A 4′-*C*-Ethynyl-2′,3′-Dideoxynucleoside Analogue Highlights the Role of the 3′-OH in Anti-HIV Active 4′-*C*-Ethynyl-2′-deoxy Nucleosides

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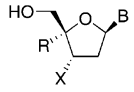
4′-*C*-Ethynyl-2′-deoxynucleosides belong to a novel class of nucleoside analogues endowed with potent activity against a wide spectrum of HIV viruses, including a variety of resistant clones. Although favorable selectivity indices were reported for several of these analogues, some concern still exists regarding the 3′-OH group and its role in cellular toxicity. To address this problem, we removed the 3′-OH group from 4′-*C*-ethynyl-2′-deoxycytidine (**1a**). This compound was chosen because of its combined high potency and low selectivity index. The removal of the 3′-OH was not straightforward; it required a different synthetic approach from the one used to synthesize the parent compound. Starting with glycidyl-4-methoxyphenyl ether, the target 4′-*C*-ethynyl-2′,3′-dideoxycytidine analogue (***rac*-1h**) was obtained after 13 steps. In a cellular assay, ***rac*-1h** was completely inactive (0.001−10 μM) against HIV_{LAI}, demonstrating the critical importance of the 3′-OH for antiviral activity. To determine whether the role of the 3′-OH was essential for the phosphorylation of the compound by cellular kinases or for inhibition of DNA polymerization, we synthesized and tested the 5′-triphosphate (***rac*-1h-TP**) for its ability to inhibit HIV reverse transcriptase (RT). ***rac*-1h-TP** was slightly more potent than AZT-5′-triphosphate against wild-type HIV RT, suggesting that the role of the 3′-OH is crucial only for the activation of the drug by cellular kinases. The lipase-catalyzed resolution of ***rac*-1h** into ***ent*-1h** (β-D-dideoxyribo) and ***ent*-14** (β-L-dideoxyribo) and the synthesis of the corresponding 5′-triphosphates established the stereochemical assignment based on HIV RT's preference for the β-D-enantiomer, which was confirmed by assaying against the M184V variant, an RT mutant with a marked preference for incorporating nucleosides in the D-configuration.

Introduction

4′-*C*-Ethynyl-2′-deoxy-β-D-nucleosides (**1a**−**e**) have been identified as some of the most potent analogues against HIV-1, including several multidrug-resistant strains and HIV-2.[1,2] Although no studies with the corresponding 5′-triphosphates have been performed to elucidate the exact mechanism of antiretroviral activity, the antiviral profile fits that of an NRTI with a mechanism of action similar to that of AZT. This means that 4′-*C*-ethynyl nucleosides suppress HIV replication at or around the step of reverse transcription. Indeed, the fact that the anti-HIV activity of the cytidine (**1a**) and guanosine (**1e**) analogues was reversed by the addition of their physiologic 2′-deoxynucleoside counterparts provides strong evidence that 4′-*C*-ethynyl-2′-deoxy-β-D-nucleosides serve as substrates for HIV RT.[2]



1a	R = C≡CH	X = OH	B = cytosine
1b	R = C≡CH	X = OH	B = 5-F-cytosine
1c	R = C≡CH	X = OH	B = adenine
1d	R = C≡CH	X = OH	B = 2,6-diaminopurine
1e	R = C≡CH	X = OH	B = guanine
1f	R = N_3	X = OH	B = thymine
1g	R = CN	X = OH	B = thymine
1h	R = C≡CH	X = H	B = cytosine

Previous reports of other 4′-substituted-2′-deoxy-β-D-nucleosides, such as 4′-azidothymidine (**1f**), have shown that following intracellular anabolism to the 5′-triphosphate, HIV reverse transcriptase (RT) efficiently incorporated the nucleotide, which prevented further chain elongation of the viral DNA.[3,4] Because these compounds bear no structural resemblance to traditional chain terminators, all of which lack the 3′-OH group, they must have a different mechanism of action. In the case of 4′-azidothymidine (**1f**), the rate of incorporation of the 5′-triphosphate by cellular polymerases

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was found to be dramatically different when compared to that of HIV RT. Although the rate of incorporation for the former was quite low, HIV RT was able to incorporate two consecutive molecules efficiently. The subsequent distortion of the growing primer brought about by this incorporation seems to prevent further DNA chain elongation, thus causing a delayed chain termination.[3,4] Other structurally similar compounds probably have comparable mechanisms of action, for example, 4′-cyanothymidine (**1g**) and the corresponding cytidine and uridine analogues, all of which are potent inhibitors of HIV replication in cell culture.[5] As a group, 4′-substituted-2′-deoxy-β-D-nucleosides are quite attractive because of the intriguing connection between their novel mechanism of action and their potent activity against multidrug-resistant (MDR) HIV-1 strains. Their efficacy against MDR strains may depend on two important factors: (1) delayed chain termination occurring beyond the polymerase active site and (2) difficulties in selecting mutants that discriminate against the incorporation of analogues that still retain the natural 3′-OH.[2]

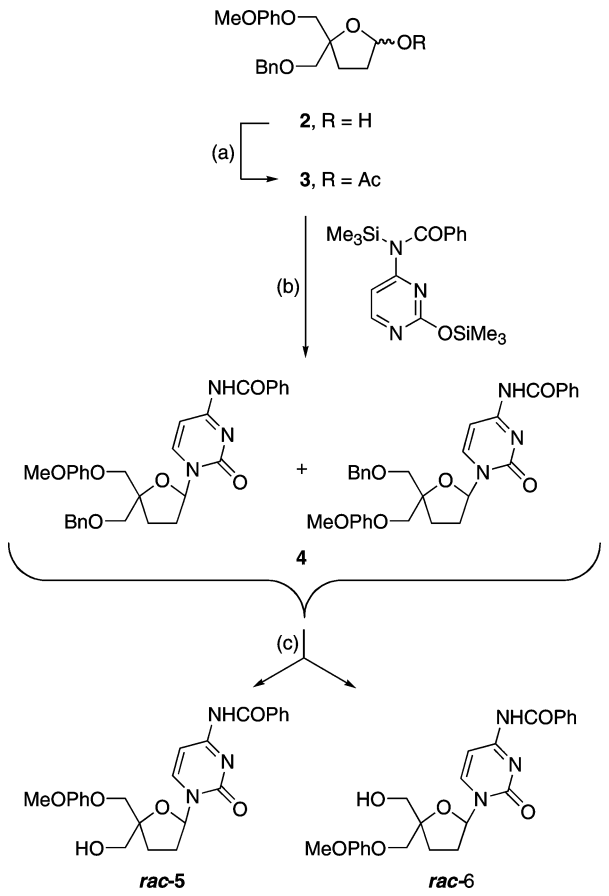
Extensive studies with other 4′-substituted-2′-deoxy-β-D-nucleosides with a thymine nucleobase and a variety of substituents, including ethyl, hydroxyethyl, and ethenyl, at the 4′-position demonstrated the superior potency of the ethynyl group.[6] Similarly, for compounds with a cytosine nucleobase, the ethynyl group as in **1a** was the most potent analogue although some host cellular toxicity was encountered.[7] Surprisingly, the 5-fluorocytosine analogue (**1b**) was less toxic and quite potent. Other important structural changes including the use of purine nucleobases, such as adenine (**1c**) and 2,6-diaminopurine (**1d**), produced compounds active against MDR HIV-1 that were less toxic than their counterparts with pyrimidine nucleobases.[1]

Because the 3′-OH in all of these 4′-substituted nucleosides could function as a key element for substrate recognition by cellular kinases and/or polymerases, we decided to investigate the importance of the 3′-OH by deleting it from the most attractive series of 4′-substituted nucleosides, the 4′-*C*-ethynyl-2′-deoxy-β-D-nucleosides. Previous studies have shown that removal of the 3′-OH from the 4′-azido (**1f**) and 4′-cyano (**1g**) analogues resulted either in a complete loss of activity or a significant reduction in potency.[5,8] However, with the structurally related 3′,4′-fused oxetane derivative with a thymine nucleobase, there was only a moderate drop in potency, suggesting that the ether oxygen could have partially fulfilled the role of the 3′-OH.[9] Despite the expected loss of activity similar to what was seen with the 4′-azido analogue (**1f**), removing the 3′-OH from 4′-*C*-ethynyl-2′-deoxy-β-D-nucleosides to generate a 4′-*C*-ethynyl-2′,3′-dideoxy-β-D-nucleoside, such as **1h**, provided the opportunity to assess the role of the 3′-OH during activation and polymerization and in generating cellular toxicity. Because the issue of toxicity was very important, cytosine analogue **1h** was selected to be the target because its progenitor **1a** combined potent activity with significant cellular toxicity.

Chemistry

The required 4-branched-2,3-dideoxysugar **2** was prepared according to a previously published method in which compound **2** was generated only as an intermediate and not fully characterized.[10] The procedure was repeated, and compound **2** was completely characterized. Acylation of the free hydroxyl group to give **3** followed by standard nucleoside synthesis with persilylated N^4-benzoylcytosine proceeded uneventfully to afford an inseparable mixture of two diastereoisomers (**4**, Scheme 1). The removal of the benzyl protecting group gave a separable mixture of ***rac*-5** and ***rac*-6**, which differ in terms of their relative stereochemistry. Because the correct stereochemical assignment was not clarified until the target compounds were reached (vide infra), the same chemical steps were performed separately on ***rac*-5** and ***rac*-6** but are shown only for one diastereoisomer (***rac*-5**, Scheme 2).

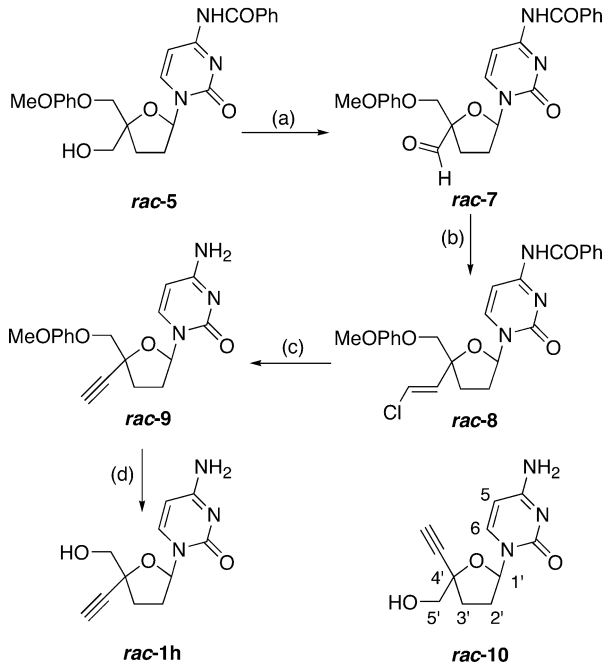
Scheme 1[a]



[a] (a) $(CH_3CO)_2O$/pyridine (86%); (b) TMSOTf, CH_2Cl_2 (100%); (c) BCl_3, CH_2Cl_2, −78 °C (***rac*-6**, 49%; ***rac*-5**, 25%).

The oxidation of the hydroxymethyl group to a formyl group (***rac*-7**) was followed by Wittig olefination with (chloromethyl)triphenylphosphonium chloride to afford chlorovinyl derivative ***rac*-8**. Without isolation, the chlorovinyl group was converted to the ethynyl group after treatment with *n*-butyllithium in tetrahydrofuran to give ***rac*-9**. Finally, deprotection of the *p*-methoxyphenyl group with ammonium cerium (IV) nitrate provided the desired target ***rac*-1h**. Following an identical approach from ***rac*-6** produced ***rac*-10**. At this stage, NMR nOe buildup data adequately determined the relative stereochemistry. In ***rac*-10**, the nOe build ups indicated a distance between H-5′a,b and H-1′ (nucleoside numbering) of 3.3 Å, whereas no nOe was observed between the same set of protons in ***rac*-1h**. Buildup

Scheme 2[a]



[a] (a) DCC, DMSO, 0 °C (93%); (b) $ClCH_2P(C_6H_5)_3Cl$, *n*-BuLi, THF, −78 °C; (c) *n*-BuLi, THF, −78 °C (48.5% two steps); (d) $(NH_4)_2Ce(NO_3)_6$, CH_3CN:H_2O, 0 °C (83%).

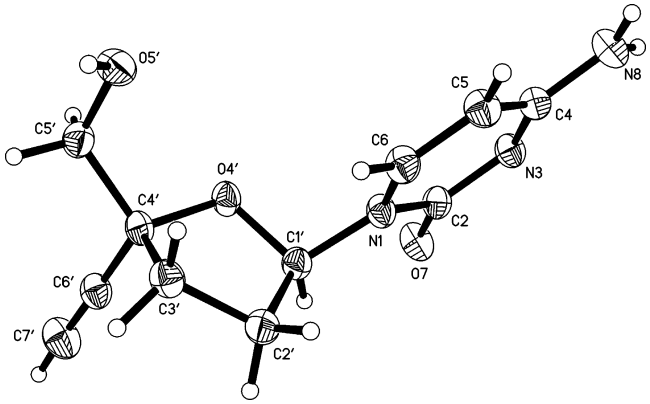
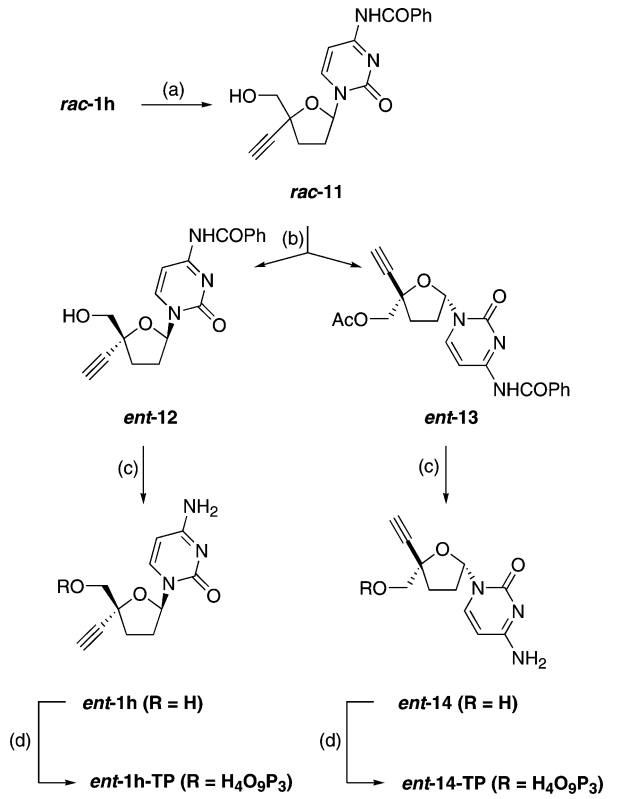


Figure 1. Displacement elliposoid plot of ***rac*-1h** drawn at the 30% probability level.

curves were obtained by irradiating the key target protons on both samples and using an internuclear distance of 2.4 Å between H-6 and H-5 (nucleoside numbering) as a reference for the calculations. During the NMR experiments, it was noticed that ***rac*-1h** crystallized readily and provided suitable crystals for X-ray analysis. As shown in Figure 1, the crystal structure of ***rac*-1h** corresponded to the desired (2*R*,5*R* or 2*S*,5*S*)-(±)-1-(2′,3′-dideoxy-4′-*C*-ethynyl-ribopentofuranosyl)cytosine.

At this point, a lipase-catalyzed approach was used to resolve ***rac*-1h**. To avoid complications with possible interference by the exocyclic amino group during trans esterification with vinyl acetate, which included acetylation or formation of a Schiff base with the released acetaldehyde, this group was protected as a benzoyl amide using the well-known transient protection method to give ***rac*-11** (Scheme 3).[11] Following a 10-day incubation of ***rac*-11** with lipase PS-C "Amano" and vinyl

Scheme 3[a]



[a] (a) i. TMSCl, pyridine, ii. PhCOCl, iii. NH_4OH (68.5%); (b) lipase PS−C Amano I, CH_2=CHOAc, $CCl_4/CHCl_3$ (***ent*-13**, 58%; ***ent*-12**, 40.5%) (c) satd NH_3/CH_3OH (70%); (d) performed by TriLink Biotechnologies, Inc., San Diego, CA (www.trilinkbiotech.com).

acetate, the less polar monoacetate (***ent*-13**) was obtained as a single enantiomer (91% ee) after flash column chromatography. The mixture of unreactive alcohols was then treated for a second time with the enzyme under the same conditions to give, after column chromatography, enantiomerically pure ***ent*-12** (98% ee). The removal of the N^4-benzoyl group from ***ent*-12** and the acetyl/N^4-benzoyl groups from ***ent*-13** gave the desired β-D-dideoxyribo targets: ***ent*-1h** and β-L-dideoxyribo enantiomer ***ent*-14**. This assignment was based on the capacity of HIV-1 RT to discriminate between the enantiomers (vide infra) and, more recently, on the synthesis of the authentic β-D-dideoxyribo enantiomer (***ent*-1h**) obtained from a chiral lactone precursor to **2**[12] using a method similar to that shown in Schemes 1 and 2. The synthesis of the corresponding 5′-triphosphates, ***ent*-1h-TP** (β-D-dideoxyribo) and ***ent*-14-TP** (β-L-dideoxyribo), was performed by conventional methods. The 5′-triphosphate of the racemate, ***rac*-1h-TP**, was also synthesized as a reference.

Biological Activity

Relative to 4′-*C*-ethynyl-2′-deoxycytidine (**1a**), dideoxy analogue ***rac*-1h** was inactive in vitro against HIV_{LAI} when tested in a concentration range between 0.001 and 10 μM in MT-2 and MT-4 cells. Under the same experimental conditions, control AZT had an IC_{50} of 0.014 μM. These results underscore the critical role of the 3′-OH group for anti-HIV activity in a cellular system. To determine whether the role of the 3′-OH was crucial for the activation step(s) or for the inhibition of

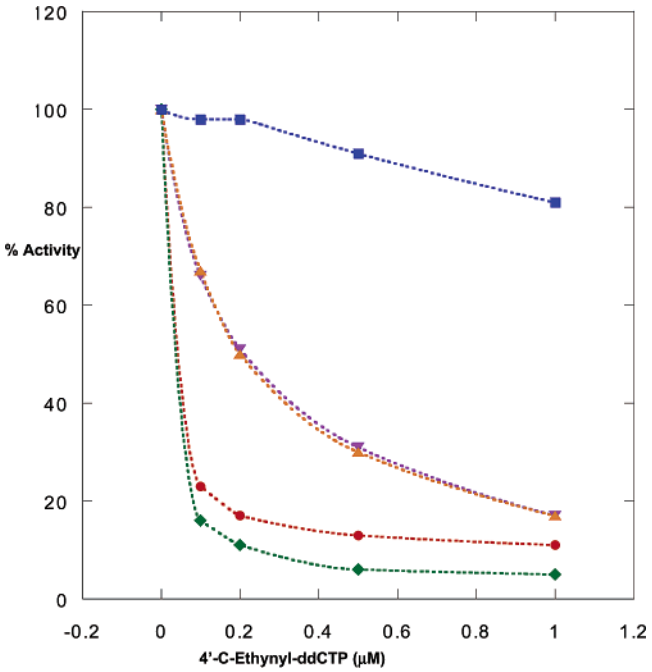


Figure 2. Inhibition of HIV RT wild type and M184V mutant strains by racemic (***rac*-1hTP**) and enantiomeric (***ent*-1hTP** and ***ent*-14TP**) 5′-triphosphates of 1-(2′,3′-dideoxy-4-*C*-ethynyl-ribofuranosyl)cytosine: ***rac*-1h-TP** (wild type = ●); ***ent*-1h-TP** (wild type = ◆); ***ent*-1h-TP** (M184V = ▼); ***ent*-14-TP** (wild type = ▲); ***ent*-14-TP** (M184V = ■).

HIV-1 viral DNA polymerization, we synthesized and tested the 5′-triphosphate (***rac*-1h-TP**) for its ability to inhibit HIV RT. Against wild-type HIV-1 RT, using a single-stranded M13mp18 DNA template and a −47 sequencing primer, ***rac*-1h-TP** was slightly more potent than AZT-5′-triphosphate (not shown), suggesting that the 3′-OH plays a critical role in the activation of the drug by cellular kinases (Figure 2, ●). We expected the enzyme to discriminate between enantiomers ***ent*-1h-TP** (*β*-D-dideoxyribo) and ***ent*-14-TP** (*β*-L-dideoxyribo) on the basis of calculations from the molecular docking of the 5′-triphosphates into the active site of HIV RT (vide infra), which predicted that the *β*-D-dideoxyribo enantiomer (***ent*-1h-TP**) would be the more active isomer. Indeed, the enantiomer presumed to be the *β*-D-dideoxyribo enantiomer (***ent*-1h-TP**) was more potent in blocking DNA synthesis by wild-type HIV-1 RT than its antipode ***ent*-14-TP** (Figure 2, ◆ versus ▲). The fact that the M184V mutant of HIV-1 RT has a greater selectivity against *β*-L-nucleosides than does wild type[13−16] also confirmed the assignment of D- and D-enantiomers: the *β*-L-dideoxyribo enantiomer (***ent*-14-TP**) was virtually inactive (Figure 2, ■), whereas the *β*-D-dideoxyribo enantiomer (***ent*-1h-TP**) strongly inhibited the M184 mutant RT despite a low level of resistance (Figure 2, ▼). This low level of resistance of M184V for the *β*-D-dideoxyribo (***ent*-1h-TP**) enantiomer is not surprising because M184V has been reported to show a low level of resistance to ddCTP.[17,18]

In light of a recent investigation reporting that the L-enantiomer of **1a** is inactive against the HIV virus in cell culture, [19] it is interesting that ***ent*-14-TP** is active against wild-type HIV-1 RT. In fact, the level of potency of ***ent*-14-TP** against wild-type HIV-1 RT is identical to that of ***ent*-1h-TP** against the M184V mutant (Figure 2, ▲ versus ▼). On the basis of these results, it would appear that the 3′-OH of the *β*-D enantiomer of **1a** is critical for phosphorylation by cellular kinases, but in the L-enantiomer, it must play a negative role by occupying an unfavorable position in the active site of the kinase. The removal of the 3′-OH from either enantiomer produces inactive compounds in cell-based assays, but to a varying degree, both 5′-triphosphates seem to be acceptable substrates for HIV-1 RT. According to a recent report, the combined removal of the 3′-OH and the flattening of the dideoxyribose ring by a 2′,3′ double bond restores anti-HIV activity in MT-2 cells.[20] This finding seems to be consistent with our own observations correlating a reduced puckering amplitude with substrate recognition by both cellular kinases and polymerases.[21]

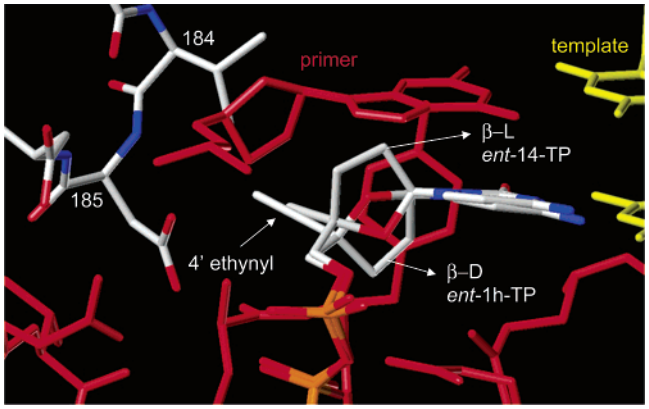


Figure 3. Superposition of ***ent*-1h-TP** and ***ent*-14-TP** at the active site of HIV RT showing a measurable deviation of the 4′-ethynyl moiety.

Crystal Structure and Docking of 5′-Triphosphates at the Active Site of HIV RT

On the basis of the torsion angles obtained from the crystal structure (Figure 1), the dideoxyribose moiety of ***rac*-1h** has a *P* value of 43.49° in the pseudorotational cycle, almost exactly in the middle of a $^{3}T_{4}$ ($P = 36°$) and 4′-*exo* ($_{4}E$, $P = 54°$) conformations. This is clearly in the Northern Hemisphere but slightly beyond the range that is observed for most conventional nucleosides (0−36°). The molecule also has a highly puckered ring with a ν_{max} of 37.10°. The X-ray coordinates of ***rac*-1h** were used to construct the 5′-triphosphates of ***ent*-1h-TP** and ***ent*-14-TP** for computer docking studies with HIV RT. When the two structures are superposed at the polymerase site of an HIV RT/DNA/nucleotide ternary complex, there is a measurable deviation in the direction of the 4′-ethynyl moiety between the two enantiomers (Figure 3). The D enantiomer (***ent*-1h-TP**) appears to fit nicely at the active site of wild-type HIV RT (Figure 4A), and consistent with the low level of resistance observed against the M184V mutant (Figure 2, ▼), the docking in Figure 4C shows some negative steric interaction with Val184. The situation with the L enantiomer (***ent*-14-TP**) would indicate that relative to ***ent*-1h-TP** the fit with respect to Met184 is somewhat tighter (Figure 4B), plus there could be some steric interactions in wild-type HIV RT with Tyr115 and/or Ala114, or with both (Figure 5). It is possible that the steric interactions of the L enantiomer with these amino acids could be alleviated either by a torsional rotation

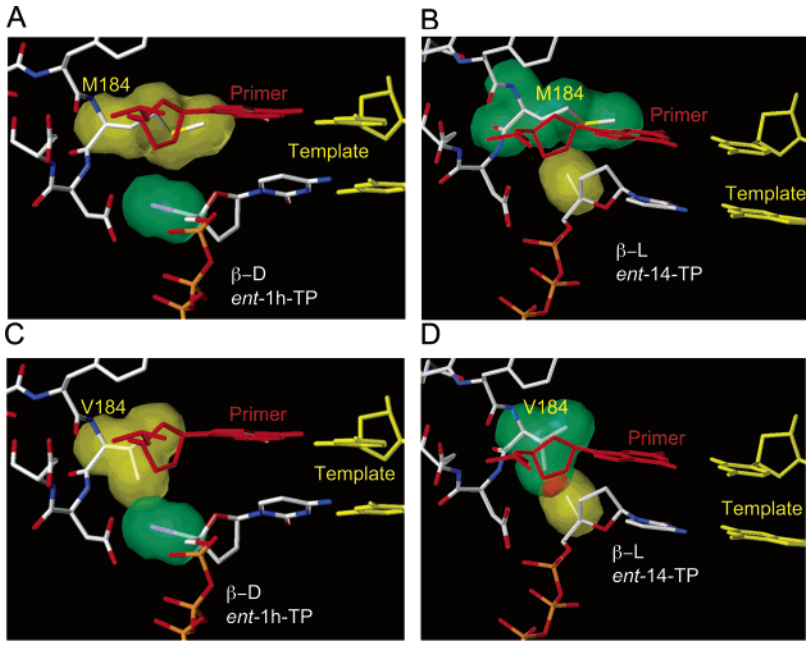


Figure 4. (A) *β*-D-enantiomer (***ent*-1h-TP**) at the active site of wild-type HIV RT. Volumes for the 4′-ethynyl group (green) and methionine184 (yellow). (B) *β*-L-enantiomer (***ent*-14-TP**) at the active site of wild-type HIV RT. Volumes for the 4′-ethynyl group (yellow) and methionine184 (green). (C) *β*-D-enantiomer (***ent*-1h-TP**) at the active site of M184V HIV RT. Volumes for the 4′-ethynyl group (green) and methionine184 (yellow). (D) *β*-L-enantiomer (***ent*-14-TP**) at the active site of M184V HIV RT. Volumes for the 4′-ethynyl group (yellow), methionine184 (green), and steric clash (orange).

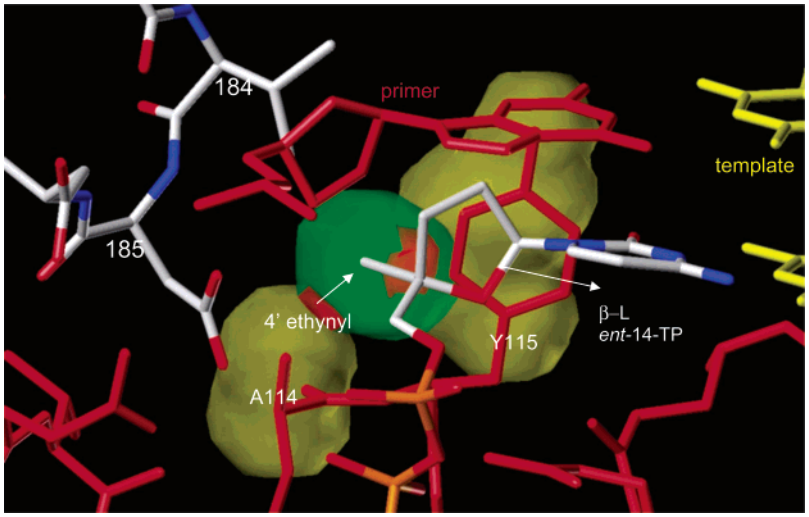


Figure 5. *β*-L-enantiomer (***ent*-14-TP**) at the active site of wild-type HIV RT. Volumes for the 4′-ethynyl group (green), alanine and tyrosine (yellow), and steric clash (orange).

of Tyr115 or a small rearrangement of the L nucleotide, explaining the lower level of potency observed for this enantiomer against wild-type HIV-1 RT (Figure 2, ▲). However, it is clear that for the M184V mutant the steric clash between the Cγ of the *β*-branched valine with the 4′-ethynyl group of ***ent*-14-TP** (Figure 4D) and possibly with the L-dideoxy ring (not shown) would prevent the L enantiomer from binding to the active site in a productive manner, explaining its lack of activity (Figure 2, ■).

Discussion

The inactivity of 1-(2′,3′-dideoxy-4′-*C*-ethynyl-ribopentofuranosyl)cytosine (***rac*-1h)** as an inhibitor of HIV replication in cultured cells highlights the importance of the 3′-OH as a key determinant for activity for the 4′-*C*-ethynyl-2′-deoxy-*β*-D-nucleosides and, by extension, for other 4′-substituted 2′-deoxynucleosides that are active against HIV (vide supra). The potent inhibition of HIV-1 RT by the 5′-triphosphate (***rac*-1h-TP**) confirms that the failure of this agent to exert good anti-HIV activity in the cell culture results from the failure of the cellular kinases to generate the requisite 5′-triphosphate. In the present work, the racemate 1-(2′,3′-dideoxy-4′-*C*-ethynyl-ribopentofuranosyl)cytosine (***rac*-1h**) was resolved into its components, *β*-D-dideoxyribo (***ent*-1h**) and *β*-L-dideoxyribo (***ent*-14**), by a lipase-catalyzed reaction. The assignment of the absolute stereochemistry was confirmed by HIV RT's preference for the natural *β*-D-configuration. This assignment was confirmed using the M184V mutant, which has a much greater preference for the D enantiomers. The synthesis of authentic ***ent*-1h** also confirmed this assignment because its 5′-triphosphate showed identical activity as

the β-D-dideoxyribo isomer separated by the lipase-catalyzed resolution (data not shown). The results presented here suggest, on the basis of the strong anti-HIV activity of the 5′-triphosphate, ***ent*-1h-TP**, that the 2′,3′-dideoxy-4′-*C*-ethynyl-β-D-ribopentofuranosyl nucleoside template is an attractive scaffold for the development of prodrugs capable of bypassing the first kinase. These types of compounds may also offer an additional advantage of having reduced toxicity because the lack of 3′-OH prevents them from being good substrates for cellular polymerases.

General Experimental Section

All chemical reagents were commercially available. Column chromatography was performed on silica gel 60, 230−240 mesh (E. Merck), and analytical TLC was performed on Analtech Uniplates silica gel GF. Routine IR and ^{1}H NMR spectra were recorded using standard methods. Positive-ion fast-bombardment mass spectra (FABMS) were obtained on a VG 7070E mass spectrometer at an accelerating voltage of 6 kV and a resolution of 2000. Glycerol was used as the sample matrix, and ionization was effected by a beam of xenon atoms. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA.

5-[(4-Methoxyphenoxy)methyl]-5-(phenylmethoxy)-methyl]oxolan-2-ol (2). This compound was reported in ref 10 but was not full characterized. We followed the same procedure and obtained **2** as an oil (mixture of anomers): ^{1}H NMR ($CDCl_3$) δ 7.30 (m, 5 H, PhH), 6.80 (m, 4 H, ArH), 5.51 (irregular t, 0.5 H, H-2), 5.46 (dd, J = 7.5, 4.2 Hz, 0.5 H, H-2), 4.60 (AB q, J = 11.8 Hz, 1 H, PhCH_2O), 4.52 (AB q, J = 12.3 Hz, 1 H, PhCH_2O), 3.80 (AB q, J = 9.2 Hz, 1 H, BnOCH_2 or ArOCH_2), 3.74 (s, 3 H, OCH_3), 3.64 (AB d, J = 1.0 Hz, 1 H, BnOCH_2 or ArOCH_2), 3.58 (d, J = 7.5 Hz, 0.5 H, BnOCH_2 or ArOCH_2), 3.46 (AB q, J = 9.5 Hz, 1 H, BnOCH_2 or ArOCH_2), 3.12 (d, J = 7.5 Hz, 0.5 H, BnOCH_2 or ArOCH_2), 1.90−2.20 (m, 4 H, H-3$_{a,b}$ and H-4$_{a,b}$); FAB-MS (relative intensity) 344 ($M^{+\bullet}$, 89). Anal. Calcd for $C_{20}H_{24}O_5$: C, 69.75; H, 7.02. Found: C, 69.60; H, 7.06.

5-[(4-Methoxyphenoxy)methyl]-5-[(phenylmethoxy)-methyl]oxolan-2-yl acetate (3). A solution of lactol **2** (6.10 g, 17.7 mmol) in dry pyridine (90 mL) was treated with acetic anhydride (4.2 mL, 44.4 mmol). After stirring at room temperature for 72 h, the reaction mixture was diluted with EtOAc (200 mL), washed with water (3 × 100 mL), dried ($MgSO_4$), and concentrated under vacuum. The crude residue was purified by flash column chromatography on silica gel using EtOAc/hexanes (1:6 → 1:3) to give 5.93 g (86.7%) of **3** as a clear oil (mixture of anomers); ^{1}H NMR ($CDCl_3$) δ 7.25 (m, 5 H, PhH), 6.80 (m, 4 H, ArH), 6.32 (m, 1 H, H-2), 4.50−4.60 (overlapping s and AB q, J = 12.4 Hz, 2 H, PhCH_2O), 3.80−4.00 (2 AB q, J = 9.4 Hz, 2 H, BnOCH_2 or ArOCH_2), 3.73 and 3.74 (s, 3 H, OCH_3), 3.48−3.62 (overlapping s and AB q, J = 9.8 Hz, 2 H, BnOCH_2 or ArOCH_2), 1.90−2.30 (m, 4 H, H-3$_{a,b}$ and H-4$_{a,b}$), 1.93 and 1.94 (s, 3 H, $OCOCH_3$); FAB-MS (relative intensity) 387 (MH^+, 25), 386 ($M^{+\bullet}$, 100). Anal. Calcd for $C_{22}H_{26}O_6$: C, 68.37; H, 6.78. Found: C, 68.38; H, 6.77.

***N*-(1-{5-[(4-Methoxyphenoxy)methyl]-5-[(phenyl-methoxy)methyl]oxolan-2-yl}-2-oxohydropyrimidin-4-yl)benzamide (4).** N^4-Benzoylcytosine (5.30 g, 24.6 mmol) was suspended in dry CH_3CN (20 mL) and was treated with bis-(trimethylsilyl)trifluoroacetamide (BSTFA, 20 mL, 75.3 mmol) under argon. After 1 h of stirring, the reaction mixture became homogeneous. The volatiles were removed under vacuum to give the silylated base as a thick oil. Separately, a solution of acetate **3** (6.29 g, 16.3 mmol) in dry $ClCH_2CH_2Cl$ (100 mL) was added to the silylated base followed by the slow addition of trimethylsilyl trifluoromethanesulfonate (TMSOTf, 4.8 mL, 26.4 mmol). The reaction mixture was stirred at room temperature for 20 h and was diluted with CH_2Cl_2 (100 mL). After cooling to 0 °C, the reaction was quenched by the addition of a saturated solution of $NaHCO_3$. The suspension that was obtained was filtered, and the organic layer was separated, dried ($MgSO_4$), and concentrated under vacuum. The crude product that was obtained was purified by flash chromatography on silica gel using EtOAc/hexanes (1:1 → pure EtOAc) to give a quantitative yield of a yellowish foam consisting of an inseparable mixture of two diastereoisomers (**4**). This material was carried out as a mixture in the following debenzylation step.

(2*R*,5*R* or 2*S*,5*S*)-*N*-(1-{5-(Hydroxymethyl)-5-[(methoxy-phenoxy)methyl]oxolan-2-yl}-2-oxohydropyrimidin-4-yl)benzamide (*rac*-5) and (2*S*,5*R* or 2*R*,5*S*)-*N*-(1-{5-(Hydroxymethyl)-5-[(methoxyphenoxy)methyl]oxolan-2-yl}-2-oxohydropyrimidin-4-yl)benzamide (*rac*-6). The inseparable mixture of diastereoisomers **4** (4.0 g, 7.38 mmol) was dissolved in anhydrous CH_2Cl_2 (40 mL), cooled to −78 °C, and treated with a solution of BCl_3 in CH_2Cl_2 (1 M, 25 mL). After stirring for 3 h, the reaction was quenched with MeOH (5 mL) and warmed to room temperature. All of the volatiles were removed under vacuum, and the residue was coevaporated three times with MeOH (10 mL). The crude product was purified by flash chromatography on silica gel using EtOAc/hexanes (2:1 → pure EtOAc) to give diastereoisomer ***rac*-5** (0.75 g, 25% yield from **3**) and diastereoisomer ***rac*-6** (1.43 g, 49% yield from **3**) as foams.

Diastereoisomer ***rac*-5**: ^{1}H NMR ($CDCl_3$) δ 8.55 (d, J = 7.6 Hz, 1 H, H-6), 7.50−8.10 (m, 6 H, H-5, PhH), 6.95 (br s, 4 H, ArH), 6.35 (m, 1 H, H-2′), 4.37 (d, J = 10.1 Hz, 1 H, HOCH_2 or ArOCH_2), 4.11 (d, J = 10.1 Hz, 1 H, HOCH_2 or ArOCH_2), 3.90 (s, 3 H, OCH_3), 3.75 (s, 2 H, HOCH_2 or ArOCH_2), 2.85 (m, 1 H, H-3′$_a$ or H-4′$_a$), 2.30 (m, 2 H, H-3′$_{a,b}$ or H-4′$_{a,b}$), 2.10 (m, 1 H, H-3′$_b$ or H-4′$_b$); FAB-MS (relative intensity) 452 (MH^+, 17), 451 ($M^{+\bullet}$, 15), 216 (bH_2^+, 100).

Diastereoisomer ***rac*-6**: ^{1}H NMR ($CDCl_3$) δ 8.38 (d, J = 7.4 Hz, 1 H, H-6), 7.40−7.92 (m, 6 H, H-5, PhH), 6.80 (br s, 4 H, ArH), 6.35 (dd, J = 6.6, 4.3 Hz, 1 H, H-2′), 3.99 (d, J = 11.7 Hz, 1 H, HOCH_2 or ArOCH_2), 3.91 (s, 2 H, HOCH_2 or ArOCH_2), 3.86 (d, J = 11.7 Hz, 1 H, HOCH_2 or ArOCH_2), 3.80 (s, 3 H, OCH_3), 2.80 (m, 1 H, H-3′$_a$ or H-4′$_a$), 2.21 (m, 2 H, H-3′$_{a,b}$ or H-4′$_{a,b}$), 2.15 (m, 1 H, H-3′$_b$ or H-4′$_b$); FAB-MS (relative intensity) 452 (MH^+, 17), 451 ($M^{+\bullet}$, 15), 216 (bH_2^+, 100).

(2*R*,5*R* or 2*S*,5*S*)-6-Amino-3-{5-ethynyl-5-[(4-methoxy-phenoxy)methyl]oxolan-2-yl}-3-hydropyrimidin-2-one (*rac*-9). A solution of ***rac*-5** (0.74 g, 1.63 mmol) and 1,3-dicyclohexylcarbodiimide (DCC, 1.02 g, 4.93 mmol) in anhydrous DMSO (40 mL) was cooled to 0 °C and treated with dichloroacetic acid (0.08 mL, 0.58 mmol). The resulting solution was stirred for 1.5 h while allowing it to reach room temperature. The precipitate formed (dicyclohexylurea, DCU) was filtered off and washed with EtOAc (50 mL), and the combined filtrate was diluted with EtOAc (200 mL) and extracted with water (3 × 100 mL) and brine (2 × 100 mL). The organic layer was separated, dried ($MgSO_4$), and concentrated under vacuum. The residue was purified by flash chromatography on silica gel using EtOAc followed by CH_2Cl_2/MeOH (40:1 → 20:1) to give 0.68 g (93.1%) of aldehyde ***rac*-7**. Thin-layer chromatography on silica gel using $CHCl_3$/*n*-BuOH/acetone (40:25:7.5) revealed some contamination with DCU, and the product was used immediately in the following reaction. A suspension of (chloromethyl)triphenylphosphonium chloride (2.15 g, 6.19 mmol) in anhydrous THF (50 mL) was cooled to −78 °C and treated with a solution of *n*-BuLi in hexanes (1.6 M, 4.0 mL). After 1 h of stirring, a solution of aldehyde ***rac*-7** (0.68 g, 1.51 mmol) in dry THF (50 mL) was added. The temperature of the bath was raised to 0 °C, and stirring continued for 3 h. Following the cautious addition of an aqueous saturated solution of H_4NCl (10 mL), the reaction mixture was extracted with EtOAc (2 × 100 mL). The combined organic layer was washed with brine (2 × 75 mL), dried ($MgSO_4$), and concentrated under vacuum. The crude product (***rac*-8**) was still contaminated with DCU and triphenylphosphine oxide. Hence, it was used as such for the following reaction. Crude ***rac*-8** was dissolved in anhydrous THF (40 mL), cooled to −78 °C, and treated slowly with a solution of *n*-BuLi in hexanes (1.6 M, 20 mL). After stirring

for 2 h, the reaction was quenched by the cautious addition of an aqueous saturated solution of H_4NCl (20 mL). The organic layer was washed with brine (2 × 75 mL), dried ($MgSO_4$), and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel using CH_2Cl_2/MeOH (40:1 → 10:1) to give ***rac*-9** (0.271 g, 48.5% yield from ***rac*-5**) as a yellowish foam; ^{1}H NMR ($CDCl_3$) δ 8.04 (d, J = 7.8 Hz, 1 H, H-4), 6.80 (m, 4 H, ArH), 6.18 (dd, J = 6.6, 3.1 Hz, H-2′), 6.08 (d, J = 7.8 Hz, 1 H, H-5), 4.37 (d, J = 10.9 Hz, 1 H, ArOCH_2), 4.10 (d, J = 10.9 Hz, 1 H, ArOCH_2), 3.80 (s, 3 H, OCH_3), 2.80 (m, 1 H, 1 H, H-3′$_a$ or H-4′$_a$), 2.60 (s, 1 H, CC*H*), 2.46 (m, 1 H, 1 H, H-3′$_b$ or H-4′$_b$), 1.98 (m, 2 H, H-3′$_{a,b}$ or H-4′$_{a,b}$); FAB-MS (relative intensity) 342 (MH^+, 40), 112 (bH_2^+, 100). Anal. Calcd for $C_{18}H_{19}N_3O_4$·0.25CH_3OH: C, 62.73; H, 5.76; N, 12.03. Found: C, 63.05; H, 5.73; N, 11.63.

(2*R*,5*R* or 2*S*,5*S*)-6-Amino-3-[5-ethynyl-5-(hydroxymethyl)oxolan-2-yl]-3-hydropyrimidin-2-one [(±)-1-(2′,3′-dideoxy-4-*C*-ethynyl-ribo-pentofuranosyl)cytosine] (*rac*-1h). A stirred solution of ***rac*-9** (0.261 g, 0.76 mmol) in acetonitrile/water (4:1, 24 mL) was cooled to 0 °C and treated with ammonium cerium (IV) nitrate (1.38 g, 2.51 mmol) for 2.5 h. The solvent was removed under vacuum, and the residue was coevaporated with MeOH (3 × 10 mL). The crude product obtained was purified once by flash chromatography on silica gel with CH_2Cl_2/MeOH (10:1 → 3:1) and twice by reverse-phase C-18 column chromatography eluting first with water and then with MeOH/water (15:1 → 5:1). The combined fractions containing the product were evaporated under vacuum and then lyophilized to give 0.148 g (83%) of product ***rac*-1h**. Recrystallization from MeOH/ether provided a crystalline solid, mp 166−167 °C; ^{1}H NMR (D_2O) δ 7.66 (d, J = 7.61 Hz, 1 H, H-4), 6.80 (dd, J = 7.5, 3.5 Hz, 1 H, H-2′), 5.90 (d, J = 7.5 Hz, 1 H, H-5), 3.70 (AB q, J = 12.3 Hz, 2 H, HOCH_2), 2.88 (s, 1 H, CC*H*), 2.58 (m, 1 H, H-3′$_a$ or H-4′$_a$), 2.05 (m, 3 H, H-3′$_b$ or H-4′$_b$ plus H-3′$_{a,b}$ or H-4′$_{a,b}$); FAB-MS (relative intensity) 236 (MH^+, 35), 112 (bH_2^+, 100); HRMS (FAB) calcd for $C_{11}H_{14}O_3N_3$: 236.1035. Found: 236.1031.

(2*S*,5*R* or 2*R*,5*S*)-6-Amino-3-[5-ethynyl-5-(hydroxymethyl)oxolan-2-yl]-3-hydropyrimidin-2-one [(±)-1-(2′,3′-dideoxy-4-*C*-ethynyl-ribo-pentofuranosyl)cytosine] (*rac*-10). This compound was obtained following the same procedure as described for the other diastereoisomer ***rac*-1h**: ^{1}H NMR (D_2O) δ 7.86 (d, J = 8.0 Hz, 1 H, H-4), 6.04 (m, 1 H, H-2′), 5.96 (d, J = 7.4 Hz, 1 H, H-5), 3.57 (AB q, J = 11.7 Hz, 2 H, HOCH_2), 3.00 (s, 1 H, CC*H*), 2.45 (m, 1 H, H-3′$_a$ or H-4′$_a$), 2.14 (m, 3 H, H-3′$_b$ or H-4′$_b$ plus H-3′$_{a,b}$ or H-4′$_{a,b}$); FAB-MS (relative intensity) 236 (MH^+, 73), 112 (bH_2^+, 100); HRMS (FAB) calcd for $C_{11}H_{14}O_3N_3$: 236.1035. Found: 236.1034.

(2*R*,5*R* or 2*S*,5*S*)-*N*-{1-[5-Ethynyl)-5-(hydroxymethyl)-oxolan-2-yl]-2-oxohydropyrimidin-4-yl}benzamide (*rac*-11). A solution of ***rac*-1h** (0.085 g, 0.361 mmol) in anhydrous pyridine (15 mL) was treated with chlorotrimethylsilane (TMSCl, 0.35 mL, 2.57 mmol) and stirred at room temperature for 20 min. Benzoyl chloride (0.23 mL, 1.93 mmol) was added, and after 2 h, the reaction was cooled to 0 °C and quenched with water (2 mL). After stirring for 15 min, concentrated NH_4-OH (5 mL) was added, the ice bath was removed, and the solution was left at room temperature for 1 h. All volatiles were removed under vacuum, and the residue was coevaporated three times with MeOH (10 mL). The final residue was purified first by flash column chromatography on silica gel (EtOAc/hexanes, 2:1 → EtOAc) followed by a second purification through a C-18 reversed-phase column (water and water/MeOH, 6:1 → 1:4) to give 0.084 g (68.5%) of ***rac*-11** as a foam; ^{1}H NMR ($CDCl_3$) δ 8.25 (d, J = 7.4 Hz, 1 H, H-6), 7.82 (d, J = 7.6 Hz, 1 H, H-5), 7.40−7.60 (m, 5 H, PhH), 6.14 (dd, J = 7.0, 3.2 Hz, 1 H, H-2′), 4.01 (d, J = 11.8 Hz, 1 H, CH_2OH), 3.80 (d, J = 11.8 Hz, 1 H, CH_2OH), 2.78 (m, 1 H, H-3′$_a$ or H-4′$_a$), 2.50 (s, 1 H, CC*H*), 2.30 (m, 1 H, H-3′$_b$ or H-4′$_b$), 2.20 (m, 1 H, H-4′$_a$ or H-3′$_a$), 2.80 (m, 1 H, H-4′$_b$ or H-3′$_b$).

Lipase-Catalyzed Resolution. A solution of ***rac*-11** (0.065 g, 0.191 mmol) in CCl_4/$CHCl_3$ (1:1, 20 mL) was treated with vinyl acetate (1 mL, 10.84 mmol) and lipase PS-C Amano I (0.190 g). The mixture was stirred at room temperature for 10 days. The enzyme was removed by filtration through a pad of Celite, and the solid cake was washed with the same solvent mixture of CCl_4 and $CHCl_3$. The combined filtrate was concentrated under reduced pressure, and the residue was purified by flash column chromatography on silica gel ($CHCl_2$/MeOH, 40:1 → 20:1) to give 0.043 g (58%) of the less polar monoacetate, ***ent*-13** (91% ee, chiral HPLC, RT = 22.38 min), and a mixture of the more polar unreactive alcohols. The mixture of unreactive alcohols was treated a second time under the same conditions in CCl_4/$CHCl_3$ (1:1, 10 mL) with the enzyme (0.10 g) and vinyl acetate (1 mL, 10.84 mmol). After flash column chromatography on silica gel under the same chromatographic conditions, 0.030 g of ***ent*-12** (98% ee, chiral HPLC, RT = 14.32 min) was obtained.

***ent*-13. (2*S*,5*S*)-*N*-{1-[5-Ethynyl)-5-(acetoxymethyl)oxolan-2-yl]-2-oxohydropyrimidin-4-yl}benzamide.** ^{1}H NMR ($CDCl_3$) δ 8.10 (d, J = 7.2 Hz, 1 H, H-6), 7.40−7.84 (m, 6 H, H-5, Ph), 6.16 (dd, J = 6.8, 2.6 Hz, 1 H, H-2′), 4.46 (d, J = 12.1 Hz, 1 H, CH_2OH), 4.34 (d, J = 12.1 Hz, 1 H, CH_2OH), 2.84 (m, 1 H, H-3′$_a$ or H-3′$_b$), 2.54 (s, 1 H, CC*H*), 2.00−2.20 (m, 3 H, H-3′$_a$ or H-3′$_b$ and H-4′$_{a,b}$), 2.10 (s, 3 H, CH_3CO).

***ent*-12: (2*R*,5*R*)-*N*-{1-[5-Ethynyl)-5-(hydroxymethyl)-oxolan-2-yl]-2-oxohydropyrimidin-4-yl}benzamide.** The ^{1}H NMR of this compound was identical to that of ***rac*-11**.

General Procedure for the Deacylation of *ent*-12 and *ent*-13. The compounds were suspended in a saturated methanolic ammonia solution and kept at room temperature in a sealed tube for 5 days. The solution was concentrated under vacuum, and the solid obtained was purified first by flash column chromatography on silica gel (CH_2Cl_2/MeOH, 10:1 → 6:1) to give a 70% yield of the final deblocked materials (***ent*-1h** and ***ent*-14**) as white foams. Both ^{1}H NMR spectra were identical to that of ***rac*-1h**. ***ent*-1h**: [a]23D + 45.84 (c 0.59, H_2O). Anal. Calcd for $C_{11}H_{13}N_3O_3$·0.33 H_2O: C, 54.76; H, 5.70; N, 17.41. Found: C, 54.52; H, 5.60; N, 17.33.

Chiral HPLC Chromatography. Chiralcel OD column with hexanes/isopropyl alcohol (1:1) as the eluant; injection volume 10 μL); flow rate 3.0 mL/min; UV detection at 220 nm.

Syntheses of 5′-Triphosphates of *rac*-1h, *ent*-1h, and *ent*-14. The custom syntheses of these 5′-triphosphates were performed by TriLink Biotechnologies, Inc. using conventional methodology. ***rac*-1h-TP** was 95.4% pure by HPLC and >95% pure by ^{31}P NMR; ***ent*-1h-TP** was 97.7% pure by HPLC and >95% pure by ^{31}P NMR; ***ent*-14-TP** was 97.08% pure by HPLC and > 5% by ^{31}P NMR. Additional information is provided as Supporting Information.

nOe Experiments. nOe buildups were not quantitative because the samples were not degassed, but the qualitative buildup data was adequate to determine the relative stereochemistry. In ***rac*-10**, the nOe data gave a distance between H-5′a,b and H-1′ (nucleoside numbering) of 3.3 Å, whereas no nOe was observed between the same set of protons in compound ***rac*-1h**. nOe buildup curves were obtained by irradiating the key target protons on both samples. An internuclear distance of 2.4 Å for the distance from H-6 to H-5 (nucleoside numbering) was used as a reference for the calculations. Data points from the integrated nOe peaks were all normalized to 100% inversion of the target before the distance calculation was carried out.

Single-Crystal X-ray Diffraction Analysis of *rac*-1h. $C_{11}H_{13}N_3O_3$·H_2O, fw = 253.26, triclinic P-1, a = 5.535(1) Å, b = 7.358(1) Å, c = 15.176(4) Å. V = 593.6(1) Å^3, Z = 2, ρ_{calcd} = 1.414 Mg/m^3, λ(Cu Kα) = 1.54178 Å, μ = 0.921 mm^{-1}, F (000) = 268, T = 293(2) K. A colorless 0.39 × 0.28 × 0.06-mm^3 crystal was used for data collection. The theta range for data collection was 2.97 to 66.59° with 3510 reflections collected. Additional information and tables of coordinates, bond distances and bond angles, and anisotropic thermal parameters have been deposited with the Crystallographic Data Centre, Cambridge, CB2, and 1EW, England.

Antiviral Activity in MT-2 and MT-4 Cells. MT-4 cells (3×10^4) were exposed to 100 $TCID_{50}$ of HIV-1_{LAI} and were cultured in the presence of ***rac*-1h** and AZT and used as a control. The EC_{50} values were determined using an MTT assay

on day 5 of the cultures. All assays were conducted in duplicate. MT-2 cells (2×10^3) were exposed to 100 $TCID_{50}$ of HIV-1_{LAI} and were cultured in the presence of ***rac*-1h** and AZT. The EC_{50} values were determined using an MTT assay on day 7 of the cultures. All assays were conducted in duplicate.

Inhibition of Wild-Type HIV-1 RT. The assay was done in duplicate as previously described.[16] Briefly, for each sample, 0.25 μg of single-stranded M13mp18 DNA (New England Biolabs) was hybridized to 0.5 μL of 1.0 OD260/mL −47 sequencing primer (New England Biolabs). The template primer was suspended in a solution containing 100.0 μL of 25 mM Tris (pH 8.0), 75 mM KCl, 8.0 mM $MgCl_2$, 100.0 μg/mL bovine serum albumin (BSA), 10.0 mM 3-[(3-cholamidopropyl)-dimethylammonio-]-1-propanesulfonate (CHAPS), 2.0 mM dithiothreitol, 10.0 μM each of dATP, dGTP, and dTTP, 5.0 μM dCTP, 2.0 μM [α-^{32}P] dCTP, and the indicated concentration of inhibitor. Extension was initiated by the addition of 1.0 μg of wild-type HIV-1 RT or RT-variant M184V. The mixture was incubated for 30 min at 37°, and the reaction was halted by the addition of 3 mL of 10% trichloroacetic acid (TCA). Precipitated DNA was collected by suction filtration through Whatman GF/C glass filters. The amount of incorporated radioactivity was determined by liquid scintillation counting. The amount of incorporated radioactivity in the absence of inhibitor was considered 100% activity. The amount of radioactivity incorporated in the presence of inhibitor was normalized to this value.

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Supporting Information Available: For ***rac*-1h**, crystallographic data, atomic coordinates, bond lengths and angles, ansiotropic displacement parameters, hydrogen coordinates and isotropic displacement parameters, torsion angles, hydrogen bonds, and a PDB file. This material is available free of charge via the Internet at http://pubs.acs.org.

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