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Bifunctional Inhibition of HIV-1 Reverse Transcriptase: A First Step in Designing a Bifunctional Triphosphate‡

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Abstract

The onset of resistance to approved anti-AIDS drugs by HIV necessitates the search for novel inhibitors of HIV-1 reverse transcriptase (RT). Developing single molecular agents concurrently occupying the nucleoside and nonnucleoside binding sites in RT is an intriguing idea but the proof-of-concept has so far been elusive. As a first step, we describe molecular modeling to guide focused chemical syntheses of conjugates having nucleoside (d4T) and nonnucleoside (TIBO) moieties tethered by a flexible polyethylene glycol (PEG) linker. A triphosphate of d4T-6PEG-TIBO conjugate was successfully synthesized that is recognized as a substrate by HIV-1 RT and incorporated into a double-stranded DNA.

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Supplementary data
Details on synthetic procedures, spectral characterization, molecular modeling, and biological assays are contained in Supporting Information.

Keywords

HIV-1; Reverse Transcriptase; Bifunctional; NRTI; NNRTI; PEG

HIV reverse transcriptase (RT) plays a pivotal role in the viral life cycle, whose multiple functions include RNA-directed and DNA-directed DNA polymerization. HIV-1 RT inhibitors, critical components of the highly active anti-retroviral therapy (HAART) against AIDS, are designed to target *either* the active site *or* a nearby hydrophobic site of the viral polymerase, but *not both*. The nucleoside analog RT inhibitors (NRTIs) are prodrug nucleosides which are transported across cellular host membranes and phosphorylated to the metabolically active nucleotides. These nucleotides serve as substrates for RT and lacking a 3′ hydroxyl group, they serve to chain-terminate DNA synthesis. This chain termination and their competition with natural deoxynucleoside triphosphates (dNTPs) yield their clinical utility[1]. Structural evidence shows that the allosteric nonnucleoside RT inhibitors (NNRTIs) bind to an inducible pocket approximately 10 Å away from the polymerase site[2,3], and steady-state kinetic studies suggest they are noncompetitive with respect to the binding of dNTP and oligonucleotides[4–8]. While NRTIs and NNRTIs are potent inhibitors of reverse transcription, resulting in an array of FDA-approved formulations, resistance at the level of RT proves to hinder their full utility–this phenomenon calls for new and improved antiviral inhibitors.

In light of viral resistance, RT has been targeted with using other strategies including dual NRTIs[9], interference of RT and DNA binding[10], among others. One approach that has previously received some attention is the concept of a “bifunctional inhibitor”. Pursuit of a “bifunctional inhibitor” for HIV-1 reverse transcriptase (a molecule which simultaneously targets both inhibitory pockets) has been both an intriguing and challenging endeavor, despite more than a decade of global research efforts[11–15].

The genesis of the idea, in part, was inspired by kinetic observations of HIV-1 RT. Biochemical studies from our lab and others[16–19] suggest that the natural dNTP and the NNRTI can simultaneously occupy their respective sites. Moreover, these studies also indicated that communication occurs between the active and the NNRTI binding sites since the inhibition of RT by NNRTIs is manifested through a remote effect on the chemical step of polymerization. At the molecular level, transient kinetic studies have shown that nonnucleoside inhibitors change the rate-limiting step of incorporation, resulting in chemistry as the slowest step[16,17]. This crosstalk between the two binding sites sets a hypothesis for designing a single molecular “bifunctional” inhibitor including an NRTI and NNRTI component tethered together to target both sites simultenaously. This innovative approach has been successful in yielding inhibitors of other enzyme systems, FKBP and stromelysin[20,21].

The attraction of a bifunctional inhibitor against HIV-1 RT is many fold. First, combining two inhibitory moieties within one molecule can have synergistic effects that may improve potency. Second, the onset of resistance may decrease as a result of simultaneous mutations that must be produced at both binding pockets. Lastly, potential therapeutic agents based on the bifunctional inhibition designed specifically for HIV-1 RT would presumably not interfere with human polymerases such as the human mitochondrial DNA polymerase γ and thus result in fewer side effects. Unfortunately, in practice, the development of an HIV-1 RT bifunctional inhibitor has been extremely challenging[11,13,14, 22–24] – attempts with d4T, AZT, and various NNRTI scaffolds have thus far not been successful to illustrate the bifunctional proof of concept. Renoud-Grappin et al. showed that a linkage to d4T did not reduce its antiviral inhibition, but further addition of the NNRTI decreased the antiviral

activity 100-fold[14]. This loss of activity can be attributed to a lack of binding of the NNRTI, as this group of inhibitors showed activity against HIV-2 RT, for which no NNRTI pocket exists. Other compounds utilizing AZT and ddC as NRTI parents illustrated NRTI-linkage is detrimental at both the C5, N3 (of AZT) and C4 positions (of ddC)[13], though the linkers chosen in this study may have influenced activity. Along with others, these two studies depict the complex structure-activity relationship that exists for putative bifunctional inhibitors, but a promising finding demonstrated that a linkage at the 5-position of d4T with a methylamino linker did not perturb activity of the NRTI in cell culture[14]. This contrasts with data from Gavriliu et al., which argues attachment of this methylamino linker indeed disrupts d4T's antiviral activity in culture[25]. Comparison of these two studies yields significant differences in the linker terminus, further supporting the complexity in addressing linkage position and linker design for an RT bifunctional inhibitor. Work from our lab preparing a bifunctional inhibitor prodrug based on phenylethylthiazolylthiourea (PETT) compounds such as HI-236 as an NNRTI showed antiviral activity in cell culture however structural data with PETT-2 NNRTIs suggested the choice of position for linkage from the NNRTI was not optimal[26,27].

In order to circumvent issues raised above and to fully understand the potential benefits of the bifunctional concept, several basic questions were addressed for the rational design. First is the choice of NRTI and the point of connection. To date, eight NRTIs have been approved for clinical use. The considerations in choosing one of these are two fold: (1) the chemical transformation to introduce properly substituted derivatives and (2) the *in vivo* transformation of the latter to its triphosphate by human kinases. N4-substitution of ddC (zalcitabine) has afforded active compounds against HIV replication, but the modifications made at this position substantially reduced potency relative to the parent compound ddC[13]. Substitution at both N-3 and C-5 of AZT (Retrovir) has lead to inactive antiviral compounds[11]. The substitutions at C-5 in d4T (stavudine, Figure 1) yield mixed results, but mostly result in inactive analogs[14,25,28–30]. Attempted modifications to AZT and d4T suggest the difficulty for human thymidine kinase (hTK) to phosphorylate 2′,3′-dideoxythymidine analogs, but there is evidence that C-5 substituted thymidine can be phosphorylated *in vivo* by thymidine kinase[31–33]. Guided by these previous studies, we chose d4T as our nucleoside because of the observed slight resistance in cell culture studies[34–36], and at the enzymatic level, it is incorporated as efficiently as the natural substrate (dTTP) with both DNA/DNA and DNA/RNA primer-templates[37].

Likewise, linkage to the NNRTI end should not interfere with the desired interactions between the inhibitor and the NNRTI pocket, namely the hydrogen bonding and aryl-aryl interactions. This is evident in the case of HEPT, an early generation NNRTI – substitution at N3 caused the loss of hydrogen bonding to the backbone carbonyl of lysine 101, diminishing its affinity for RT[11], while derivatives of HEPT modified at position N1 preserved key interactions and activity[38]. The connection to N3 of TSAO-T also resulted in active compounds, presumably because of preserved hydrogen bonding between the 4-amino group and the backbone carbonyl of lysine 101[11]. At the time that initial compounds were developed for our study, only a few NNRTI structures were available and thus choosing an NNRTI depended on this limited structural data. Structures that were available gave insight into using 8-Cl-TIBO as our NNRTI of choice, since a plausible linkage could be attached[39,40].

Design of a bifunctional HIV-1 RT inhibitor was guided by molecular modeling that benefitted from the availability of emerging x-ray crystallographic and biochemical data for RT. Based on the rational design, an iterative and systematic synthetic effort was undertaken to prepare, purify and characterize several generations of small molecule compounds. These compounds were then tested in biochemical and cellular assays to develop a bifunctional

structure-activity relationship. With d4T and 8-Cl-TIBO as our initial building blocks to illustrate our "proof of concept," here we present the first evidence of DNA incorporation of a rationally designed and fully synthesized bifunctional nucleoside triphosphate by HIV-1 RT.

Thymidine-PEG-TIBO Inhibitors

To begin our design, we chose polyethylene glycol[9] as our linker (Figure 1), for its low toxicity in biological venues and for its amphiphilic nature, thus giving it favorable solubility in the cellular environment. We also chose to begin using a thymidine analog as our NRTI, to avoid potential synthetic pitfalls with other NRTIs. And at the time this study was initiated in our lab, structural evidence suggested that a bifunctional concept could be achieved with the NNRTI 8-Cl-TIBO[39,40], with linkage stemming from the 8-position (Figure 1). To aid in the design of the inhibitor, modeling was performed to not only to verify that linkage could be achieved on position 8 of TIBO, but to also estimate the number of PEG units needed to bridge the two pockets. 8-Cl-TIBO was modeled into the ternary RT structure (solved with natural substrate dTTP)[24], which suggested that the 8-position of TIBO was directed towards the dNTP pocket, and that approximately six PEG units could connect the NRTI to the NNRTI as shown in Figure 2.

Though the 5-position is a debatable site for derivatization (as stated earlier), linkage precedence guided the attachment of linker to this position[12,14,33,41], as thymidine kinase can phosphorylate C-5 substituted pyrimidines and that d4T with long linkers remained active against HIV. We thus set out to synthesize the thymidine-6PEG-TIBO bifunctional inhibitor. As illustrated in Scheme 1, we chose a modular strategy for linker attachment to allow a variation in linker length and composition. Shown in Scheme 1 is the synthesis of the bifunctional molecule, where an ester linkage was introduced into the linker region for chemical feasibility. Molecules with four, five, or six PEG monomers were synthesized to develop a structure-activity relationship (SAR) for this group of inhibitors.

d4T-PEG-TIBO Inhibitors

We moved to synthetic efforts employing d4T as our NRTI moiety (Scheme 2). As mentioned earlier, d4T was chosen as our NRTI because of its similar kinetics of incorporation relative to natural thymidine and the low-level resistance developed against this NRTI. Modeling also suggested that we could pursue attachment of PEG to the 5-position of d4T, with the assumption that d4TTP bound in a similar manner to natural dTTP – a reasonable supposition based on the incorporation kinetics of d4TTP. An initial evaluation of antiviral activities of these compounds was carried out by examining their ability to inhibit HIV-1 replication in MT-2 cell culture. Table 1 shows the antiviral potency (EC_{50}) and general cellular toxicity (IC_{50}) for the bifunctional compounds **16a–c**, containing 4, 5, and 6 PEG linkers. As illustrated in Table 1, these compounds showed some modest antiviral activity in the mid-micromolar range and the bifunctional molecule linked with four PEG units (16a) was most inhibitory, though the bifunctional inhibitors altogether fell short in illustrating a proof of concept. In trying to rationalize the poor activity a number of questions arise: (1) Was the nucleoside moiety in the bifunctional compounds phosphorylated as would be required for activity at the cellular level?; (2) was the ester functionality cleaved by cellular proteases to yield a cleaved inhibitor; and (3) was the linker length not optimal and/or did linker attachment perturb binding? As an initial step, we addressed the question of phosphorylation by synthesizing the protected monophosphate of d4T-4PEG-TIBO, with protecting groups utilized in stampidine, a prodrug formulation of d4T[42]. This approach did not yield an improvement in activity (data not shown). Synthesis of the corresponding triphosphate of d4T-4PEG-TIBO as a substrate was attempted to

address the phosphorylation requirement for in vitro incorporation assays catalyzed by HIV-1 RT. However, a triphosphate product could not be synthesized (data not shown).

Though N3 substituted derivatives of d4T have been shown to be less active than d4T itself[43], we attempted to synthesize a bifunctional inhibitor with N3 substitution (Scheme 3). Chemistry at this position was less problematic than C5 derivatization and a bifunctional inhibitor that displayed "proof of concept" might overcome the loss of activity that might be observed with N3 linkage. This includes the caveat that disrupted Watson-Crick base pairing may be a potential obstacle for activity with N3 substitution. The first compound of this series is shown in Figure 3A, where the PEG linker was directly attached to the N3 position of d4U without use of an ester moiety. This was deliberately planned through chemical synthesis to avoid ester cleavage in the cell as this may have been one problem associated with the lack of activity with the d4T-PEG-series. d4U was initially chosen for chemical feasibility and because the substitution now resides on N3, the linkage was lengthened by 2 PEG units. Estimation for PEG linker length was provided by modeling studies. In cell culture, this bifunctional compound, **22**, did not illustrate a "proof of concept", and we observed a minor increase in cell toxicity that could be generated by potential off-target interactions through the bifunctional inhibitor (Table 1). For nucleoside analogs, the first phosphorylation limits the rate of triphosphate formation[44], and thus we again attempted to synthesize the prodrug version of N3-d4U-6PEG-TIBO (using stampidine formulation, see above). Again, this modification did not improve activity (data not shown) – suggesting that subsequent phosphorylations might also pose an obstacle to antiviral activity. Since our two initial prototypes were not showing significant activity, we asked if linkage at either the NRTI or NNRTI end might be affecting activity. The TIBO-6PEG fragment (Figure 3B) was tested in our cell culture assay. As shown in Table 1, the addition of linker significantly affects the activity of TIBO, decreasing activity by ~ 2000 fold. The perturbation upon TIBO's activity certainly may account for our large hit on bifunctional activity.

The MT-2 cell culture inhibition assay illustrated the pitfalls with our strategy, but it does not directly address potential interactions of inhibitors with RT. To focus on RT interactions, the triphosphate of N3-d4U-6PEG-TIBO (Figure 4A) was successfully synthesized and tested for incorporation with RT. Figure 4B shows a gel comparing the incorporation of the triphosphate of N3-d4U-6PEG-TIBO with that of natural dTTP. While the reaction using N3-d4U-6PEG-TIBO triphosphate showed a higher migrating product band, the migration appeared to be similar to that for a *D24-mer, which corresponds to a natural single deoxynucleotide incorporation band size (Figure 4B). This observation indicates the decomposition of the N3-d4U-6PEG-TIBO triphosphate into d4UTP serving as a substrate for RT, since the expected product mobility of the incorporated bifunctional inhibitor band size might be expected to be higher than the incorporated dTTP band size. We believe that the cleavage of the PEG linker along with the TIBO from the nucleotide part could occur due to the thermal instability of the bifunctional inhibitor either during the reaction time at 37°C or after the incorporation process when the samples were running on a denaturing gel. Because the temperature of the gel apparatus goes up to 55°C. Addition to this thermal denaturation, attaching the linker to the N3 position compels the nucleotide part to be in the unfavourable syn conformation that could impart additional strain on the inhibitor structure, and thus result in a higher propensity towards decomposition. This is supported by the observation that a very faint higher migrating band can be observed (Supplementary figure 1).

In the light of these findings, we synthesized a new bifunctional inhibitor (a 4PEG linker attached to the C5 position of d4T and a different NNRTI drug) in order to compare the incorporation efficiency with the TIBO bifunctional (Supplementary figure 2). The incorporation reaction of N3-d4U-6PEG-TIBO triphosphate at the room temperature

resulted in a major denaturation band of the bifunctional product due to its intrinsic chemical and physical properties (Supplementary figure 1). However, the new bifunctional inhibitor (d4TTP-4PEG-TMC) was successfully incorporated by RT showing a much higher migration band corresponding to the size of the inhibitor with trace amounts of degredation product band likely owing to increased compound stability.

Implications for Future Research

Molecular modeling, as demonstrated in this study, should ecome an indispensable tool in the design of bifunctional RT inhibitors, so the probability of problems, such as wrong connection sites or inadequate linker length, can be minimized, and downstream synthetic efforts can be more focused. It should be appreciated, however, that the inhibitors designed from molecular modeling are “rough sketches”, not exact molecular “blueprints” to be taken literally. Rather, they should serve as the starting point from which structures can be refined based on experimental tests. For example, though structural and modeling studies suggested that the 8-position of 8-Cl-TIBO would be a favorable site for linkage, they should not preclude other positions, such as C-9, as feasible, even preferable, anchoring points. The ultimate support of the dual-binding of the bifunctional inhibitor relies on the availability of x-ray structural evidence of RT complexed with the inhibitor.

The effect of linker to the binding of the bifunctional inhibitor is underappreciated. More than an inert, weightless tether, the linker itself can significantly alter the way the bifunctional inhibitors bind to RT, and derail the intended interaction altogether. The 2000-fold decrease in activity of TIBO-6PEG vs. TIBO is one such example. On the other hand, the linker can be a source of positive contribution to binding. Creative use of diverse structures as linker that can bind to RT can add an unexplored dimension to bifunctional inhibitor design.

Synthetic difficulties remain a significant bottleneck in the preparation of the triphosphates of bifunctional inhibitor, as we encountered in numerous occurrences in our endeavors. Our successful synthesis of the triphosphate of N3-d4U-6PEG-TIBO and its small degree of incorporation by RT due to its thermal instability, however, illustrate a first step toward a successful RT bifunctional strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HIV-1	human immunodeficiency virus type 1
RT	reverse transcriptase
NRTIs	nucleoside RT inhibitors
NNRTIs	nonnucleoside RT inhibitors
dNTP	deoxynucleoside triphosphate

dNMP	3′-deoxynucleoside monophosphate
PP_i	pyrophosphate
AZT	β-D-(+)-3′-azido-3′-deoxythymidine
d4T	β-D-(+)-2′,3′-didehydro-3′-deoxythymidine
K_d	Dissociation constant
WT	wild type
TEAB	triethylammonium bicarbonate
MOI	multiplicity of infection
DMSO	dimethyl sulfoxide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PEG	polyethylene glycol
8-Cl-TIBO	8-chloro tetrahydroimidazobenzodiazepinone
SAR	structure-activity relationship
MW	molecular weight
TBDMS	tert-butyldimethylsilyl
Tris	tris(hydroxymethyl)aminomethane

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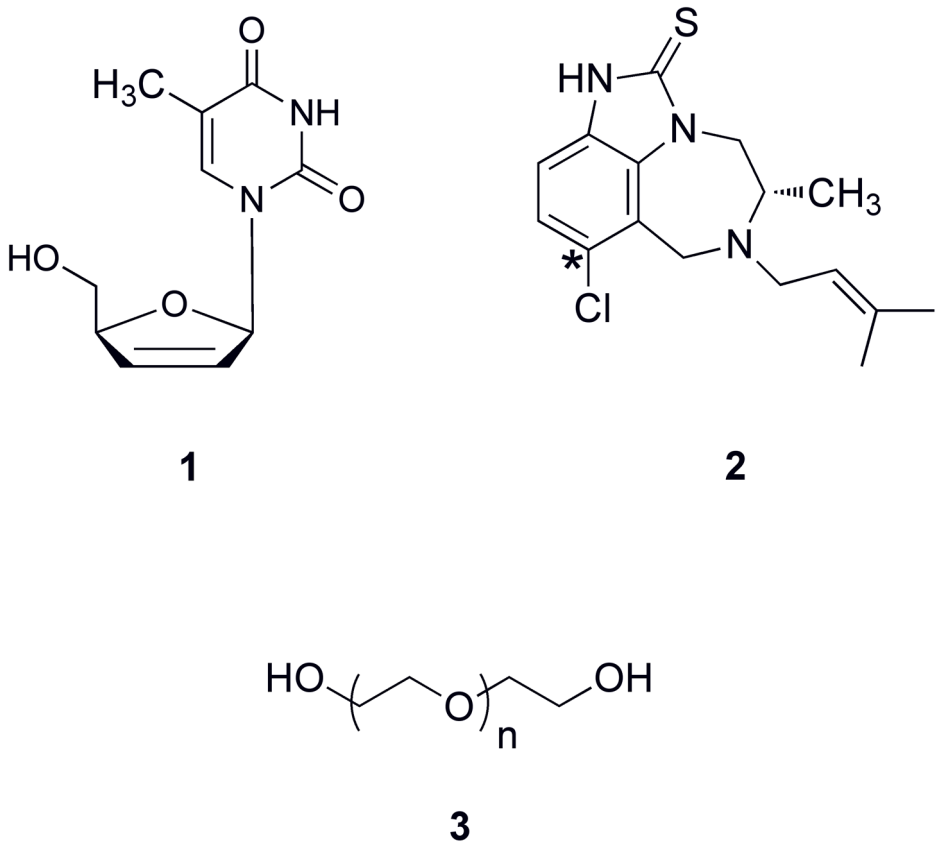


Figure 1. Bifunctional Building Blocks

Structures of d4T (**1**), 8-Cl-TIBO (**2**), and PEG (**3**). The 8-position of 8-Cl-TIBO is indicated with a (*).

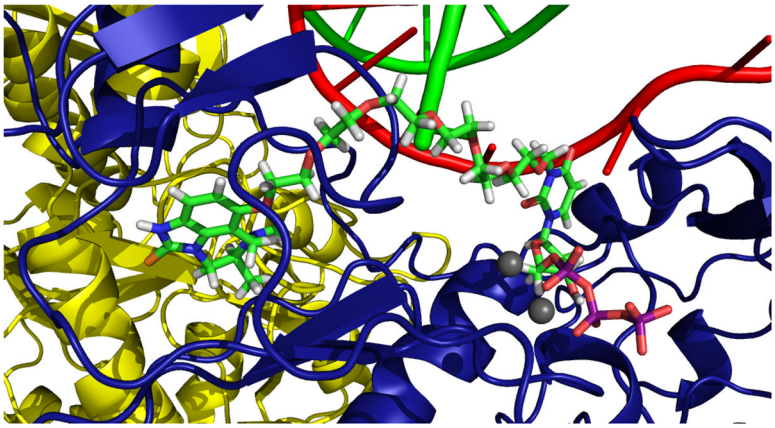
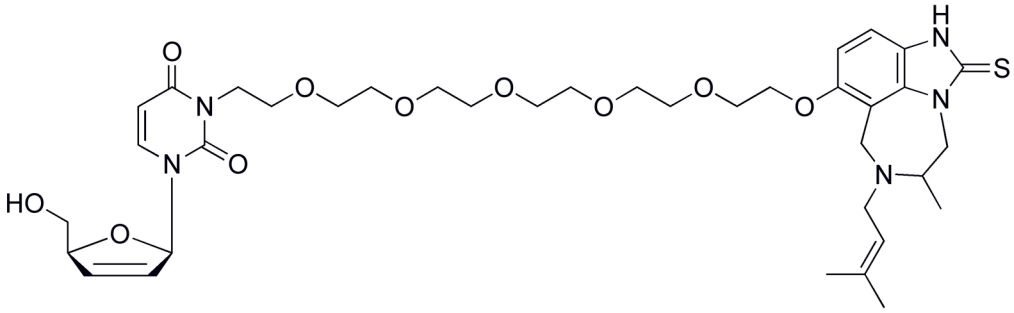


Figure 2. Modeled Fit of dTTP-PEG-TIBO

Using coordinates from the previously published ternary RT structure (RT, primer/template, and dTTP; pdb 1RTD)[24] and the RT structure solved with 8-Cl TIBO (pdb 1HNV)[40], the NNRTI pocket from 1HNV was aligned and superimposed onto 1RTD (see Experimental Section). In this hybrid modeled structure, the chlorine at position 8 of TIBO is clearly oriented towards the dTTP in the active site. The straight-line distance between 8-Cl of TIBO and 5-methyl of dTTP is 17.10 Å. Shown in blue and yellow are the p66 and p51 subunits of RT, respectively, and the green and red curved lines represent primer and template, respectively. The modeled bifunctional inhibitor is shown in ball and stick representation, and the grey spheres symbolize the active site magnesium ions.

A



B

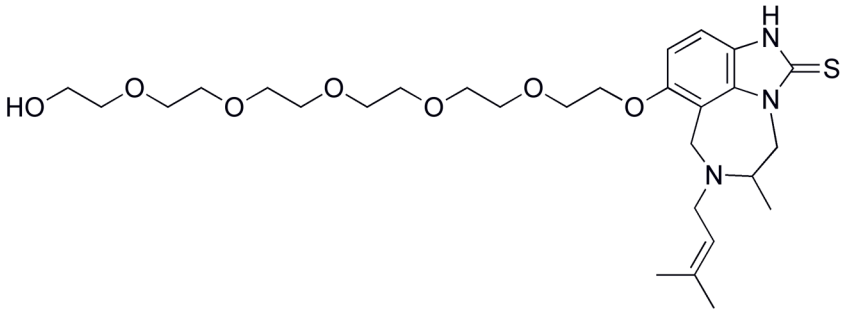


Figure 3. Structures of the synthesized bifunctional inhibitors

(A) Structure of N3-d4U-6PEG-TIBO. (B) Structure of TIBO-6PEG.

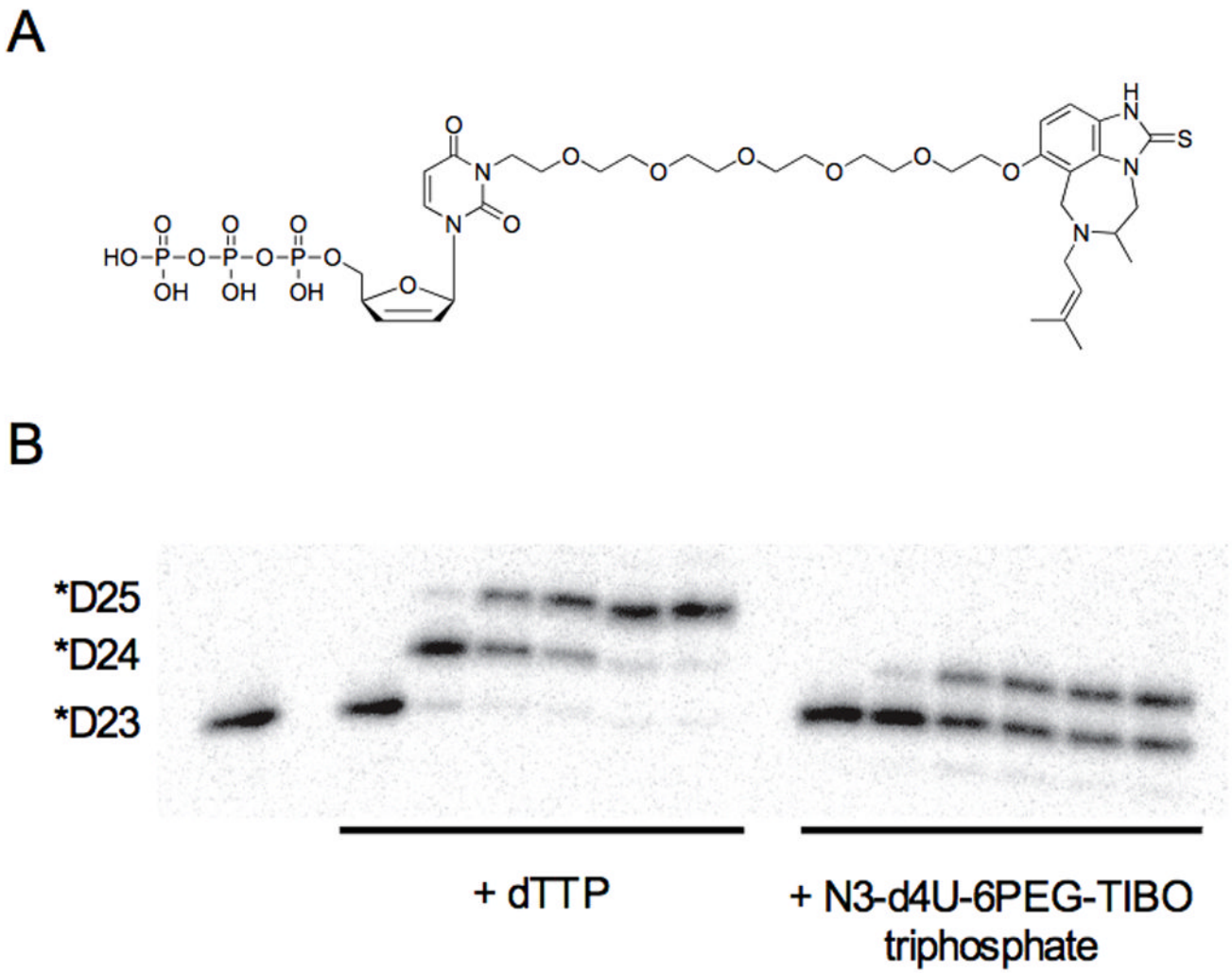
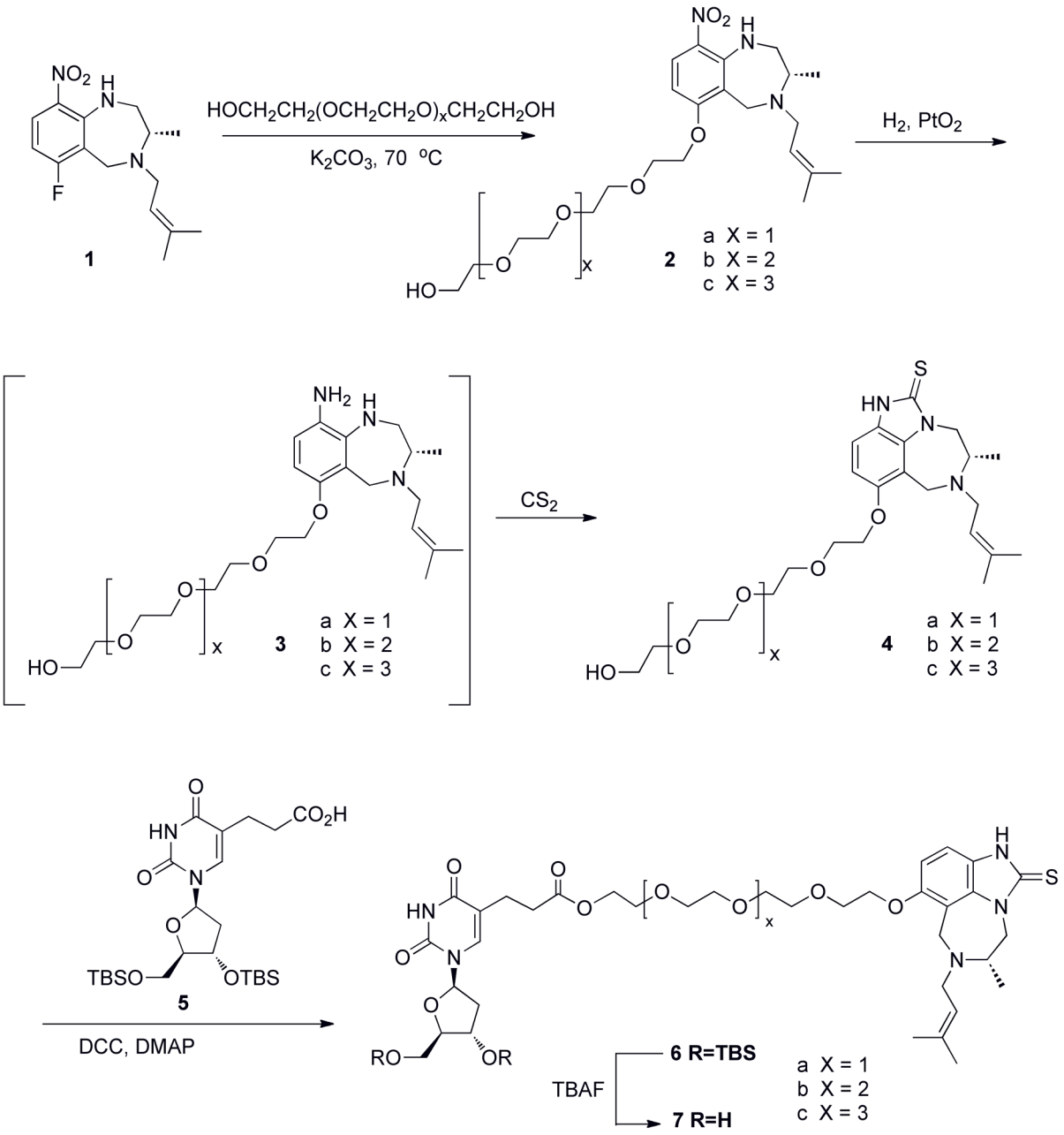
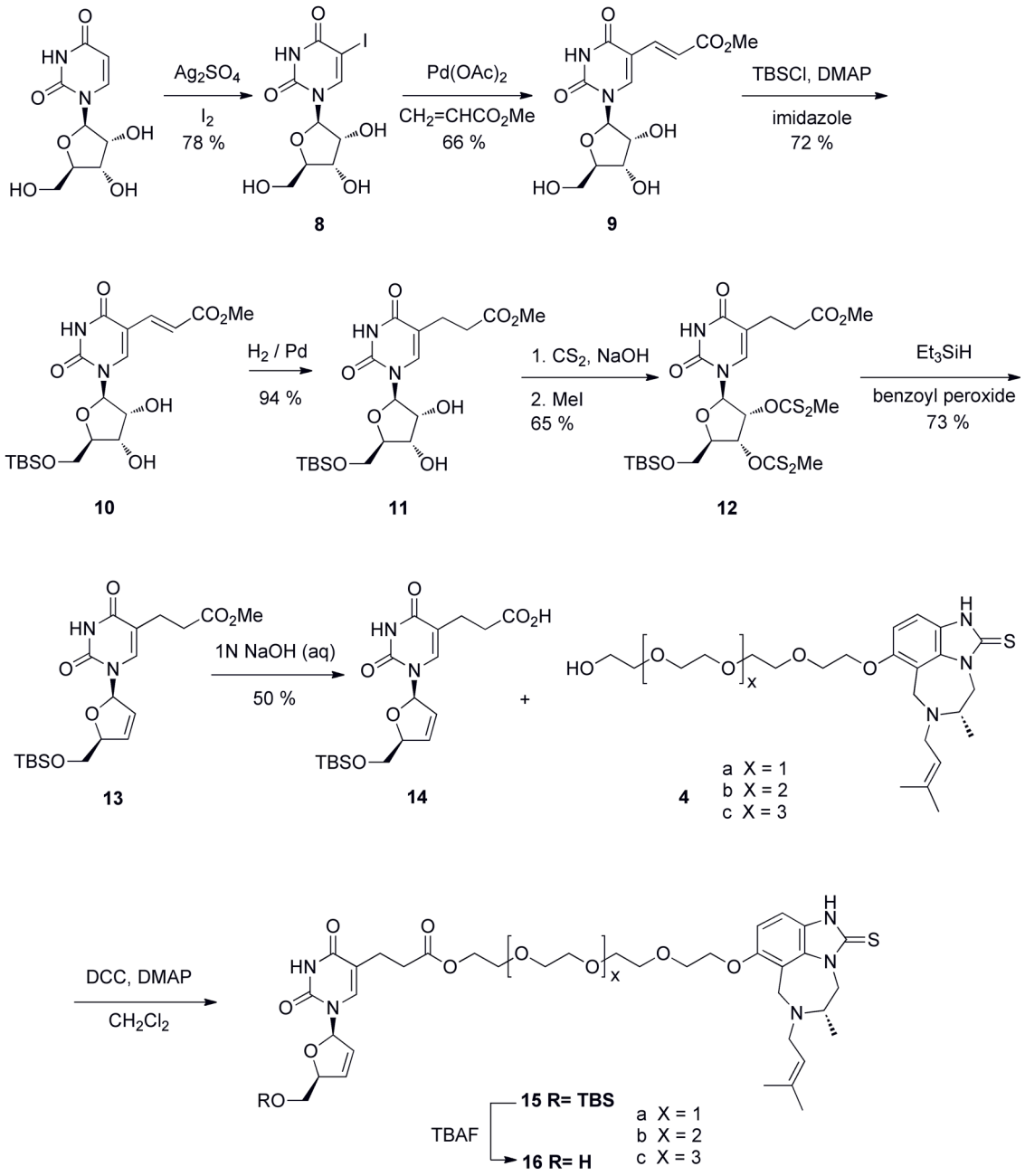


Figure 4. Incorporation of triphosphate of N3-d4U-6PEG-TIBO

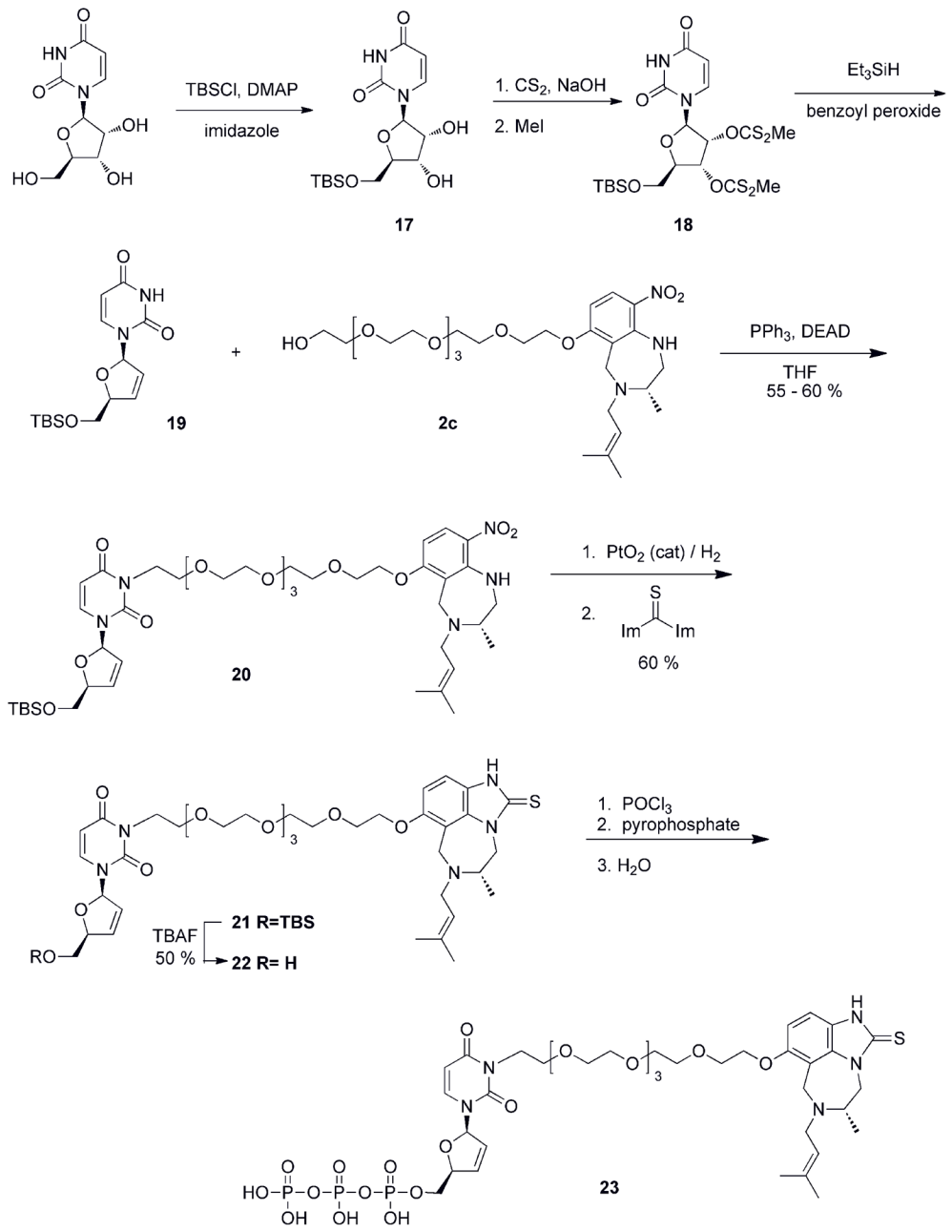
(A) Structure of triphosphate of N3-d4U-6PEG-TIBO (**23**). (B) Acrylamide gel showing incorporation of dTTP (100 μM) and triphosphate of N3-d4U-6PEG-TIBO (150 μM) over a time course under single turnover conditions (250 nM RT, 50 nM D23/D36 primer/template). Time points are t = 0, 30 sec, 5 min, 10 min, 30 min, and 60 min. Note the misincorporation of a second molecule of dTTP during this time course.



Scheme 1.
Synthesis of Thymidine-PEG-TIBO conjugates.



Scheme 2.
Synthesis of d4T-PEG-TIBO bifunctional RT inhibitors.



Scheme 3.
Synthesis of N3-d4U-6PEG-TIBO.

Table 1

Activity of Potential Bifunctional Inhibitors in HIV-infected Cell Culture

Compound	EC_{50}	IC_{50}
d4T	2.2 μM	> 100 μM
8-Cl-TIBO	14 nM	> 100 nM
d4T-4PEG-TIBO **(16a)**	45 μM	> 100 μM
d4T-5PEG-TIBO **(16b)**	100 μM	> 100 μM
d4T-6PEG-TIBO **(16c)**	120 μM	> 100 μM
N3-d4U-6PEG-TIBO **(22)**	20 μM	38 μM
TIBO-6PEG **(2c)**	27 μM	> 100 μM

Compounds were tested for their in vitro effect on viral replication. EC_{50} – antiviral potency; IC_{50} – cellular toxicity.