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# Synthesis of a Universal 5-Nitroindole Ribonucleotide and Incorporation into RNA by a Viral RNA-Dependent RNA Polymerase

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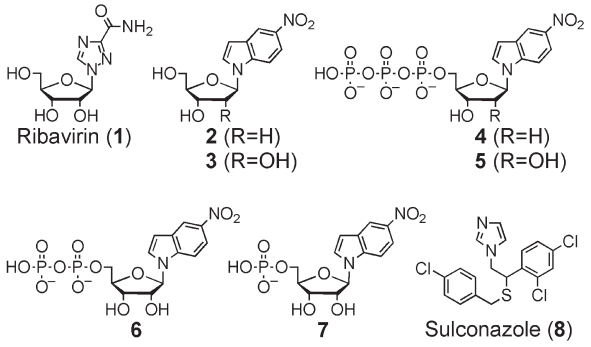
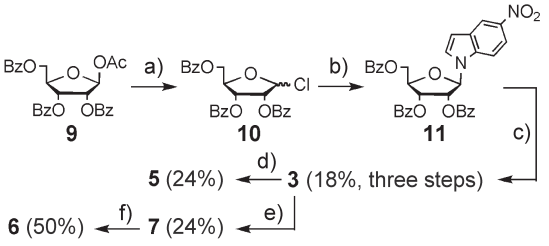
Small molecules that mimic natural nucleosides and nucleotides comprise a major class of antiviral agents. A new approach to the design of these compounds focuses on the generation of lethal mutagens:[1,2] compounds that further accelerate the high rate of viral mutagenesis[3,4] to confer antiviral effects. By incorporating artificial nucleobases with degenerate base-pairing abilities into viral genomes, lethal mutagens increase viral genomic mutagenesis to intolerable levels during replication, a process termed "error catastrophe", which results in the loss of viral viability.[5,6] The antiviral drug ribavirin (**1**) is one such lethal mutagen effective against the RNA viruses poliovirus (PV)[7] and hepatitis C virus.[8] Ribavirin is converted intracellularly to the 5′-triphosphate (RTP), which is a substrate for viral RNA-dependent RNA polymerases (RdRP). By mimicking the natural purines, RTP is misincorporated opposite pyrimidines in the enzyme-bound viral RNA template. The incorporated nucleobase of ribavirin promotes genomic mutatagenesis by templating C and U during subsequent rounds of viral replication; this facilitates error catastrophe and loss of viral viability.[7,9–11]




As part of our efforts to identify more efficacious antiviral lethal mutagens, we report the synthesis, X-ray structure, and antiviral evaluation of the 5-nitroindole-containing ribonucleoside **3**, and incorporation of the related ribonucleotide **5** into RNA by a viral RdRP. These compounds represent RNA analogues of the previously reported "universal" *deoxy*ribonucleoside **2**, a compound shown to base pair with all four natural DNA pseudobases.[12,13] By eliminating strong hydrogen-bond donors/acceptors, and possessing a large aromatic $\pi$ system, 5-nitroindole **2** stabilizes DNA duplexes by aromatic $\pi$-stacking interactions with adjacent DNA bases.[14,15] The utility of **2** has been demonstrated in applications that range from incorporation into DNA hairpins,[16,17] primers for PCR and DNA sequencing,[13,18,19] detection of single nucleotide polymorphisms,[20,21] and (pseudobase) incorporation into peptide nucleic acids.[22]

We hypothesized that ribonucleoside analogues with universal base-pairing properties might possess enhanced antiviral activity relative to ribavirin (a purine mimic) by accelerating lethal viral mutagenesis. We demonstrate here that 5-nitroindole ribonucleotide **5** is universally incorporated opposite each native RNA base by a viral (poliovirus) RdRP ($3D^{pol}$). Although triphosphate **5** becomes incorporated into RNA by poliovirus $3D^{pol}$ more slowly than ribavirin triphosphate (RTP), **5** represents a much more potent inhibitor of this viral enzyme, and nucleoside **3** exhibits antiviral activity in cell culture.

5-Nitroindole ribonucleoside **3** and phosphorylated analogues **5**–**7** were synthesized as shown in Scheme 1. Commercially available **9** was chlorinated with $TiCl_4$,[23] treated with the sodium salt of 5-nitroindole, and globally deprotected with $NH_3$ in MeOH to yield the target nucleoside **3**. Triphosphate **5** was prepared by using the well-precedented phosphorylation conditions shown.[24] Monophosphate **7** was synthesized by modification of the conditions used to prepare **5**,[25] except the initially formed phosphodichloridate (step i) was immediately hydrolyzed with aqueous triethylammonium bicarbonate (TEAB). Diphosphate **6** was elaborated from monophosphate **7** by CDI activation of the $\alpha$-phosphate to yield a phosphoimidazolidate intermediate,[26] which was subsequently displaced with phosphate to install the $\beta$-phosphate of **6**. The structure and stereochemistry of **3** were confirmed by X-ray crystallography (Figure 1).[27] Other methods reported to yield **3** were




**Scheme 1.** a) $TiCl_4$, $CH_2Cl_2$, 23 °C, 2 h; b) 5-nitroindole, NaH, MeCN, 2 °C–23 °C, 22 h; c) $NH_3$, MeOH, 50 °C (sealed tube), 12 h; d) i. $POCl_3$, $(CH_3O)_3P{=}O$, Proton Sponge, 2 °C, 2 h; ii. $Bu_3N$, tributylammonium pyrophosphate, DMF, 2 °C, 2 min; TEAB hydrolysis; e) $POCl_3$, $(CH_3O)_3P{=}O$, Proton Sponge, 2 °C, 2 h; TEAB hydrolysis; f) i. $Bu_3N$, CDI, DMF, 23 °C, 3 h; MeOH; ii. anhydrous $H_3PO_4$, $Bu_3N$, DMF, 23 °C, 12 h. Proton Sponge: 1,8-bis(dimethylamino)naphthalene; TEAB: triethylammonium bicarbonate; CDI: carbonyl diimidazole.

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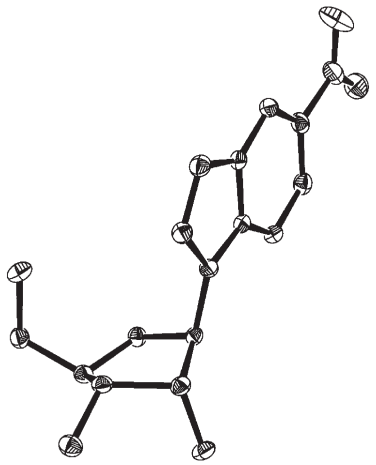

**Figure 1.** X-ray structure of **3** (ORTEP representation, 50% probability).[27]

attempted but did not yield the desired compound[28] or did not provide the pure β-anomer.[29]

To investigate whether the 5-nitroindole pseudobase was recognized and incorporated universally by a viral RdRP, we subjected triphosphate **5** to a primer-extension assay[30] with recombinant poliovirus RdRP ($3D^{pol}$).[31] This assay provides kinetic and thermodynamic measurements of nucleotide incorporation into a symmetrical primer/template (designated s/s) by viral RdRPs. As shown in Figure 2, the incorporation of **5**

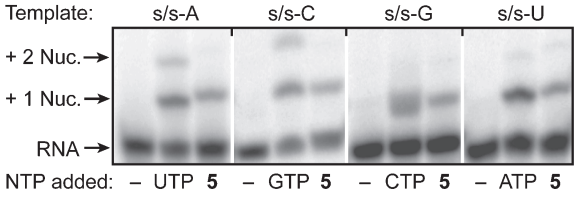


**Figure 2.** Incorporation of ribonucleotides into double-stranded RNA by poliovirus RdRP ($3D^{pol}$). Complexes of s/s-N RNA template (N represents the first templating nucleobase of the RNA primer) and $3D^{pol}$ were incubated for 90 s, the complementary NTP (0.1 mM) was added, and the reaction was allowed to proceed for 15 min at 30 °C; Nuc.: nucleotide.

into RNA by $3D^{pol}$ was evaluated across the four naturally occurring nucleotides (A, C, G, U) in the RNA template. Detailed kinetic analysis of incorporation of **5** into s/s-U afforded the data listed in Table 1. These experiments revealed that triphosphate **5** was incorporated across all four natural templating nucleotides and demonstrate its ability to function as a universal base. Kinetic measurements with the s/s-U template revealed **5** was incorporated tenfold more slowly than ribavirin triphosphate, yet tenfold more rapidly than a previously reported structurally-related 3-nitropyrrole ribonucleotide.[32] Although the kinetic data suggest that natural nucleoside triphosphates (NTPs) will be incorporated much more efficiently than **5** into viral RNA by PV RdRP, the sensitivity of viral genomes to even subtle increases in mutation frequency[10] renders them vulnerable to even low levels of incorporation of lethal mutagens. For example, ribavirin triphosphate incorporates into viral RNA with a frequency similar to natural nucleotide misincorporation.[7]

**Table 1.** Kinetics of incorporation into dsRNA and inhibition of PV $3D^{pol}$ by nucleoside 5′-phosphates.[a]

| N5′-P[b] | $K_{d,app}$ [μM][c] | $k_{pol}$ [$s^{-1}$][c] | $K_i$ [μM][d] |
|---|---|---|---|
| **5** | 9.9 ± 1.5 | 0.00136 ± 0.00006 | 30 |
| **6** | n.d.[e] | n.d.[e] | 50 |
| RTP[b] | 496 ± 21[f] | 0.014 ± 0.001[f] | 150 |

[a] See the Supporting Information for experimental details. [b] N5′-P: nucleoside 5′-phosphate; RTP: ribavirin 5′-triphosphate. [c] Kinetic analysis of nucleotide incorporation into s/s-U template (Figure 2) by recombinant PV $3D^{pol}$ measured by using the primer-extension assay.[30,31] [d] Stopped-flow, fluorescence-based kinetic analysis of nucleotide incorporation inhibition by NTPs (Figure 4). [e] Not determined. [f] Previously reported values.[7]

Previous studies that measured incorporation of 5-nitroindole 2′-deoxyribonucleotide (**4**) opposite native DNA bases by DNA polymerases (pol α and *E. coli* KF) revealed that **4** functions as a universal DNA nucleotide. However, further extension past the templated 5-nitroindole pseudobase was not observed for either polymerase; this suggests that **4** also functions as a chain terminator.[33,34] To examine whether **5** functions as a chain terminator following viral incorporation by RdRP, an additional primer-extension assay with PV RdRP and the s/s-U RNA template was performed. In this experiment, enzyme-bound $Mg^{2+}$ was replaced with $Mn^{2+}$ to decrease RdRP fidelity and overcome the slow rate of incorporation of **5** relative to the rate of enzyme dissociation.[35] Extension of the RNA primer/template by up to three nucleotides (Figure 3) was observed under these conditions; this is consistent with a lack of chain termination activity.

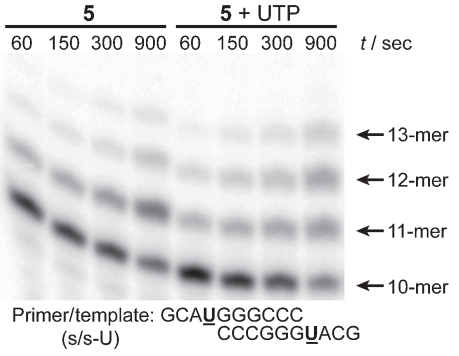


**Figure 3.** Chain-termination assay. The s/s-U template was used for primer extension by the PV RdRP ($3D^{pol}$) in the presence of $Mn^{2+}$. Ribonucleotide **5** (1 mM) and UTP (10 μM) were provided as substrates for the extension reaction.

PV RdRP incorporated **5** into RNA very slowly relative to the "correct" nucleotides[36] and with a low apparent $K_d$ (Table 1). If this $K_d$ represents binding to the enzyme active site, then **5** should inhibit elongation catalyzed by the RdRP. To probe for polymerase inhibition by **5**, primer extension by PV RdRP was assayed by using stopped-flow kinetics with the s/s-U RNA

  

template, which contained 2-aminopurine (2AP) as a fluorescent probe. Inhibitors of incorporation of the natural substrate ATP were analyzed (Figure 4). Inhibition constants ($K_i$) were determined by simulation against a competitive inhibition model

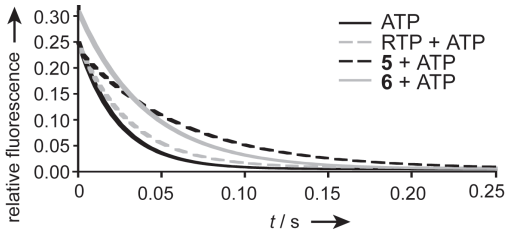


**Figure 4.** Stopped-flow kinetic analyses of inhibition of incorporation of ATP catalyzed by PV RdRP. Best-fit lines (nonlinear regression to a single exponential) of raw fluorescence data (see the Supporting Information) are shown. [$3D^{pol}$] = 0.5 μM; [s/s-U-2AP] = 0.25 μM (duplex); [ATP], [RTP], [**5**], and [**6**] = 100 μM. Data for **6** were extrapolated to 0.25 s (last data point collected at 0.20 s).

(Figure S1). As shown in Table 1, ribonucleotide **5** inhibited PV RdRP ($3D^{pol}$) approximately five times more potently than RTP. To investigate whether related 5′-phosphates **6** and **7** could similarly inhibit $3D^{pol}$, we subjected both compounds to the same assay conditions. Interestingly, diphosphate **6** was found to strongly inhibit PV RdRP ($K_i$ = 50 μM) whereas monophosphate **7** and nucleoside **3** lacked any detectable inhibitory activity. These experiments suggest that the diphosphate group is a critical determinant of inhibition, and the terminal γ-phosphate substituent plays a relatively minor role in binding and inhibition of $3D^{pol}$ by phosphorylated analogues of **3**.

Human HeLa cells infected with poliovirus were treated with ribonucleoside **3** and ribavirin (**1**) to examine the biological activities of these compounds in cell culture. Antiviral activity was compared with effects on proliferation of the host cell line (Figure 5). Importantly, both **3** and **1** substantially reduced the titer of poliovirus. Moreover, coadministration of the cytochrome P-450 inhibitor sulconazole[37] (**8**) with **3** magnified the antiviral activity of **3**, presumably by affecting metabolism of the nitroindole base. However, **8** did not affect the activity of **1**. The combination of **3** (1 mM) and **8** (10 μM) reduced viral titer by over two orders of magnitude; this surpasses the antiviral activity of **1** by approximately fivefold at this concentration. Only a slight effect on the proliferation of the HeLa host cells was observed at the highest dose evaluated.

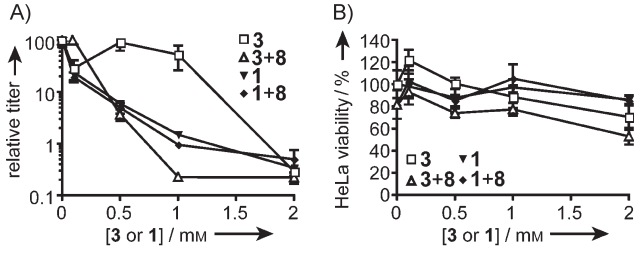


**Figure 5.** Antiviral and antiproliferative activity of **3** compared with ribavirin (**1**) in the presence and absence of sulconazole (**8**; 10 μM). A) Effects on the titer of poliovirus in infected HeLa cells (7 h treatment). B) Cytotoxicity of compounds to HeLa cells measured by using Trypan blue exclusion assay (7 h treatment).

We conclude that PV RdRP can incorporate a ribonucleotide that bears the 5-nitroindole pseudobase into RNA opposite each templating base. Although the rate of incorporation of triphosphate **5** into RNA by PV RdRP was slower than RTP and natural nucleoside triphosphates, both **5** and diphosphate **6** were much more potent inhibitors of this enzyme. Ribonucleoside **3** reduced the titer of poliovirus in cell culture, and this compound represents a promising lead for the development of novel antiviral lethal mutagens and related inhibitors of viral RdRPs.

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