specifically noted. Tetrahydrofuran and diethyl ether were distilled from sodium benzophenone ketyl under nitrogen. Acetonitrile was distilled from calcium hydride under nitrogen. *N*,*N*-Dimethylformamide was dried by treatment with 4−8 mesh, 13× molecular sieves. Thin-layer chromatography (TLC) was performed on glass-backed silica gel-coated plates coated with silica gel 60 F_{254} (EM Science, 250 μm thick). ICN SiliTech Silica Gel (32−63 μm) was employed in column chromatography. HPLC purification was performed on a Hewlett-Packard 1100 series instrument equipped with a Zorbax 300SB-C18 semipreparative column (9.4 mm × 250 mm, 5 μm). The HPLC gradient for purification of **4** (2 mL/min flow rate) was as follows: 2 to 15% acetonitrile in 0.1 M triethylammonium bicarbonate (TEAB) (pH 7.5) from 0 to 30 min, 15 to 40% acetonitrile in 0.1 M TEAB from 30 to 45 min, and 40 to 70% acetonitrile and 0 to 20% MeOH in 0.1 M TEAB from 45 to 50 min. Bruker CDPX-300, DPX-300, AMX-360, DRX-400, and AMX-2-500 MHz spectrometers were employed in nuclear magnetic resonance (NMR) spectroscopy. 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) (*30*) and 85% H_3PO_4 (0 ppm), respectively. Mass spectral data was obtained from either The University of Texas at Austin Mass Spectrometry Facility (ESI and CI) or The Pennsylvania State University Mass Spectrometry Facility (ESI). Elemental analyses were performed by Midwest Microlab, LLC (Indianapolis, IN). Melting points are uncorrected.

Synthesis of 1-(5-O-tert-Butyldimethylsilyl-2,3-O-isopropylidene-β-D-ribofuranosyl)-3-nitropyrrole (9). The stereoselective chlorination of **6** was performed as previously described (*31*−*35*). Briefly, anhydrous CCl_4 (0.75 mL, 7.77 mmol) was added to a solution of **6** (1.5113 g, 4.96 mmol) in anhydrous THF (16 mL). The mixture was cooled to −72 °C, and hexamethylphosphorus triamide (HMPT, 1.2 mL, 6.60 mmol) was added slowly via syringe. The reaction mixture was stirred at −70 °C for 2 h. Periodically, the mixture was briefly warmed to 25 °C to solubilize the formed gel phase, and then recooled to −70 °C as needed to enable stirring. After 2 h, the reaction mixture was concentrated in vacuo (25 °C) and vented to atmospheric pressure with dry argon. This chloro sugar (crude **7**) was triturated with anhydrous diethyl ether (2 × 10 mL), concentrated in vacuo (25 °C), and vented to atmospheric pressure with dry argon. The resulting orange-brown solid (crude **7**) was dissolved in anhydrous acetonitrile (3 mL) and employed immediately in the subsequent coupling step. To a solution of 3-nitropyrrole (1.0847 g, 9.68 mmol) (*36*) (**8**) in anhydrous acetonitrile (15 mL) cooled to 2 °C was added NaH (95%, 175.6 mg, 7.32 mmol). To the resulting bright yellow solution was added the chloro sugar (crude **7**) via cannula, with additional anhydrous acetonitrile (2 mL) used in the transfer. The reaction mixture was then warmed to room temperature and stirred for 40 h. The reaction mixture was poured into dH_2O (60 mL) and extracted with CH_2Cl_2 (3 × 60 mL). The organic layers were combined, diluted with CH_2Cl_2 (to 300 mL), and washed with saturated NH_4Cl (aqueous, 1 × 300 mL) and saturated NaCl (aqueous, 1 × 300 mL). The organic layer was dried over $MgSO_4$, filtered through a pad of Celite, and concentrated in vacuo. Column chromatography (10% Et_2O in hexanes) provided the β anomer **9** (393.9 mg, 20%) as a light yellow oil. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 7.75 (m, 1H, ArH-2), 6.78 (dd, J = 2.44 Hz, J = 3.30 Hz, 1H, ArH-5), 6.70 (dd, J = 1.80 Hz, J = 3.31 Hz, 1H, ArH-4), 5.59 (d, J = 3.22 Hz, 1H, H-1), 4.79 (dd, J = 2.18 Hz, J = 6.05 Hz, 1H, H-3), 4.64 (dd, J = 3.22 Hz, J = 6.05 Hz,

1H, H-2), 4.37 (dd, $J = 2.52$ Hz, $J = 4.98$ Hz, 1H, H-4), 3.87−3.73 (m, 2H, H-5$_{a,b}$), 1.55 [s, 3H, C(CH$_3$)$_2$], 1.32 [s, 3H, C(CH$_3$)$_2$], 0.85 [s, 9H, C(CH$_3$)$_3$], 0.05 (s, 3H, SiCH$_3$), 0.04 (s, 3H, SiCH$_3$). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 137.40, 119.24, 118.76, 114.14, 105.88, 95.22, 86.21, 86.14, 81.04, 63.44, 27.20, 25.77, 25.19, 18.26, −5.61, −5.71. IR (film): ν_{max} 2930.7, 2857.9, 1535.6, 1494.3, 1385.1, 1299.1, 1126.0, 1086.0, 835.4 cm^{-1}. Mass calcd for $C_{18}H_{30}N_2O_6Si$: 398.19. Found $[M + H]^+$: 399.1 (16%, ESI$^+$). Anal. Calcd for $C_{18}H_{30}N_2O_6Si$: C, 54.25; H, 7.59; N, 7.03. Found: C, 54.38; H, 7.62; N, 6.99.

*Synthesis of 1-β-D-Ribofuranosyl-3-nitropyrrole (**3**).* To 1-(5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-β-D-ribofuranosyl)-3-nitropyrrole (**9**, 331.7 mg, 0.83 mmol) was added a solution of trifluoroacetic acid and dH_2O (1:1, 10 mL) (*37*, *38*). The reaction mixture was stirred for 40 min at 25 °C, concentrated in vacuo, and triturated with MeOH (3 × 10 mL). Column chromatography (5% MeOH in CH_2Cl_2) afforded **3** (196.3 mg, 97%) as a pale-yellow solid. ^{1}H NMR (MeOH-d_4, 400.13 MHz): δ 8.01 (m, 1H, ArH-2), 7.04 (dd, $J = 2.44$ Hz, $J = 3.32$ Hz, 1H, ArH-5), 6.72 (dd, $J = 1.83$ Hz, $J = 3.35$ Hz, 1H, ArH-4), 5.54 (d, $J = 4.86$ Hz, 1H, H-1), 4.20 (m, 2H, H-2, H-3), 4.06 (dd, $J = 3.28$ Hz, $J = 6.58$ Hz, 1H, H-4), 3.83−3.71 (m, 2H, H-5$_{a,b}$). ^{13}C NMR (MeOH-d_4, 75.40 MHz): δ 138.42, 121.56, 121.13, 106.20, 94.22, 87.14, 77.72, 72.04, 62.76. IR (KBr): ν_{max} 3345.9, 2944.0, 1528.6, 1480.0, 1386.3, 1293.5, 1126.5, 1048.0 cm^{-1}. Mp: 139−141 °C. Mass calcd for $C_9H_{12}N_2O_6$: 244.07. Found $[M + H]^+$: 245 (100%, CI$^+$). Anal. Calcd for $C_9H_{12}N_2O_6$: C, 44.27; H, 4.95; N, 11.47. Found: C, 44.11; H, 4.97; N, 11.19.

*Synthesis of 1-(5′-Triphosphate-β-D-ribofuranosyl)-3-nitropyrrole (**4**).* This compound was prepared via modification of a previously reported procedure (*39*−*45*). Briefly, a solution containing **3** (14.4 mg, 0.059 mmol), Proton-Sponge (21.3 mg, 0.099 mmol), and trimethyl phosphate (0.6 mL, 5.127 mmol) was cooled to 2 °C, and $POCl_3$ (11 μL, 0.118 mmol) was added dropwise. The resulting solution gradually turned lavender in color and was stirred for 2 h. Tributylamine (0.06 mL, 0.252 mmol) was injected into this mixture, followed by a solution of tributylammonium pyrophosphate (147.8 mg) in DMF (0.5 mL). The reaction mixture was stirred for 2 min, and then the reaction was quenched by the addition of TEAB (6 mL, 0.2 M, pH 8). The mixture was warmed to 25 °C, diluted with dH_2O (5 mL), and lyophilized. The crude reaction products were purified by RP-HPLC to afford the triphosphate as the triethylammonium salt (retention time, 18.8 min). Excess salts were removed by loading this material onto a Waters Light C18 Sep-Pak syringe column and eluting dropwise with dH_2O (material off the column was fractionally collected; the first few drops contained excess salts followed by elution of **4**). Residual **4** was eluted with acetonitrile. Fractions containing the triphosphate (identified as UV-active material at 254 nm) were pooled and lyophilized. The triphosphate was converted from the triethylammonium salt to the sodium salt by passing it through a DOWEX 50WX8-100 ion exchange column [Na^+ form, activated by equilibration with saturated $NaHCO_3$ (aqueous) followed by removal of salts with dH_2O], and fractions containing **4** were combined and lyophilized. The resulting material was then resuspended in dH_2O, filtered through an Acrodisc syringe filter (0.2 μm), and lyophilized to afford **4** (15.7 mg, 47% yield) as a yellow-brown powder. ^{1}H NMR (D_2O, 500.13 MHz): δ 8.09 (m, 1H, ArH-2), 7.17 (m, 1H, ArH-5), 6.89 (dd, $J = 1.69$ Hz, $J = 3.37$ Hz, 1H, ArH-4), 5.69 (d, $J = 6.39$ Hz, 1H, H-1), 4.56−4.47 (m, 2H, H-2, H-3), 4.37 (m, 1H, H-4), 4.29−4.20 (m, 2H, H-5$_{a,b}$). ^{13}C NMR (D_2O, 125.76 MHz): δ 139.76, 125.65, 123.60, 109.50, 94.99, 87.40, 78.16, 73.85, 68.72. ^{31}P NMR (D_2O, 145.79 MHz): δ −9.94 (br s), −10.61 (d), −22.08 (br s). UV: λ_{max} (H_2O) 200 nm ($\epsilon = 3431$ M^{-1} cm^{-1}). Mass calcd for $C_9H_{11}N_2Na_4O_{15}P_3$: 571.90. Found $[M - Na]^-$: 548.7 (100%, ESI$^-$, acetonitrile, H_2O, 1% AcOH). Found $[M - 4Na + 3H]^-$: 483 (100%, ESI$^-$, MeOH, H_2O, TEA).

Propagation of Cells and Procedures for Poliovirus Infections. HeLa S3 cells were propagated in D-MEM/F-12 medium (Invitrogen) supplemented with dialyzed fetal bovine serum (2%, Invitrogen). For cytotoxicity assays, cells (2×10^4) were plated in each well of a 24-well dish 24 h before addition of the compound (**3** or **1**). A final volume of 0.5 mL of medium was used. After the cells had been exposed for 6 days, viable cells were evaluated for Trypan Blue exclusion and counted with a hemacytometer. For poliovirus infection assays, cells were plated in 24-well dishes (1×10^5 cells/well) 48 h before the experiment. Compounds (**3** or **1**) were preloaded 24 h before the experiment, and cells were infected with 2000 PFU poliovirus per well. Upon reaching 100% cytopathic effect (CPE), virus was harvested by freeze−thaw, and serial dilutions were plaqued on six-well dishes of confluent HeLa S3 cells. After 72 h, cells were stained with Crystal Violet (0.2% in 20% ethanol) to visualize plaques. Time to 100% CPE was recorded as the number of days required for Mahoney strain poliovirus (2000 PFU) to visibly cause complete cell death.

Expression and Purification of CHO Adenosine Kinase. A previously reported Chinese hamster ovary (CHO) adenosine kinase (AK) expression vector (GenBank accession number P55262) was employed (*46*). This AK gene is approximately 90% homologous to the human AK gene, and the CHO AK was purified to ∼90% purity for kinase assays. *Escherichia coli* BL21(DE3) was transformed with the pET15b CHO AK expression plasmid and grown overnight at 25 °C in NZCYM medium (Invitrogen Life Technologies, 100 mL) supplemented with 100 μg/mL ampicillin (A100). This culture was used to inoculate NZCYM medium (1 L supplemented with A100) to an OD_{600} of 0.05. The cells were then grown at 37 °C to an OD_{600} of 1.0, cooled to 25 °C, and induced by addition of isopropyl 1-thio-β-galactopyranoside (500 μM final concentration). After 4 h, the cells were harvested by centrifugation using a Sorvall GS-3 rotor (6000 rpm for 10 min), washed once with TE (200 mL, 10 mM Tris, 1 mM EDTA), and harvested by centrifugation. This cell paste corresponded to 7−9 g/L of culture. The cells were frozen (−80 °C) for storage prior to protein purification. The frozen cells were thawed and suspended in lysis buffer [100 mM potassium phosphate (pH 8.0), 0.5 mM EDTA, 20% glycerol, 10 mM 2-mercaptoethanol, 5.6 μg/mL pepstatin A, and 4.0 μg/mL leupeptin] at 4 mL/g of cell weight. The suspended cells were disrupted by extrusion through a French pressure cell (1000 psi), and PMSF (2 mM final concentration) was then added to the cell lysate. The lysate was centrifuged in a Beckman 60-Ti rotor (30 000 rpm). This clarified lysate was diluted with buffer A [50 mM Tris (pH 8.0), 20% glycerol, 1 mM 2-mercaptoethanol, 0.1% NP-40,

and 500 mM NaCl] to a protein concentration of 1 mg/mL and applied to a Ni−NTA column (Qiagen, 1 mL) at 100 μL/min, which was equilibrated with buffer A. The column was then washed with 10 column volumes of buffer B [50 mM Tris (pH 8.0), 20% glycerol, 1 mM 2-mercaptoethanol 0.1% NP-40, 500 mM NaCl, and 5 mM imidazole]. The protein was eluted with 3 column volumes of buffer C [50 mM Tris (pH 8.0), 20% glycerol, 1 mM 2-mercaptoethanol, 0.1% NP-40, 500 mM NaCl, and 500 mM imidazole]. An SDS−PAGE gel illustrating this purification is provided in the Supporting Information. The protein concentration was determined from the absorbance at 280 nm in guanidine-HCl (6 M, pH 7.0) using a molar extinction coefficient of 25 460 M^{-1} cm^{-1}. The purified protein was stored at −80 °C.

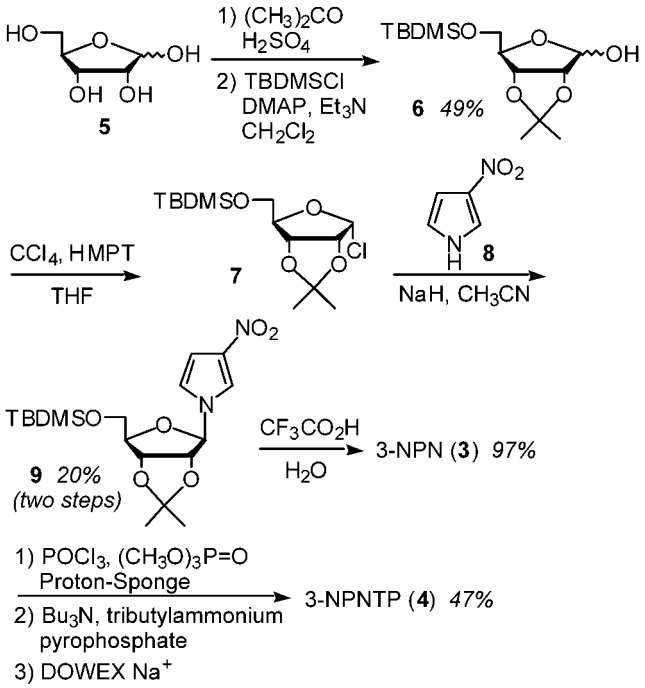
Phosphorylation Assay. The assay conditions were modified from a previous report (*46*) wherein [2,8-^{3}H]adenosine was replaced with nonradioactive adenosine and the isotope that was used was [α-^{32}P]ATP. Additionally, thin-layer chromatography was substituted for glass filter binding. Reactions were carried out at 37 °C for the indicated reaction times. Each reaction mixture contained HEPES (100 mM, pH 7.5), adenosine (1 μM, control) or **1** or **3** (500 μM), ATP (1 μM), $MgCl_2$ (1.5 mM), and [α-^{32}P]ATP (0.001 μM, NEN Life Science Products). Adenosine kinase (0.015 μM) and ddH_2O were added to afford a final volume of 50 μL. Reactions were quenched by the addition of EDTA (0.5 M) to a final concentration of 80 mM. Samples (1 μL) of the quenched reaction mixtures were spotted on a TLC PEI-cellulose F plate (EM Science) and resolved with potassium phosphate buffer (0.3 M, pH 7.0). The TLC plate was removed from the buffer, air-dried, and exposed to a phosphorimager screen. Phosphorimager and ImageQuant software from Molecular Dynamics were used for imaging and quantitation, respectively. KaleidaGraph software (Synergy) was employed for further data analysis.

3-NPNTP Incorporation by 3D^{pol}. The 3D^{pol} polymerase was expressed and purified as previously reported (*47*). All primer extension experiments were carried out as previously described (*48*). Complexes of 3D^{pol}-sym/sub were preassembled for at least 3 min and then mixed with the appropriate nucleotide to initiate the reactions. Reactions were quenched by the addition of 50 mM EDTA.

RESULTS

Chemical Synthesis of 3-NPN (3) and 3-NPNTP (4). The synthesis of the 3-nitropyrrole nucleoside (3-NPN, **3**) employed glycosylation methodology described by Ramasamy et al. for the synthesis of the analogous 3-cyanopyrrole nucleoside (*37*) (Scheme 1). This methodology installed on ribose the known heterocyclic base 3-nitropyrrole (**8**), which was prepared as previously reported (*36*) from commercially available 1-(triisopropylsilyl)pyrrole. The previously reported protected ribose derivative **6** was synthesized in two steps from D-ribose (**5**) (*38*, *49*−*53*). The stereoselective chlorination of protected sugar **6** with CCl_4 and hexamethylphosphorus triamide (HMPT) (*31*−*35*) was followed by coupling with the anion derived from 3-nitropyrrole to yield the desired β-substituted 3-nitropyrrole nucleoside **9** in 20% yield from alcohol **6**. This compound was assigned as the β anomer on the basis of the characteristic difference in the chemical

Scheme 1: Synthesis of 3-NPN (**3**) and 3-NPNTP (**4**)



shift (δ 0.23 ppm) between the isopropylidene methyl substituents of β-substituted nucleosides observed by ^{1}H NMR spectroscopy (*37*, *54*, *55*). Final removal of protecting groups with 50% trifluoroacetic acid in water (*37*, *38*) provided the free 3-nitropyrrole nucleoside **3**. As shown in Figure 3, the structure of 3-NPN (**3**) was confirmed by small molecule X-ray crystallography.

As shown in Scheme 1, the triphosphate derivative of **3** (3-NPNTP, **4**) was also prepared for investigation of the kinetics of incorporation of this non-natural nucleoside by poliovirus RNA-dependent RNA polymerase (3D^{pol}). This compound was synthesized by adaptation of previously described chemical methodology (*39*−*45*) that initially involved conversion of **3** into the corresponding phosphorodichloridate by treatment with $POCl_3$, trimethyl phosphate, and Proton-Sponge. This unstable intermediate was immediately reacted with tributylamine and tributylammonium pyrophosphate in DMF to afford the triphosphate **4** as the triethylammonium salt. Subsequent HPLC purification and

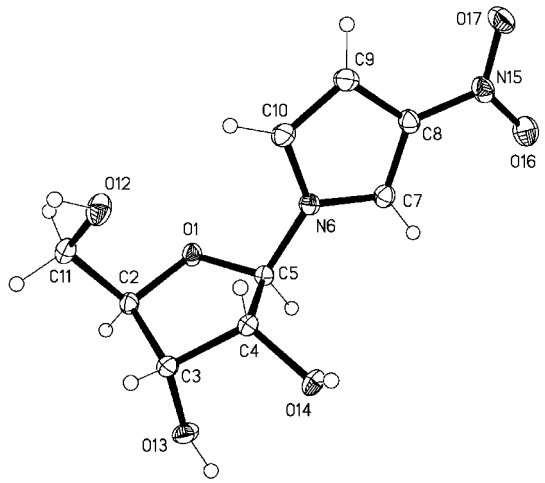


FIGURE 3: ORTEPII plot of the X-ray structure of 1-β-D-ribofuranosyl-3-nitropyrrole (3-NPN, **3**).

Table 1: Effects of 3-NPN (**3**) and Ribavirin (**1**) on Poliovirus-Infected HeLa Cells[a]

nucleoside	cell viability (%)	time to 100% CPE (days)	reduction in titer (*x*-fold)
none	100	2	−
3-NPN (100 μM)	106	2	−
3-NPN (1000 μM)	54	2	−
ribavirin (100 μM)	28	3	14
ribavirin (1000 μM)	3	6	1900

[a] Cell viability represents the number of live HeLa S3 cells after treatment for 6 days compared to an untreated control (100% viability). Cells were plated at a density of 2×10^4 cells/well 24 h before treatment with compounds; data are the averages of at least four wells. The average number of days required for 2000 PFU poliovirus to completely kill a confluent HeLa S3 monolayer is shown as the time to 100% CPE. Titer was determined by a plaque assay after 100% CPE was reached. Changes in titer of <2-fold relative to control are denoted −.

ion exchange chromatography provided the tetrasodium salt of 3-NPNTP (**4**).

*Evaluation of Cytotoxicity of 3-NPN (**3**) toward HeLa S3 Cells in Culture.* An evaluation of the cytotoxicity of 3-NPN (**3**) to HeLa S3 cells grown in culture revealed surprisingly large populations of viable cells following treatment for 6 days. As shown in Table 1, HeLa cells grown for 6 days in the presence of 100 μM 3-NPN (**3**) showed no decrease in cell viability. A 10-fold increase in the concentration of **3** elicited a slight 36% decrease in cell viability compared with controls. In contrast, 1000 μM ribavirin (**1**) exhibited significant cytostatic effects, and the number of viable cells after 6 days was only 3% of the control wells.

*Evaluation of 3-NPN (**3**) against Poliovirus.* The ability of 3-NPN (**3**) to function as an antiviral agent was evaluated using poliovirus as a model RNA virus. To compare the antiviral activity of 3-NPN (**3**) to that of ribavirin (**1**), HeLa S3 cell monolayers were treated with either 3-NPN or ribavirin for 24 h and then infected with the Mahoney strain of poliovirus (2000 PFU). The time required for lysis of the monolayer was measured. As indicated in Table 1, 1000 μM 3-NPN showed no antiviral activity, whereas ribavirin at this concentration delayed the cytopathic effects of the virus by 4 days. Additionally, 3-NPN did not significantly reduce viral titer at any concentration, whereas 1000 μM ribavirin reduced the poliovirus titer by 1900-fold.

*Phosphorylation of 3-NPN (**3**) by Adenosine Kinase.* The 3-NPN nucleoside must be phosphorylated intracellularly to become a substrate of poliovirus RdRP that can subsequently be incorporated into the viral genome. In general, the rate-limiting step for conversion to the triphosphate is production of the monophosphate. To investigate whether the absence of antiviral activity associated with 3-NPN might relate to poor phosphorylation by adenosine kinase, 3-NPN and ribavirin were compared as substrates for this enzyme. Conversion of ATP to ADP by the kinase was used as an indirect measure of the level of phosphorylation of the nucleoside under investigation. As shown in Figures 4 and 5, these experiments demonstrated that both ribavirin and 3-NPN were phosphorylated equivalently by adenosine kinase, with an efficiency at least 20-fold lower than that of adenosine.

*Kinetics of 3-NPNTP (**4**) Incorporation by the Poliovirus Polymerase 3D^{pol}.* The kinetics of incorporation of 3-NPNTP (**4**) by 3D^{pol} were assessed using a well-precedented primer

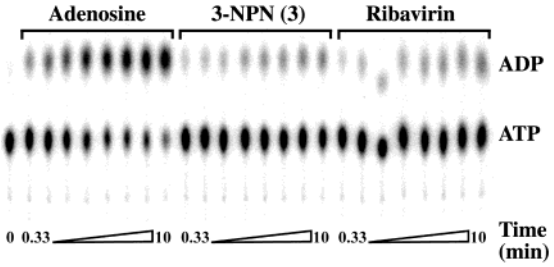


FIGURE 4: Phosphorylation of the ribonucleosides adenosine, 3-NPN (**3**), and ribavirin (**1**) by adenosine kinase. Substrate efficiency was determined by analysis of the conversion of ATP to ADP by thin-layer chromatography.

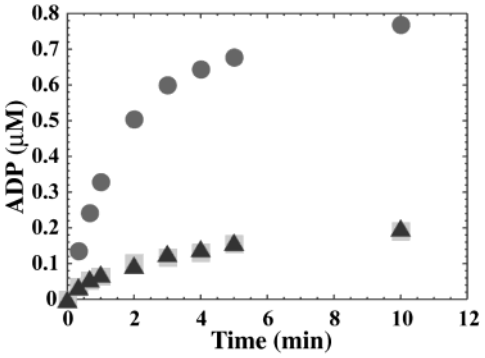


FIGURE 5: Quantitative comparison of phosphorylation of adenosine, 3-NPN, and ribavirin by adenosine kinase: adenosine (●), ribavirin (■), and 3-NPN (**3**) (▲).

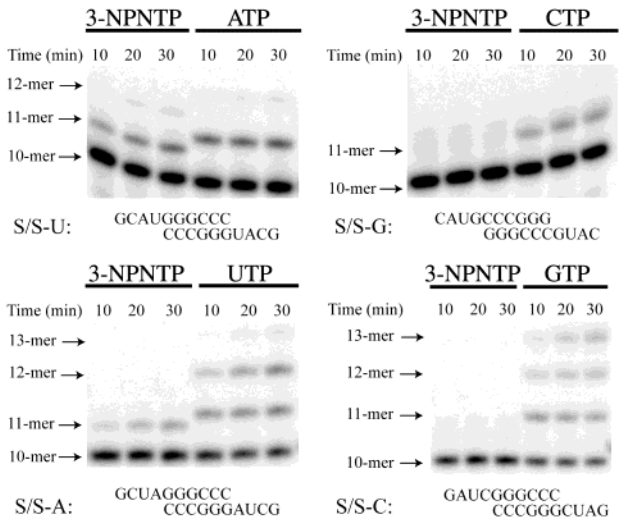


FIGURE 6: Incorporation of monophosphates derived from 3-NPNTP (**4**) opposite complementary nucleotides (N) in symmetrical substrates (S/S-N) in vitro. Oligoribonucleotide substrates (10-mers) were end labeled with ^{32}P and treated with poliovirus polymerase and 3-NPNTP, and products were separated by denaturing PAGE.

extension assay on synthetic symmetrical primer/template substrates (S/S) (*3*, *48*, *56*). Incubation of the polymerase with the S/S RNA gives a stable, elongation-competent complex. Subsequent addition of the appropriate nucleoside triphosphate triggers incorporation catalyzed by the polymerase. This primer extension assay has been shown to permit detailed kinetic and thermodynamic analyses of incorporation of correct and incorrect ribonucleotides, deoxyribonucleotides, and nucleotide analogues (*3*, *48*). Surprisingly, as shown in Figure 6, these experiments revealed that incorporation of the monophosphate derived from 3-NPNTP

Table 2: Kinetics of 3-NPNTP Incorporation into Synthetic RNA Templates by Poliovirus RdRP[a]

	S/S-U	S/S-A
K_m (μM)	19.2 ± 7.1	46.1 ± 15.5
k_{cat} (s^{-1})	0.000120 ± 0.000001	0.00010 ± 0.00001
IC_{50} (μM)	76.3 ± 18.4	26.0 ± 3.0

[a] S/S-U is the template for quantification of incorporation opposite U. S/S-A is the template for quantification of incorporation opposite A.

(**4**) was templated only by adenosine (S/S-A) and uracil (S/S-U). No incorporation into the S/S-G or S/S-C templates was observed (Figure 6). Furthermore, the rate of reaction mediated by 3-NPNTP (**4**) opposite S/S-U and S/S-A was much slower than that of the correct nucleotide (Table 2) and 100-fold slower than that of ribavirin triphosphate (*3*). After the first round of incorporation with 3-NPNTP (**4**), the $3D^{pol}$ polymerase incorporated additional 3-NPNTP-derived monophosphates (Figure 6), suggesting that 3-NPMP is not a chain-terminating agent. As shown in Table 2, quantification of the kinetics of 3-NPNTP (**4**) incorporation into primer/template substrates enabled determination of K_m, k_{cat}, and IC_{50} values. The IC_{50} values reflect concentrations of 3-NPNTP (**4**) required to inhibit incorporation of AMP into S/S-U and UMP into S/S-A.

DISCUSSION

Ribavirin (**1**) can increase the frequency of mutations in RNA virus genomes because of the ambiguous pairing capacity of its pseudobase. The pseudobase of ribavirin (**1**) mimics both adenine and guanine and misincorporates opposite U and C in the viral template. We report the synthesis and antiviral evaluation of a highly structurally similar analogue (3-NPN, **3**) designed on the basis of the universal DNA nucleoside (**2**) (*13, 14*) that hybridizes to all four DNA bases in the context of duplex DNA. We hypothesized that 3-NPN would be converted intracellularly into the triphosphate (3-NPNTP, **4**) and cause more significant mutagenesis to the viral genome in the presence of poliovirus RdRP by mimicking both purines and pyrimidines.

A structural comparison of the X-ray crystal structures of 3-NPN (**3**) and ribavirin (**1**) (*57*) is shown in Figure 7. As anticipated from the NMR data, the configuration of 3-NPN at C-1′ is β. Crystallography revealed that the bases of 3-NPN and ribavirin exhibit identical anti conformations, and the only significant differences between these two molecules lie in the conformation of the sugar moiety. The sugar conformation of 3-NPN is *C*$^{2'}$*-endo*, whereas the conformation of the sugar of ribavirin is *C*$^{3'}$*-endo*. Although the *C*$^{2'}$*-endo* conformation is the sugar pucker observed in B-form DNA and the *C*$^{3'}$*-endo* conformation characterizes A-form RNA, in solution both of these conformations are in rapid equilibrium and are separated by an energy barrier of less than 5 kcal/mol. Calculation of the root-mean-square similarities between 3-NPN and ribavirin revealed a deviation of 0.83 Å overall, and this value primarily reflected differences in sugar atom positions (Figure 7). This comparison indicated that 3-NPN and ribavirin are practically identical in overall structure in the solid state, while differing substantially in hydrogen bonding ability. Interestingly, solution phase studies with ribavirin have revealed a preference for the syn conformation of the heterocyclic base (*58, 59*). On the basis of the substantial structural similarities, the observed differences in biological activity are presumably not a result of conformational effects.

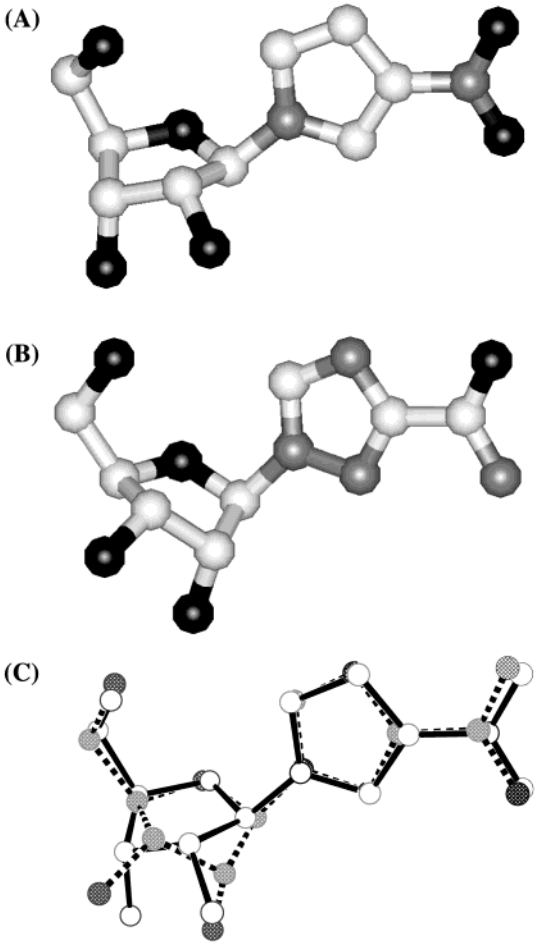


FIGURE 7: Comparison of X-ray crystal structures of 3-NPN (**3**) and ribavirin (**1**). Atom labels for panels A (3-NPN) and B (ribavirin): light gray for carbon, dark gray for nitrogen, and black for oxygen. (C) Structural overlay for calculation of root-mean-square (rms) deviations of atom positions: solid bonds and empty circles for 3-NPN and dashed bonds and shaded circles for ribavirin.

In contrast to ribavirin, 3-NPN did not exhibit antiviral activity in cell culture against poliovirus. Additionally, 3-NPN was significantly less cytostatic than ribavirin toward HeLa S3 cells grown in culture, as only a 36% reduction in cell viability was observed at 1 mM. Although many factors might contribute to this lack of biological activity, two processes critical for antiviral activity were specifically investigated in vitro: phosphorylation of 3-NPN by adenosine kinase, a critical step required for conversion to the triphosphate in cells, and utilization of 3-NPNTP by the poliovirus RdRP ($3D^{pol}$).

Ribavirin is a known substrate of adenosine kinase (*60*). Comparison of this drug with 3-NPN revealed that both compounds are phosphorylated at equivalent rates by this enzyme. Hence, production of the monophosphate should not be a rate-limiting step in cells, and the monophosphate of 3-NPN should be converted into the active triphosphate (3-NPNTP) in cells.

Synthetic 3-NPNTP was prepared to analyze the kinetics of incorporation by poliovirus RdRP ($3D^{pol}$). A primer extension assay (*48*) confirmed that 3-NPNTP is a mutagenic

substrate of $3D^{pol}$, and this compound was found to mimic both the purine A and the pyrimidine U during incorporation into a synthetic RNA template. However, the absence of incorporation opposite G and C was surprising given that the corresponding DNA analogue (**2**) is a universal base that substitutes for all four DNA bases in a DNA template. Previous studies of the triphosphate derivative of the DNA nucleoside **2** have shown polymerase specific differences in substrate incorporation. This DNA nucleoside triphosphate has been shown to be templated by all four nucleobases using the exonuclease-free Klenow fragment of *E. coli* DNA polymerase I (*61*), whereas Taq DNA polymerase incorporates this triphosphate opposite only A and T in a DNA template (*62*). Consequently, the 3-nitropyrrole pseudobase can be considered universal within a hybridization context, but is recognized by some polymerases as a mimic of A and T (or A and U for $3D^{pol}$).

Ribavirin is incorporated into viral RNA with rates equivalent to that of an incorrect nucleotide, which is ca. 3000−6000-fold slower than incorporation of a correct nucleotide (*3*). During viral replication in the presence of clinically relevant concentrations of ribavirin, poliovirus polymerase incorporates fewer than three ribavirin molecules into its genome (ca. 7500 nucleotides) per replication cycle. This incorporation causes error catastrophe due to resulting A to G and G to A transition mutations (*3*). Analysis of the kinetics of primer extension by $3D^{pol}$ in the presence of 3-NPNTP revealed that this compound is incorporated into synthetic RNA ~100-fold more slowly than ribavirin triphosphate. Hence, even under optimal conditions for incorporation of 3-NPNTP by poliovirus, fewer than 0.03 molecule of this compound would be introduced per genome per replication cycle. This decreased rate of incorporation is sufficient to explain the diminished antiviral activity observed with 3-NPN, even if this compound provides universal base-pairing specificity once incorporated into the RNA template. Additionally, it is possible that the inability of 3-NPNTP to function in cells may be a consequence of the inability of this compound to inhibit enzymes of the de novo purine biosynthetic pathway. However, inhibition of IMP dehydrogenase by RMP is not sufficient to confer antiviral activity (*3*). Whether reduction of intracellular GTP pools augments the antiviral activity of ribavirin is unclear, but previous reports have demonstrated no correlation between the concentration of ribavirin required for maximal antiviral activity and that required for maximal (2-fold) reduction of intracellular GTP pools (*11*).

The results presented here indicate that highly hydrophobic nucleoside analogues lacking hydrogen bonding functionality exhibit slow rates of incorporation by viral RNA polymerases. Studies of universal DNA nucleosides suggest that low incorporation rates may relate to overly stable π-stacking interactions with aromatic amino acid residues in the active site of DNA polymerases that prevent efficient ejection after incorporation (*12*). Therefore, the ability of a pseudobase to hybridize to all four nucleobases is not sufficient for defining the pseudobase as a universal analogue for the viral RdRP. The pursuit of improved mutagenic antiviral nucleosides will benefit from a more detailed understanding of the factors that influence base pairing specificity at the polymerase active site and the fate of non-natural ribonucleotides in cells.

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SUPPORTING INFORMATION AVAILABLE

SDS−PAGE data on purified adenosine kinase and crystallographic data for 3-NPN (**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

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