Antiviral Amphipathic Oligo- and Polyribonucleotides: Analogue Development and Biological Studies

Robyn M. Hyde,[†] Arthur D. Broom,*[,†] and Robert W. Buckheit, Jr.[‡]

Department of Medicinal Chemistry, 201 Skaggs Hall, University of Utah, 30 South 2000 East, Salt Lake City, Utah 84112-5820, and TherImmune Research Corporation, 620 Professional Drive, Gaithersburg, Maryland 20879

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A series of novel N1 alkylated purine nucleic acids were polymerized either enzymatically or by automated synthesis to further establish the SAR requirements for HIV, RT, and HCMV activity. Out of the series, two constructs, 2′-*O*-methyl-1-allylinosinic acid phosphorothioate 33-mer (**16**) and an oligomer incorporating 1-propyl-6-thioinosinic acid residues (**20**), were found to be highly active under all three assay conditions. SAR studies indicate that sulfur incorporation, high molecular weight, and low steric bulk at N1 all can be important for activity.

Introduction

The human immunodeficiency virus (HIV) is a genetically diverse infectious agent, and its ability to rapidly develop resistance toward standard front line treatment has left physicians scrambling for new therapeutic options. Because of mutational changes within the HIV genome, mostly resulting from the infidelity of reverse transcriptase (RT),[1,2] mutant particles are able to withstand drug pressure, survive, and proliferate. It has been documented that some resistant strains are in fact more fit than wild-type HIV.[3] Only three classes of drugs, the nucleoside reverse transcriptase inhibitors (NRTIs), the non-nucleoside RT inhibitors (NNRTIs) and the protease inhibitors (PIs) are currently approved for use in the U.S. Numerous resistant mutants to each of these drugs have been characterized.[4] For several drugs it has been shown that a single mutation will greatly diminish efficacy.[5,6] Salvage treatment after initial treatment failure is not always effective because within each of the three classes of FDA-approved drugs there is significant cross-resistance.[7,8] The change in treatment regimen from one drug to another within the same class may not always lead to viral suppression. Because of the impressive mutation ability of HIV, it is imperative that drugs with diverse structures and unique targets be developed. For both first and second line management of HIV infections, new drugs are needed that lack cross-resistance with existing agents and are slow to select resistant clones.

Previous work identified nucleic acid polymers, especially those incorporating thiolated purines, that were active against viral RT.[9−11] It was hypothesized that these constructs act as inhibitors of RT by binding as "antitemplates" within the active site and blocking the transcription of viral RNA.[12] Studies in this lab focused on the development of other novel oligo- and polyribonucleotides as anti-HIV agents and identified several potent inhibitors including the lead compound poly(1-methyl-6-thioinosinic acid) (**1**, Figure 1). As a group, these "senseless" polymers, so-called because of their lack of Watson−Crick hydrogen bonding potential, form a new class of anti-HIV agents. Their nucleic acid macromolecular structure and proposed mechanism(s) of action significantly differ from drugs currently used. The pursuit of these macromolecules as opposed to small molecules is warranted because (1) the proposed mechanism of action will be less likely to select for resistant strains and (2) they have demonstrated equipotent activity against HIV and human cytomegalovirus (HCMV). A significant advantage over current HCMV therapy would be realized if there were the ability to treat both HIV and HCMV with a single agent. HCMV is the most prevalent opportunistic infection (OI) observed in HIV patients,[13,14] and current therapy suffers from toxicity limitations.[15,16]

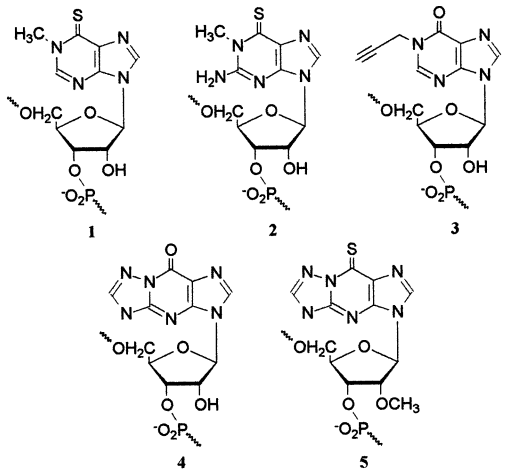

Figure 1. Anti-HIV active oligo- and polyribonucleotides.

Until recently, the SAR that has guided analogue development of the "senseless" macromolecules emphasized the requirement for amphipathicity and the ability to form secondary structure in solution and highlighted the preference for long polymer length and sulfur incorporation.[17−19] Unexpectedly, it was discovered that poly(1-propargylinosinic acid) (**3**) had submicromolar

* To whom correspondence should be addressed. Phone: (801)581-5764. Fax: (801)581-7087. E-mail: arthur.broom@hsc.utah.edu.
[†] University of Utah.
[‡] TherImmune Research Corporation.

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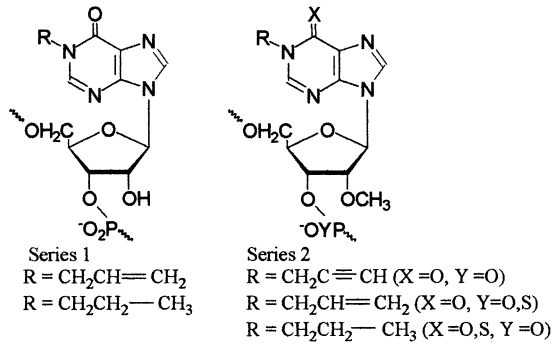


Figure 2. Target oligo- and polyribonucleotides.

activity against HIV though it lacked both secondary structure and sulfur.[20]

Project Rationale

Proposed for this study were two series of compounds based on derivative **3** that would be used to further investigate the SAR of inosinic acid analogues. The first series would include polymers (~300-mers) prepared enzymatically with polynucleotide phosphorylase (PNPase) from nucleoside diphosphate derivatives modified at the N1 position with either the allyl or propyl group. The second series would include oligomers (33-mers) prepared by automated synthesis from protected nucleoside derivatives with N1 modifications of propargyl, allyl, or propyl and additional modifications at the 2′,6 and nonbridging oxygen positions (Figure 2).

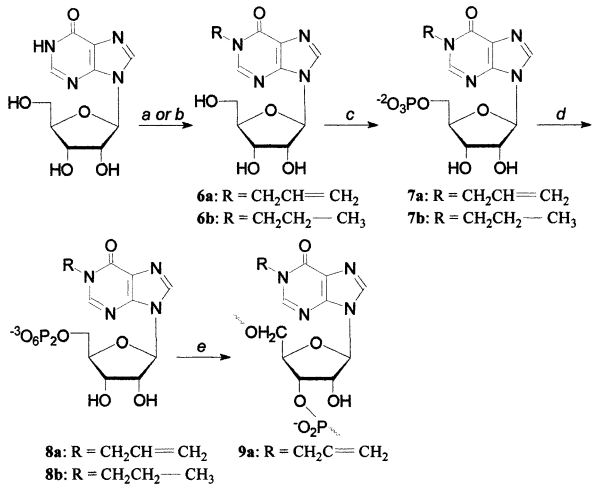
Although the established SAR suggested that the polymers of series 1 would be intrinsically more active than the oligomers of series 2, the synthesis and biological evaluation of oligomers were added to the project to further study this factor and to exploit the advantages of automated synthesis. Automated synthesis of polymers is less expensive, and the chemistry involved has lower substrate specificity. It was thought that although some activity may be sacrificed by having a shortened polymer, the reduction in activity may be overcome by the incorporation of other groups into the base, sugar, and backbone of the oligomers that would not be possible if PNPase were used for polymerization. Proposed were 2′-*O*-methyl and phosphorothioate substitutions to increase the stability of the prepared oligoribonucleotides[21,22] and to improve the delivery kinetics and half-lives of the drugs. Also proposed was a 6-thio substitution to satisfy the SAR preference for sulfur. Earlier attempts to incorporate sulfur at the C6 position of the propargyl[20] or allyl[23] derivatives failed, but it was expected that the synthesis of 2′-*O*-methyl-1-propyl-6-thioinosinic acid would be possible.

Chemistry

Except for those derivatives incorporating sulfur, the strategies used to synthesize each polymer in series 1 and oligomer in series 2 were identical; only the alkylating reagent was changed to afford the different analogues (Schemes 1 and 2). Polymer synthesis began with the alkylation of inosine under basic conditions with either allyl bromide or propyl bromide to give 1-allylinosine (**6a**) and 1-propylinosine (**6b**), respectively. Each nucleoside was converted to its 5′-monophosphate derivative in reaction with phosphoryl chloride in chilled tributylamine.[24] Activation of the 5′-monophosphate with carbonyldiimidazole followed by treatment with tri(*n*-butyl)ammonium phosphate gave the 5′-diphosphate derivatives **8a** and **8b**.[25] Polymerization with PNPase[20] at basic pH afforded poly(1-allylinosinic acid) (**9a**) with an approximate molecular weight of 1×10^5 as estimated by size exclusion HPLC.[18] 1-Propylinosine diphosphate (**8b**) was not a substrate for PNPase, and poly(1-propylinosinic acid) was not formed.

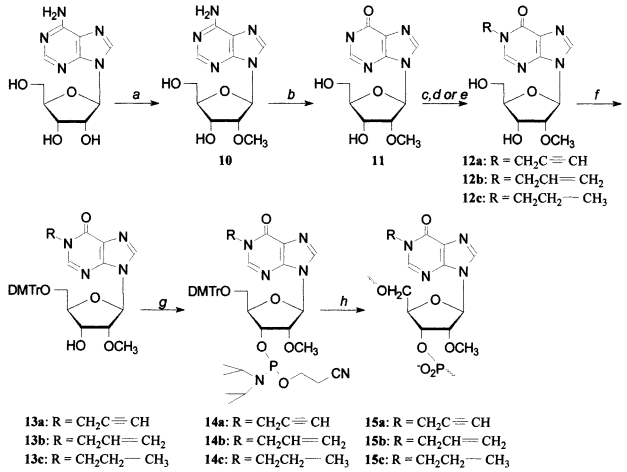
The starting material for oligonucleotide synthesis, 2′-*O*-methylinosine (**11**), was obtained in two steps. Adenosine was alkylated with iodomethane after a 1 h NaH treatment in dimethylformamide[26] to give 2′-*O*-methyladenosine (**10**). This purified product was diazotized with nitrous acid generated in situ and solvo-

Scheme 1[a]



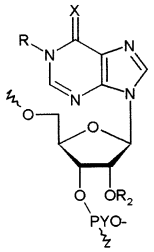
[a] Reagents: (a) dimethylacetamide, 1,8-dizabicyclo[5.4.0]undec-7-ene, allyl bromide; (b) dimethylacetamide, 1,8-dizabicyclo[5.4.0]undec-7-ene, propyl bromide; (c) $POCl_3$, $PO(OEt_5)$; (d) dimethylformamide, carbonyl diimidazole, $(nBuNH_3^+)OPO_3H_2^-$; (e) *E. coli* PNPase, Tris-HCl, pH 9, 37 °C.

Scheme 2[a]



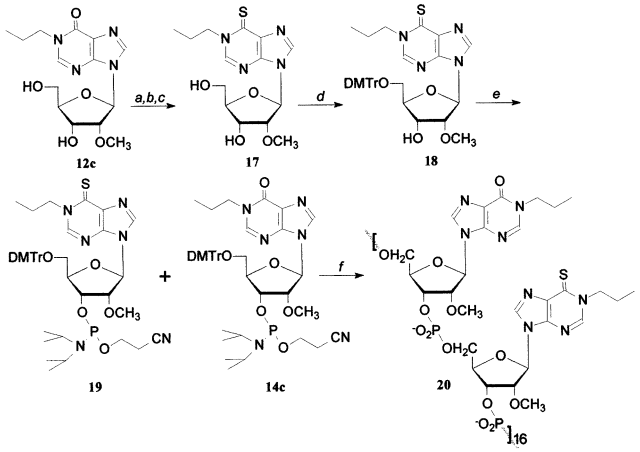
[a] Reagents: (a) dimethylformamide, NaH, iodomethane; (b) H_2O, acetic acid, sodium nitrite; (c) dimethylacetamide, 1,8-dizabicyclo[5.4.0]undec-7-ene, propargyl bromide; (d) dimethylacetamide, 1,8-dizabicyclo[5.4.0]undec-7-ene, allyl bromide; (e) dimethylacetamide, 1,8-dizabicyclo[5.4.0]undec-7-ene, propyl bromide; (f) pyridine, DMTrCl; (g) acetonitrile, diisopropylammonium tetrazolide, 2-cyanoethyl tetraisopropylphosphorodiamidite; (h) automated RNA synthesis.

Table 1. SAR Relationship



compd	R	R_2	X	Y	amphipathicity	2° structure	sulfur	chain length
3[20]	CH_2CCH	H	O	O	X			~300-mer
9a	CH_2CHCH_2	H	O	O	X			~300-mer
15a	CH_2CCH	CH_3	O	O	X	X		33-mer
15b	CH_2CHCH_2	CH_3	O	O	X	X		33-mer
15c	$CH_2CH_2CH_3$	CH_3	O	O	X	X		33-mer
16	CH_2CHCH_2	CH_3	O	S	X		X	33-mer
20	$CH_2CH_2CH_3$	CH_3	S	O	X		X	33-mer

Scheme 3[a]



[a] Reagents: (a) pyridine, acetic anhydride; (b) pyridine, Lawesson's reagent, reflux; (c) methanolic ammonia; (d) pyridine, DMTrCl; (e) acetonitrile, diisopropylammonium tetrazolide, 2-cyanoethyl tetraisopropylphosphorodiamidite; (f) automated RNA synthesis.

lyzed to give **11**.[27] Starting material **11** was alkylated using propargyl, allyl, or propyl bromide under basic conditions to give **12a**, **12b**, and **12c**, respectively. Each modified nucleoside was then prepared for automated synthesis by protecting the 5′-hydroxyl group with dimethoxytrityl and derivatizing the 3′-hydroxyl as its cyanoethyldiisopropylphosphoroamidite.[28] The 33-mer oligomers were synthesized by automated synthesis from a solid support resin conjugated to a 3′-thymidine using a 25 min coupling time and oxidation with *tert*-butyl peroxide. Oligomer **16** with a phosphorothioate backbone was made by automated synthesis similarly to **15b** except a Beaucage reagent[29] was used for oxidation instead of *tert*-butyl peroxide. All oligomers were removed from the resin by a 1 h treatment with ammonium hydroxide at room temperature and purified from their failure sequences.[30]

To synthesize the 6-thio nucleoside precursor 2′-*O*-methyl-1-propyl-6-thioinosine (**17**), **12c** was treated with acetic anhydride to protect the 3′ and 5′ hydroxyl groups and dry crude product was refluxed in pyridine with Lawesson's reagent (Scheme 3). After removal of excess pyridine from the reaction at termination, the crude oil was dissolved in methanol and treated with methanolic ammonia under pressure to remove the acetate protecting groups. Nucleoside was then derivatized at the 3′ and 5′ positions appropriately for automated oligonucleotide synthesis. Owing to low yields at each of the synthetic steps, there was not enough product to synthesize a thio homopolymer, and instead, a copolymer using both 2′-*O*-methylinosinic acid and 2′-*O*-methyl-6-thioinosinc acid residues was made. Automated synthesis was conducted as before.

Table 2. Activity of Target Compounds against HIV in CEM-SS Cells and against Cell-Free RT

compd	HIV EC_{50} (μM)	RT IC_{50} (μM)
3[20]	0.13	0.0223
9a	0.94	9.6
15a	0.26	0.15
15b	0.32	50.5
15c	4.97	6.7
16	0.61	0.039
20	0.14	0.82

Results and Discussion

Anti-HIV Activity. In comparison with the previously established SAR, each of the constructs made for this project had two of the highlighted structural characteristics. Each was amphipathic, having a hydrophilic backbone and hydrophobic base, and each had one additional characteristic (Table 1). None of the macromolecules fully adhered to the established SAR. It is not known if the secondary structure observed in the oligomers **15a**−**c** is significant. Though melting transitions were observed, the T_m was below 11 °C and was not highly cooperative.[31]

All senseless macromolecules were tested for their ability to protect cells from the pathogenicity of HIV. Additionally, they were tested for their ability to inhibit the polymerization activity of RT in a cell-free environment. As shown in Table 2, most constructs had submicromolar activity against HIV in a CEM-SS cell line using wild-type RF virions initially acquired from the NIH AIDS Research and Reference Reagent Program. The exception was 2′-*O*-methyl-1-propylinosinic acid 33-mer (**15c**) that had activity roughly equivalent to 5 μM. Fitting with the SAR, the polymer **3** was more active than the oligomers **15a**−**c**; however, activity was rescued in the oligomer series by the incorporation of sulfur

Table 3. Activity of Target Compounds against HCMV in MRC-5 Cells

compd	EC_{50} (μM)
ganciclovir	9.4
3[20]	0.019
15a	0.56
15b	inactive
15c	inactive
20	1.3

into the construct. Of those compounds made for this project, the sulfur compounds **16** and **20** were the most active.

In general, the propargyl analogues were more active than the allyl or propyl analogues. It is more likely that the effective space occupied by each group contributed to the activity difference rather than to the degree of hydrophobicity. The propargyl group has limited flexibility and therefore a smaller effective steric volume than the two other groups. Correspondingly, the propargyl derivatives were the most active. This may indicate that the mechanism of action requires the base to fit into a constrained active site. This observation would be consistent with the hypothesis that these constructs act as RT inhibitors.

On the other hand, although the whole-cell activities of the most active constructs were approximately equivalent, the RT activity was not. Generally, the RT activity was poor except for constructs **15a** and **16**. This may indicate that the current compounds act primarily by mechanisms of action other than RT inhibition. Clearly, **9a**, **15b**, **15c**, and **20** cannot act as RT inhibitors in the cell. The IC_{50} values were higher than the EC_{50} values, which precludes them from being realistic RT inhibitors in the in vivo system.

Each of the polymers was relatively nontoxic to cells. Though the CD_{50} has not been well explored for each compound, cell viability was only observed to drop below 96% for **15a** (50%, 50.3 μM) and **15c** (77%, 16 μM).[32]

Anti-HCMV Activity. A very curious quality about the "senseless" compounds is that they have potent activity against HCMV. HCMV, a DNA virus, has little in common with HIV, an RNA virus. Though the two viruses are enveloped and have similar viral entry processes, the remainder of the life cycle is quite different. Of the senseless macromolecules tested, **15a** and **20** had activity against HCMV and that activity was nearly equipotent with HIV activity. Even though **20** was only weakly active, it was still severalfold more potent than ganciclovir, the current first-line therapeutic agent (Table 3).

Until now, the SAR profile for anti-HCMV activity of the senseless compounds has been loosely related to the SAR for the anti-HIV activity. Of the previously made compounds, those that had anti-HIV activity also had anti-HCMV activity. This study demonstrates for the first time dissimilarity in antiviral activity. Both **15b** and **15c** had quite good anti-HIV activity; however, they were inactive against HCMV. The most reasonable explanation is that the cellular target has a more stringent requirement regarding maximal steric volume allowed at the N1 position of the purine. This cannot be the whole story, though, since the inclusion of sulfur in the purine with **25** salvages the activity. Until further work is done that elucidates the mechanism of action for the anti-HCMV activity, full understanding of the SAR is not possible.

Conclusions

Though the inosinic acid based compounds synthesized for this project did not strictly adhere to the previously established SAR, the most active compounds retained significant anti-viral activity. The data do not clearly support RT inhibition, however, which was the prevailing hypothesis regarding the mechanism of action. The previously established SAR may have identified those compounds that do primarily act by RT inhibition. These active constructs that are at variance with the SAR may primarily act by some other mechanism of action. There is some indication that the mechanism of action does include a specific interaction because steric bulk at N1 limits the inhibitory capabilities of the constructs. Additionally, these studies show that sulfur is important, though it is not known exactly what role sulfur plays in any interaction. Other mechanisms of action have been proposed for HIV activity such as inhibition of virion attachment and uptake and interference with viral uncoating.[33] Further analogue development and viral assays will be required to fully understand the SAR and mechanism of action.

Experimental Section

General. Pyridine was dried by distillation over CaH_2. Anhydrous dimethylacetamide (DMAC), dimethylformamide (DMF), and methanol (MeOH) were purchased from Aldrich. All other chemicals were purchased as ACS or reagent grade and used without further purification. Thin-layer chromatography was conducted on silica gel coated Whatman aluminum backed flexible plates containing fluorescent indicator F_{254}. Spots were visualized with short- and long-wavelength UV as appropriate. Slide-A-Lyzer cassettes (MWCO 10,000) from Pierce were used for dialysis. Gravity and flash silica gel chromatography were performed with particle size 63−200 gel from Selecto Scientific and 170−400 from Fisher, respectively. Adsorption chromatography was performed with Amberlite XAD-4 resin from Sigma. Reverse-phase chromatography was performed with Poly-Pac II cartridges from Glen Research. Cation exchange and anion exchange chromatography were performed with Bio-Rad resins AG 50W-X8, 200−400 mesh and AG 1-X8, 200−400 mesh, respectively. Gel filtration chromatography was performed with Bio-Gel P2 (fine) from Bio-Rad. Ion exchange and gel filtration chromatography used Bio-Rad Econo-Columns, Wiz pump/diluter/dispenser, ISCO type 6 optical unit (254 nm) and ISCO UA-5 absorbance monitor. HPLC chromatography was preformed with a Hitachi L6200 pump equipped with a L3000 photodiode array. Size exclusion and reverse-phase HPLC were accomplished with the Bio-Rad Bio-Sil SEC-125 (Bio-Sil 125 guard) and Rainin Microsorb-MV 100 Å C8 columns, respectively. High-resolution mass spectra were recorded with a MAT 95 spectrometer. Proton, carbon, and phosphorus NMR were recorded with either a Brucker AF200 MHz, Varian Mercury 400 MHz FT-NMR or Unity 500 MHz NMR spectrometer. Phosphorus NMR spectra were referenced to an external phosphoric acid standard. UV spectra and T_m measurements were recorded with a Hewlett-Packard 8452A diode array spectrometer equipped with a Peltier variable-temperature controller.

HPLC Protocol. Size exclusion (Bio-Rad Sec-125) parameters are the following: 100%; phase a, 0.05 M phosphate buffer + 0.15 M NaCl + 0.01 M NaN_3. Ion exchange (partisil-SAX) parameters are the following: 0−100%, 50 min; phase a, 0.01 M acetic acid, 6 mM potassium phosphate monobasic, pH 5; phase b, 0.6 M potassium phosphate monobasic, pH 4. Reverse-phase (C8) parameters are the following: 0−60%, 45 min; phase a, acetonitrile; phase b, 0.1 M ammonium acetate.

1-Allyllinosine (6a). Allyl bromide (8.0 mL, 91.5 mmol) and 1,8-dizabicyclo[5.4.0]undec-7-ene (DBU) (14.0 mL, 91.9 mmol) were added to a suspension of inosine (18.7 g, 69.78 mmol) in DMAC (400 mL). The reaction mixture was stirred at room temperate under argon overnight, at which time TLC in chloroform/methanol (85:15) demonstrated the disappearance of starting material. The reaction was terminated by addition to 3.5 L of ether/hexane (1:1). The resulting suspension was placed at −15 °C overnight. While still chilled, the solvent was decanted from the resulting gum that was then dissolved in methanol. The solution was evaporated under reduced pressure onto silica gel. The product was isolated by silica gel chromatography using chloroform/methanol (95:5). Isolated yield was 18.0 g (83.7%) of a white foam. UV ϵ_{max} 9403 at 250 nm; ^{1}H NMR (DMSO-d_6) δ 8.38 (1H, s, H2), 8.36 (1H, s, H8), 6.00 (1H, m, CH), 5.86 (1H, d, J = 5.8 Hz, H1′), 5.48 (1H, d, J = 6.1 Hz, 2′-OH), 5.20 (1H, d, J = 4.8 Hz, 3′-OH), 5.10 (2H, m, CH_2), 5.06 (1H, m, 5′-OH), 4.64, 4.63 (2H, d, J = 5.5, CH_2), 4.48 (1H, q, J = 16.7, H2′), 4.13 (1H, q, J = 13.3, H3′), 3.94 (1H, q, J = 11.6, H4′), 3.63 (1H, m, H5′), 3.56 (1H, m, H5″); FABMS (glycerol and EtOAc), m/z (MH^+) calcd for $C_{13}H_{17}N_4O_5$ 309.119 89, found 309.118 16; HPLC retention time 32.5 min (C18, 0−15%, 25 min, phase a 0.05 M phosphate buffer, phase b MeOH); homogeneous by HPLC.

1-Allylinosine-5′-monophosphate (7a). The dry solid **6a** (10 g, 32.5 mmol) was converted to **11b** as described earlier by Yoshikawa,[24] yielding 14.1 g (83.0%) of white solid. The product was used without further purification. Samples for HRMS were obtained by cation exchange chromatography conversion of the compound to its NH_4^+ salt. HRMS (glycerol and EtOAc) m/z (M − $3NH_3$ − H) calcd for $C_{13}H_{16}PN_4O_8$ 387.070 58, found 387.068 70.

1-Allylinosine-5′-diphosphate (8a). Monophosphate **7a** (7 g, 13.4 mmol) was converted to **8a** as described by Hoard and Ott.[25] The reaction mixture was quenched by doubling the volume with MeOH, and using minimal heat, the solution was evaporated to dryness. The resulting residue was dissolved in 200 mL of H_2O, and after addition of 8.4 g of lithium acetate, the pH was adjusted to 12.0 with 3 N LiOH while being chilled in an ice bath. The solution was immediately filtered through Celite, and the filtrate was adjusted to pH 7.0 with 1 N HCl while chilled in an ice bath. After the filtrate was concentrated to approximately 100 mL, **8a** was precipitated with addition of 1 L of ethanol/acetone (1:1). The precipitate was isolated by centrifugation and dried in a vacuum oven at room temperature. Crude diphosphate was purified by anion exchange chromatography using LiCl (0−0.3 M) in 0.003 M HCl. Pooled fractions containing product as identified by ion-exchange HPLC were adjusted to pH 7.0 with 1 M HCl and concentrated to approximately 300 mL by rotary evaporation. Diphosphate was precipitated in 2.4 L of ethanol/acetone (1:1) and, following 48 h of refrigeration, was isolated by centrifugation and dried in a vacuum oven at room temperature. When H_2O was used as eluent, diphosphate was converted to its Na^+ salt by cation exchange chromatography and, after lyophilization, desalted by gel filtration. The product was isolated as a white fluffy solid (1.1 g, 15.4%). Samples for HRMS were obtained by cation exchange chromatography conversion of the compound to its NH_4^+ salt. HRMS (glycerol and MeOH) m/z (M − $3NH_3$ − H) calcd for $C_{13}H_{16}PN_4O_8$ 467.036 91, found 467.036 26; HPLC retention time 25.7 min (ion exchange protocol); homogeneous by HPLC.

Poly(1-allylinosinic acid) (9a). A solution comprising the following components was incubated at 37 °C for 6 h with gentle rocking: 1.0 mL of Tris-HCl (pH 9.0, 2 M); 1.0 mL of $MgCl_2$ (0.1 M); 1.0 mL of 2-mercaptoethanol (2%); 4.0 mL of H_2O; 75 IU of PNPase (*E. coli*) in 0.6 mL of buffer containing 50% glycerol, 5 mM Tris, and 150 mg of diphosphate **8a**. After incubation was complete as determined by size exclusion HPLC,[18] the reaction mixture was diluted to 20 mL with H_2O and adjusted to pH 7.0 with 0.1 M HCl. All protein was removed from the aqueous reaction mixture by several extractions with chloroform/isoamyl alcohol (5:2). Extraction was repeated until no precipitated protein was apparent at the aqueous/organic interface. The aqueous solution was dialyzed against 0.1 M NaCl (12 L, 24 h) and H_2O (24 L, 48 h). Lyophilization of the aqueous solution gave poly(1-allylinosinic acid) (11.5 mg, 10.1%) as a fluffy, white solid. UV ϵ_{max} 7477 at 250 nm (0.1 M NaCl, 0.1 M phosphate buffer, pH 6.8); HPLC retention time 7.3 min (size exclusion protocol); homogeneous by HPLC.

2′-*O*-Methyladenosine (10). The reaction of adenosine (18 g, 67.4 mmol) in 430 mL of DMF treated with NaH (2.9 g) and iodomethane (3.1 mL, 50.0 mmol) as described earlier[34] yielded 12.6 g (66.5%) of a white foam. FABMS (glycerol and H_2O) m/z (MH^+) calcd for $C_{11}H_{16}N_5O_4$ 282.1, found 282.

2′-*O*-Methylinosine (11). 2′-*O*-Methyladenosine **10** (7.6 g, 26.9 mmol) was reacted with 400 mL of H_2O, 18.2 mL of acetic acid, and 14.6 g of sodium nitrite (211.6 mmol) to give **11** as described earlier.[35] The product was isolated by adsorption chromatography using ethanol (0−30%). The isolated yield was 6.1 g (80.4%) of a white solid. FABMS (thioglycerol [1% TFA], MeOH), m/z ($M + Na^+$) calcd for $C_{11}H_{14}N_4O_5Na$ 305.1, found 305.

2′-*O*-Methyl-1-propargylinosine (12a). To a solution of 2′-*O*-methylinosine (**11**) (6.0 g, 18.8 mmol) in 120 mL of DMAC were added DBU (3.0 mL, 19.7 mmol) and propargyl bromide (2.4 mL, 16.6 mmol) dropwise. The solution was stirred at room temperature for 18 h, at which time TLC showed complete disappearance of starting material. The reaction mixture was treated generally as for **6a**. The isolated yield was 4.5 g (75.2%) of a white foam. Purity was determined by comparison of the peak height of starting material H1′ and product H1′ observed in the NMR spectra. UV ϵ_{max} 6424 at 250 nm; ^{1}H NMR (DMSO-d_6) δ 8.51 (1H, s, H2), 8.42 (1H, s, H8), 5.98 (1H, d, J = 5.8 Hz, H1′), 5.29 (1H, d, J = 5.5 Hz, 3′-OH), 5.10 (1H, t, J = 10.9 Hz, 5′-OH), 4.84, 4.83 (2H, d, J = 2.7, CH_2), 4.30 (1H, q, J = 8.5, H2′), 4.26 (1H, t, J = 10.6, H3′), 3.96 (1H, q, J = 11.3, H4′), 3.55 (1H, m, H5′), 3.53 (3H, m, OCH3), 3.45 (1H, m, H5″), 3.40 (1H, t, J = 5.1, CH); HRMS (glycerol and MeOH) m/z (MH^+) calcd for $C_{14}H_{17}N_4O_5$ 321.119 89, found 321.119 99; purity 95% by NMR.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-1-propargylinosine (13a). To a solution of 4.5 g of dry product (**12a**) (14.1 mmol) in 66 mL of pyridine was added 6.0 g of DMTrCl (17.8 mmol). The reaction mixture was vigorously stirred for 6 h until TLC in $CHCl_3$/MeOH (97:3) showed disappearance of starting material. One volume of MeOH was added and evaporated to a thick oil. The oil was dissolved in 100 mL of $CHCl_3$, washed three times with 15 mL of both saturated $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated onto silica gel. The product was purified by flash chromatography under argon by gradient elution of ethyl acetate from 0% to 100% in hexanes in the constant presence of 1% triethylamine. Product was recovered at 100% ethyl acetate and evaporated to a white foam by coevaporation with CH_2Cl_2. The isolated yield was 3.2 g (36.5%). HRMS (glycerol and MeOH), m/z (MH^+) calcd for $C_{35}H_{35}N_4O_7$ 623.250 57, found 623.251 62; R_f = 0.80 ($CHCl_3$/MeOH, 95:5); R_f = 0.30 (EtOAC/hexanes, 9:1); single spot by TLC.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-1-allylinosine (13b). The product was prepared in two steps generally as described for **12a** and **13a**. Dry product **11** (5.0 g. 17.9 mmol) was suspended in 100 mL of DMAC. To the reaction mixture were added DBU (2.7 mL, 17.7 mmol) and allyl bromide (2.2 mL, 25.17 mmol). The reaction mixture was stirred vigorously under argon overnight and was then doubled in volume with methanol and evaporated by oil pump onto silica gel. Product was eluted from a silica gel column with $CHCl_3$/MeOH (95:5).

Oil (**12b**) was coevaporated three times with pyridine to remove all traces of water. To the suspension of oil in pyridine (85 mL) was added dimethoxytrityl chloride (7.8 g, 23.0 mmol). The reaction was completed, and the product was purified as described for **13a**. The isolated yield was 4.8 g (43.0%). HRMS (glycerol [1% TFA] and MeOH), m/z (MH^+) calcd for $C_{35}H_{37}N_4O_7$ 625.266 22, found 625.263 98; R_f = 0.42 ($CHCl_3$/MeOH, 95:5); R_f = 0.20 (EtOAC/hexanes, 9:1); homogeneous by TLC.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-1-propylinosine (13c). Product was prepared from **11** (3.0 g. 10.6 mmol) as described for **13b**. Isolated yield was 2.3 g (34.7%). FABMS (glycerol and MeOH), *m/z* (MH^+) calcd for $C_{35}H_{39}N_4O_6$ 627.264 12, found 627.266 88; $R_f = 0.40$ ($CHCl_3$/MeOH, 95:5); $R_f = 0.18$ (EtOAC/hexanes, 9:1); single spot TLC.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-1-propargylinosine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosophoramidite (14a). To a vigorously stirring solution of product (1.2 g, 1.93 mmol) (**13a**) in 15 mL of acetonitrile was added 91.3 mg of diisopropylammonium tetrazolide and 1 g of 2-cyanoethyl tetraisopropylphophorodiamidite (3.22 mmol). The salt was prepared by adding diisopropylamine (1 mL, 7.14 mmol) to a solution of tetrazole (206 mg, 2.94 mmol) in 11 mL of dry acetonitrile and isolated by filtering the precipitate that formed after 10 min. The reaction mixture was stirred overnight under argon and verified to be complete by TLC in $CHCl_3$/MeOH (97:3). The reaction volume was doubled with saturated $NaHCO_3$ and extracted three times with 25 mL of ethyl acetate. The organic layer was washed two times with 25 mL of brine and once with H_2O, dried over anhydrous Na_2SO_4, and evaporated to a thick oil. The product was purified by flash chromatography under argon by gradient elution of ethyl acetate from 0% to 100% in hexanes in the constant presence of 1% triethylamine. Product was recovered at 80−100% ethyl acetate and evaporated to a white foam by coevaporation with CH_2Cl_2. The isolated yield was 1.3 g (81.9%). ^{31}P NMR (200 MHz, CD_3CN) δ 151.96, 151.18; HRMS [3-nitrobenzyl alcohol (NBA) and $CHCl_3$], *m/z* (MH^+) calcd for $C_{44}H_{52}PN_6O_8$ 823.358 43, found 823.361 59; $R_f = 0.72$, 0.6 ($CHCl_3$/MeOH, 95:5); $R_f = 0.75$, 0.67 (EtOAC/hexanes, 9:1)

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-1-allylinosine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosophoramidite (14b). Dry product **13b** (2.5 g, 4.0 mmol) was treated as described for **14a**. The isolated yield was 2.3 g (69.8%) of a white fluffy foam. ^{31}P NMR (DMSO-d_6) δ 150.71, 150.53; HRMS (glycerol) *m/z* (MH^+) calcd for $C_{44}H_{54}PN_6O_8$ 825.374 08, found 825.372 07; $R_f = 0.69$, 0.58 ($CHCl_3$/MeOH, 95:5); $R_f = 0.60$, 0.55 (EtOAC/hexanes, 9:1).

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-1-propylinosine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosophoramidite (14c). The product was prepared from **13c** (1.0 g, 1.6 mmol) generally as described for **14a** and **14b**. The isolated yield was 1.2 g (90.7%). ^{31}P NMR (DMSO-d_6) δ 150.72, 150.52; HRMS (NBA and EtOAc) *m/z* (MH^+) calcd for $C_{44}H_{56}PN_6O_8$ 827.389 73, found 827.390 97; $R_f = 0.67$, 0.60 ($CHCl_3$/MeOH, 95:5); $R_f = 0.65$, 0.52 (EtOAC/hexanes, 9:1).

2′-*O*-Methyl-1-propargylinosinic Acid 33-mer (15a). 33-mer oligo was synthesized using **14a** on a 1 μmol scale with an automated DNA synthesizer. The oligomer chain was initially extended from a 3′-T residue attached to a controlled pore glass (CPG) solid support. Modifications to the standard protocol included extension of the coupling time to 25 min, oxidation of the backbone with *tert*-butyl peroxide, and removal of product from the solid support by a 1 h treatment with NH_4OH at room temperature. Crude DMTr-ON product **15a** was dried by speed evacuation at room temperature and purified by reverse-phase chromatography. Collected product was lyophilized and converted to its Na^+ salt by ion exchange chromatography with sodium acetate (1.0 M) and by dialysis with 0.1 M NaCl (24 h, 8 L). The oligomer was washed (H_2O, 48 h, 16 L) and lyophilized. The isolated yield was 6.7 mg (54.2% based on a theoretical 1 μm yield) of a white fluffy solid. The product was collected for MALDI by reverse-phase HPLC chromatography. UV ϵ_{max} 8015 at 250 nm; MALDI (M + 2Na) calcd 124 88, found 124 92; HPLC retention time 23.3 min (reverse-phase protocol); homogeneous by HPLC.

2′-*O*-Methyl-1-allylinosinic Acid 33-mer (15b). 33-mer was synthesized using **14b** on a 1 μmol scale with an automated DNA synthesizer as described for **15a**. The isolated yield was 6.9 mg (55.2%) of a fluffy white solid. The sample for MALDI evaluation was collected by HPLC (reverse-phase protocol). UV ϵ_{max} 6855 at 250 nm. MALDI (M + 3Na) calcd 125 69, found 12 570.5; HPLC retention time 20.2 min (reverse phase protocol); homogeneous by HPLC.

2′-*O*-Methyl-1-propylinosinic Acid 33-mer (15c). 33-mer was synthesized using **14c** on a 1 μmol scale with an automated DNA synthesizer as described for **15a** and **15b**. The isolated yield was 7.9 mg of fluffy white solid (62.7%). UV ϵ_{max} 7060 at 252; MALDI calcd 12 565, found 12 565.9; HPLC retention time 24.4 min (C8 protocol); homogeneous by HPLC.

2′-*O*-Methyl-1-allylinosine Phosphorothioate 33-mer (16). 33-mer was synthesized with **14b** on a 1 μmol scale with an automated DNA synthesizer as described for **15a**; however, tetraethylthiuram disulfide was used for oxidation to create the phosphorothioate backbone. The isolated yield was 6.4 mg (38.1%) of a white fluffy solid. UV ϵ_{max} 8775 at 252 nm; MALDI (M + H) calcd 13 048, found ∼13 000. HPLC retention time 22.7 min (broad peak) (reverse-phase protocol); homogeneous by HPLC.

2′-*O*-Methyl-1-propyl-6-thioinosine (17). To a solution of 2′-*O*-Me-1-propylinosine (**17c**, 2.1 g, 6.5 mmol) dissolved in 25 mL of pyridine was added 10.5 mL of acetic anhydride. TLC in $CHCl_3$/MeOH (90:10) indicated that the reaction was complete in 3 h. Crude reaction mixture was coevaporated three times with toluene and then brought up in 50 mL of $CHCl_3$. The organic layer was washed three times with 15 mL of 0.1 N HCl and twice with 15 mL of H_2O. The aqueous solution was back-extracted once with 15 mL of $CHCl_3$, and then the entire $CHCl_3$ solution was dried over Na_2SO_4 and rotary-evaporated to a thick oil.

Protected nucleoside was coevaporated three times with dry pyridine and then dissolved in 50 mL of pyridine and refluxed with 1 g of Lawesson's reagent (2.5 mmol) overnight. TLC in $CHCL_3$/MeOH (90:10) showed the disappearance of all staring material. Once cooled, the reaction mixture was evaporated three times with toluene to remove all pyridine. The resulting gum was dissolved in 50 mL of methanol, and insoluble black material was removed by filtration. The filtrate was evaporated, dissolved in 100 mL of MeOH, doubled in volume with methanolic ammonia, placed in a Parr bomb, and sealed. The bomb was left sealed for 48 h, and then pressure was slowly released. The methanol solution was dried onto silica gel. Product was purified by silica gel chromatography in a gradient from 0% to 2% MeOH in $CHCl_3$. The isolated yield was 0.6 g (27.1%) of a light-brown solid. Purity was determined by comparison of the peak height of starting material H1′ and product H1′ observed in the NMR spectra. UV ϵ_{max} 10 745 at 234 nm, 24 970 at 322 nm; ^1H NMR (DMSO-d_6) δ 8.79 (1H, s, H2), 8.54 (1H, s, H8), 5.99 (1H, d, $J = 5.5$ Hz, H1′), 5.29 (1H, d, $J = 5.5$ Hz, 3′-OH), 5.10 (1H, t, $J = 11.3$ Hz, 5′-OH), 4.51, 4.49 (2H, t, $J = 14.7$, CH_2), 4.31 (1H, q, $J = 13.7$, H2′), 4.26 (1H, t, $J = 10.6$, H3′), 3.96 (1H, q, $J = 11.3$, H4′), 3.64 (1H, m, H5′), 3.56 (1H, m, H5″), 3.33 (3H, s, OCH_3), 1.81, 1.78 (2H, q, $J = 22.9$, CH_2), 0.92, 0.90, 0.88 (3H, t, $J = 14.7$, CH_3); FABMS (glycerol [1% TFA] and MeOH) *m/z* (MH^+) calcd for $C_{14}H_{20}N_4O_4S$ 341.128 35, found 341.128 75; purity 86% by NMR.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-1-propyl-6-thioinosine (18). Dimethoxytrityl chloride (0.65 g, 1.9 mmol) was added to a suspension of the starting material **17** (0.50 g, 1.5 mmol) in 20 mL of pyridine, and the reaction was carried out as generally described for **13c**. The isolated yield was 0.5 g (51.9%) of a light-brown foam. FABMS (glycerol [1% TFA] and MeOH) *m/z* (MH^+) calcd for $C_{35}H_{39}SN_4O_6$ 643.259 03, found 643.257 93; $R_f = 0.43$ ($CHCl_3$/MeOH, 95:5); $R_f = 0.60$ (EtOAC/hexanes, 9:1); single spot by TLC.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-1-propyl-6-thioinosine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosophoramidite (19). Product **18** (0.5 g, 0.80 mmol) in acetonitrile (15 mL) was treated as generally described for **14c** with 60 mg of diisopropylammonium tetrazolide salt and 0.5 g of 2-cyanoethyl tetraisopropylphophorodiamidite (1.61 mmol). The isolated yield was 0.4 g (59.4%) of a light-brown foam. ^{31}P NMR (DMSO-d_6) δ 150.71, 150.57; HRMS (glycerol) *m/z* (MH^+) calcd for $C_{44}H_{56}PSN_6O_7$ 843.366 88, found 843.368 46; $R_f = 0.69$, 0.62 ($CHCl_3$/MeOH, 95:5); $R_f = 0.5$, 0.39 (EtOAC/hexanes, 9:1); purity >95% by TLC.

2′-*O*-Methyl-1-propyl-6-thioinosinic Acid/2′-*O*-Methyl-1-propylinosinic Acid Copolymer 33-mer (20). 33-mer was synthesized as described for **16c** using both **14c** and **19** on a 1 μmol scale with an automated DNA synthesizer. Isolated yield was 6.2 mg (48.4%) of a fluffy light-orange solid. MALDI (M + 4Na) calcd 12 912, found 12 928; HPLC retention time 27.7 min (reverse-phase protocol); homogeneous by HPLC.

Degradation to Starting Nucleoside. To solutions of **9a**, **15a**−**c**, **16**, and **20** in 0.1 M NaCl (100 μL, 2 mg/mL) was added 65 μL of a solution comprising the following: 33 μL of Tris-HCl (2 M, pH 9.0), 44 μL of $MgCl_2$ (0.1 M), 44 μL of venom phosphodiesterase, and 22 μL of alkaline phosphatase. The solution was incubated at 37 °C with gentle rocking for 18 h and then diluted to 5 mL with NaCl (0.1 M). TLC in chloroform/methanol (85:15) confirmed degradation of **9a** to **6a** as the sole nucleoside product. TLC of the oligomers **15a**−**c**, **16**, and **20** confirmed degradation to their respective nucleoside precursors including the 3′-T residue.

Biological Assays. Anti-HIV. Southern Research Institute. CEM-SS cells passaged in T-75 flasks were split (1:2) 24 h prior to the time of infection. Just prior to infection, cells were washed twice with media, pelleted, and resuspend with media to a concentration of 5×10^4 cells per milliliter. Cell viability was evaluated by trypan blue exclusion, and any culture found to have less than 95% viability was discarded. For HIV analysis, an amount of 50 μL of the cell culture was added to drug-containing wells and to cell control wells in a 96-well plate.

For the standard HIV assay, HIV-1_{RF} particles initially acquired from the NIH AIDS Research and Reference Reagent Program were grown in CEM-SS cells for stock pools that were subsequently frozen at −80 °C. Just prior to infection, the particles were slowly thawed, suspended in media, and diluted to a concentration that would effect 85−95% cell kill in 6 days. For analysis, an amount of 50 μL was added to drug-containing wells and virus control wells in a 96-well plate. Additional tests with other virus strains are conducted by the same protocol just using the other particles for infection.

For HIV analysis, the test compound was brought up in 0.1 M NaCl, PBS, or H_2O to predetermined concentrations. The compound was aliquoted into wells at 0.32, 1.0, 3.2, 10, 32, and 100 μL. Drug was added to wells to provide duplicate measurements of cells + drug and drug + media and triplicate measurements of cells + drug + media at each drug concentration.

Cell viability was determined at the end of the testing period by staining with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS). Briefly, 20 μL of MTS was added to all wells and incubated overnight. The sealed plates were inverted several times, and dye absorbance was read at 490 nm with a plate reader. Calculations from the absorbance readings provided the percent cytopathic effect (CPE) reduction, percent cell viability, IC_{50}, and therapeutic concentration 50% (TC_{50}) values.

Anti-RT Assay. Southern Research Center. Reverse transcriptase activity was determined using generally the process described earlier by Boyer et al.[36]

Anti-HCMV Activity. Institute for Antiviral Research, Utah State University. Anti-HCMV assays were performed as generally described earlier.[37,38,39]

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