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[d4U]-Spacer-[HI-236] double-drug inhibitors of HIV-1 reverse-transcriptase

Yassir Younis [a], Roger Hunter [a,*], Clare I. Muhanji [a], Ian Hale [a], Rajinder Singh [a], Christopher M. Bailey [b], Todd J. Sullivan [b], Karen S. Anderson [b]

[a] Department of Chemistry, University of Cape Town, Rondebosch 7701, Cape Town, South Africa
[b] Department of Pharmacology, School of Medicine, Yale University, New Haven, CT 06520, USA

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ABSTRACT

Four double-drug HIV NRTI/NNRTI inhibitors **15a–d** of the type [d4U]-spacer-[HI-236] in which the spacer is varied as 1-butynyl (**15a**), propargyl-1-PEG (**15b**), propargyl-2-PEG (**15c**) and propargyl-4-PEG (**15d**) have been synthesized and biologically evaluated as RT inhibitors against HIV-1. The key step in their synthesis involved a Sonogashira coupling of 5-iodo d4U's benzoate with an alkynylated tethered HI-236 precursor followed by introduction of the HI-236 thiourea functionality. Biological evaluation in both cell-culture (MT-2 cells) as well as using an in vitro RT assay revealed **15a–c** to be all more active than d4T. However, overall the results indicate the derivatives are acting as chain-extended NNRTIs in which for **15b–d** the nucleoside component is likely situated outside of the pocket but with no evidence for any synergistic double binding between the NRTI and NNRTI sites. This is attributed, in part, to the lack of phosphorylation of the nucleoside component of the double-drug as a result of kinase recognition failure, which is not improved upon with the phosphoramidate of **15d** incorporating a 4-PEG spacer.

1. Introduction

HIV reverse-transcriptase (RT)[1] is a multifunctional enzyme responsible for the catalytic transformation of single-stranded HIV viral RNA into double-stranded DNA (dsDNA). It continues to be the principal drug-target for chemotherapy using combination therapy HAART[2] (Highly Active Antiretroviral Therapy), in which RT inhibitors (RTIs) provide at least two of the three anti-HIV drugs[3] used. Two types of RTIs have emerged as NRTIs[3] (Nucleoside Inhibitors), which act as competitive inhibitors of DNA polymerization, and NNRTIs (Non-Nucleoside Inhibitors),[4] which inhibit allosterically by binding into a hydrophobic pocket adjacent to the DNA substrate binding site.[3] The close proximity (10–15 Å) of the two RTI sites inspired Arnold[5] in 1993 and Steitz[6] in 1994 to propose the double-drug concept for the first time. They postulated that a molecular entity comprising one of each type of inhibitor joined by a spacer might be able to interact simultaneously with the two sites on RT and in so doing provide a super-inhibitor via additive binding.[7] Biochemical support to realize such a concept was subsequently provided by Anderson[8a] Goody[8b] and Wainberg[8c] who showed that binding of an NNRTI does not inhibit the binding processes of an NRTI. These research contributions effectively provided inspiration for a number of synthetic medicinal chemistry research efforts towards realizing such a concept, and in 1995 Camarasa[9] published the first example of a bifunctional NRTI/NNRTI anti-HIV double-drug containing a non-cleavable spacer based on an [AZT]-spacer-[TSAO-T] or [AZT]-spacer-[HEPT] combination joined at N-3 of the base of each drug. TSAO-T[10] derivatives interact with amino acids situated in both HIV-1 RT subunits (p51 and p66) at the dimer interface. A likely important interaction is with the carboxyl group of Glu 138, which lies at the entrance to the NNRTI pocket in the p51 sub-unit. Camarasa varied both the drug combinations as well as the tether length and did achieve some IC_{50} inhibition values in the 0.06–0.55 μM range against HIV-1. However, it was concluded that the double-drugs inhibited as extended NNRTIs with no contribution apparent from the NRTI component. In a subsequent paper,[11] the study was broadened to accommodate other NRTI's like d4T as well as different attachment points on the drugs, but still without demonstrating any activity at the NRTI site. In the following ten years or so, reports on both cleavable,[12] in which the spacer is designed to be hydrolytically labile in order to release the individual drugs, as well as other non-cleavable double-drugs,[13] also known as mixed site inhibitors,[13b] appeared in the literature but similarly without evidence of true synergy between the two inhibitors. For the cleavable type, systems have become extended to different drug targets[14] other than RT, whereas for the non-cleavable type, RT remains to date as the only target[15] studied for HIV in view of the uniqueness of having the NRTI and NNRTI sites in close proximity. Challenges

* Corresponding author. Tel.: +27 21 650 2544; fax: + 27 21 650 5195.
E-mail address: Roger.Hunter@uct.ac.za (R. Hunter).

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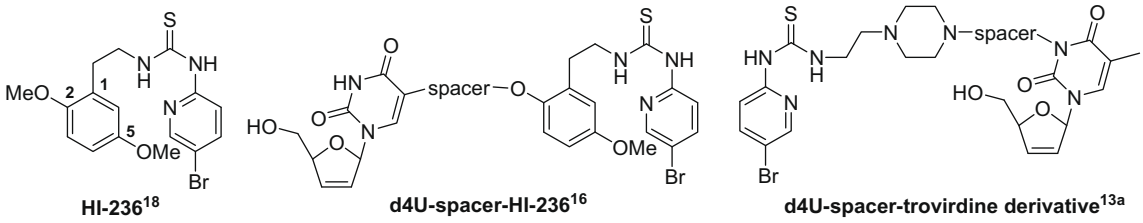


Figure 1. Structures of HI-236 and d4U-spacer-PETT double-drugs.

regarding the successful realization of a truly synergistic double-drug RTI in the non-cleavable class have been primarily twofold as: (i) where on the drugs to attach the linker—this has suffered from a lack of modeling data, and, (ii) the lack of phosphorylation of the NRTI nucleoside pro-drug contained within the double-drug in vitro. Recently, we have reported on our own efforts in the field of non-cleavable HIV double-drugs using a d4U/HI-236 system. Our first prototype[16] with a relatively short butynyl tether against HIV-1 (IIIB) in MT-2 cell culture using an MTT assay returned an EC_{50} of 250 nM. This was only a fivefold reduction of the NNRTI (HI-236) EC_{50} and eightfold more potent than d4T (2 μM). Encouraged by this result we went on in a subsequent paper[17] to explore the influence of tether type and length on the anti-HIV activity of C-2 aryl O-tethered HI-236 derivatives in order to establish the feasibility of projecting a tether out of the NNRTI pocket. A 2-PEG-propynyl substituent returned an EC_{50} of 390 nM suggesting feasibility of the concept. In this paper we extend these studies and report on results pertaining to a small family of [d4U]-spacer-[HI-236] double-drugs as well as a phosphoramidate pro-drug of one of them.

2. Double-drug design aspects

Regarding design aspects, d4U was chosen as the nucleoside in view of its synthetic versatility as well as its proven activity (as a d4T unit if not substituted at C-5 in the double-drug) in other double-drug systems.[11] A water-friendly PEG (polyethylene glycol) spacer that could be varied in length and easily introduced via standard substitution methodology was chosen for the tether. The choice of HI-236[18] as the NNRTI has been delineated earlier[17] but essentially, apart from its potency, synthetic accessibility and ease of manipulation, its flexibility as a second-generation NNRTI was considered to be a crucial parameter for achieving communication between the two sites. Regarding the all-important issue of where to attach the tether, two important decisions clearly had to be made. C-5 on the pyrimidine base was chosen as the attachment point to the nucleoside drug as this was not only synthetically readily accessible but was also considered to offer minimal interference with both base-pairing as well as hindrance around the C-5 site. These views were based on the work of Ruth and Cheng,[19] and the choice of the C-5 connecting group as alkynyl was considered to offer an attractive exit from the substrate binding site in view of its directionality away from the developing DNA strand. An alkynyl connection to the nucleoside also presented itself as an attractive synthetic option via the well-known and versatile Sonogashira Pd(0) cross-coupling protocol, a well-known methodology in the nucleoside field.[11,12d,20] This left the all-important choice of NNRTI attachment. It was significant to us that Ladurée[13a] had failed to observe any appreciable activity with their trovirdine derivative-based double-drug, which is shown against our double-drug design together with HI-236 in Figure 1, both double-drugs containing a non-cleavable linker.

Not withstanding the different (C-5 vs N-3) attachments to the individual nucleosides, the '*para*-like' configuration of the tethers attached to the piperazine nitrogens in Ladurée's case was felt to be extremely significant. In HI-236, the piperazine ring of the trovirdine derivative is replaced by a trisubstituted phenyl group in which the C-5 (see Fig. 1 for numbering) methoxy group *meta* to the HI-236 thiourea tether at C-1 forges an important C–H/π interaction with the conserved Trp229 residue at the back of the NNRTI pocket. This allows the C-2 phenolic methoxyl to point its methyl group down towards the floor of the cavity where residues like V106 reside. Modeling reported in our 2008 paper[17] illustrates such an arrangement as shown in Figure 2 (seen from either end of the pocket) for a derivative in which the C-2 methyl is replaced by a methoxycarbonylmethylene grouping. Importantly, it suggests that a C-2 *ortho*-substituent to the C-1 thiourea tether allows

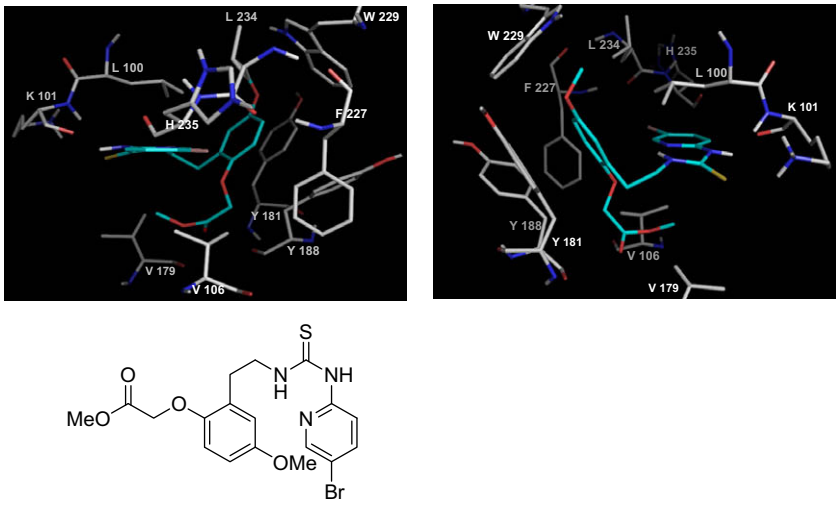


Figure 2. A C-2 O-alkylated HI-236 derivative modeled in the NNRTI pocket.[17]

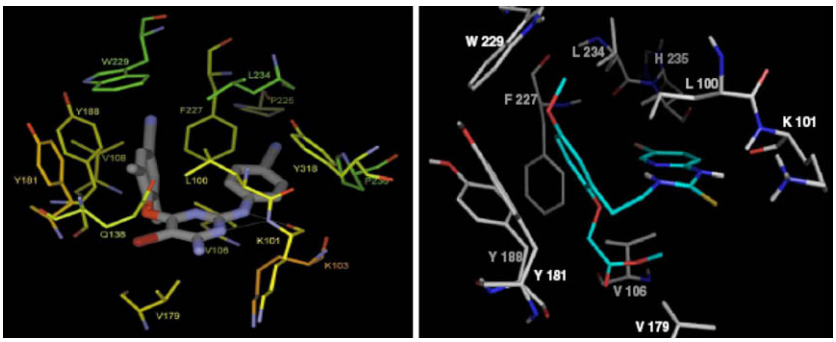


Figure 3. TMC125 in the NNRTI pocket[21] showing Y181, Glu138 and Val179 compared to our modeling[17] of an HI-236 derivative from Figure 2.

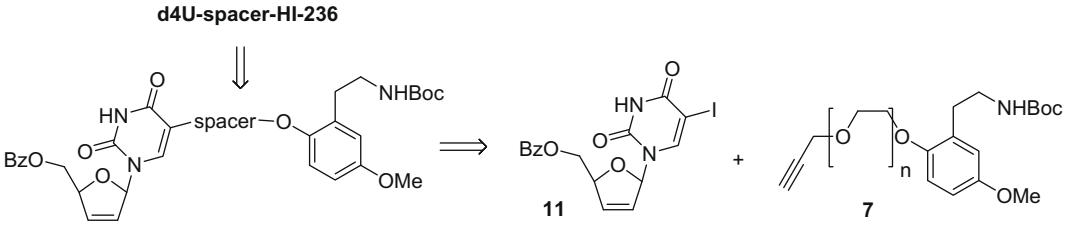


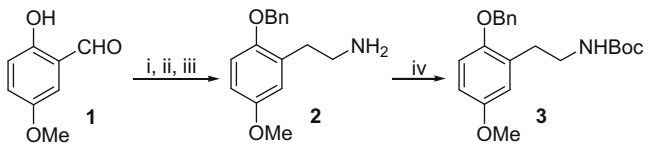
Figure 4. Retrosynthetic analysis of the d4U-spacer-HI-236 target.

an exit possibility from the pocket and may well explain why Laduree's '*para*-like' arrangement on the piperazine in his d4U-spacer-trovirdine derivative double-drug failed to accommodate the tethered grouping to the nucleoside into the pocket. In this regard, one must bear in mind the importance of the directing role that the bromopyridyl group of the thiourea plays via tight hydrogen bonding towards the front of the pocket in Wing 1 with K101.[17,18a]

Thus, based on these ideas and coupled with results from the earlier work mentioned previously,[16,17] we thought it feasible that a tethered [d4U]-spacer-[HI-236] might exit the pocket into the solvent channel close to Glu138[21] (p51 sub-unit shown in Fig. 3) and preferably closer to Tyr181 rather than Val179. On the assumption that the NNRTI would bind first, the NRTI of the double-drug would have to make its way to the substrate binding site around the corner behind the hydrophobic back of the NNRTI pocket near to the conserved Trp229. Figure 3 depicts the NNRTI TMC125[21] bound into the NNRTI pocket and helps to clarify this important issue.

3. Chemistry

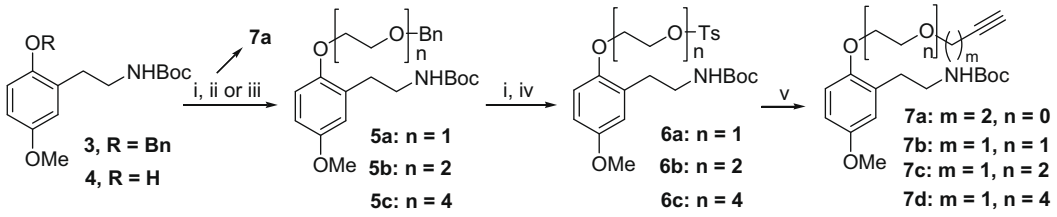
Typical of the art of total synthesis, the timing of key bond connections in the synthesis of the double-drugs proved to be crucial. A completely convergent synthesis via coupling of a tethered HI-236 alkyne to a protected derivative of 5-iodo-d4U using a Sonogashira Pd(0) coupling as the key and final step failed to give a significant yield of product, presumably due to interference from the nucleophilic thiourea sulfur. Thus, it was deemed necessary to bring the key coupling step forward in the sequence and introduce the HI-236 thiourea functionality late, and this approach gratifyingly turned out to be successful. Thus, the synthesis involved synthesis of two halves, a coupling step and an end-game as intimated in the retrosynthetic analysis shown in Figure 4.



Scheme 1. Reagents and conditions: (i) BnBr, K_2CO_3, EtOH, 80 °C; (ii) CH_3NO_2, NH_4OAc, 70 °C; (iii) $LiAlH_4$, THF, 70 °C; (iv) $(Boc)_2O$, CH_3CN, rt.

For the right-hand tethered derivatives **7**, the synthesis started with commercially available 2-hydroxy-5-methoxybenzaldehyde **1**, which following a three-step sequence described by Glennon[16,22] involving phenolic hydroxyl protection with benzyl, a Henry aldol reaction and LAH-mediated reduction of both the double bond and the nitro group afforded amine **2** in gram quantities, Scheme 1. For practical reasons it was easier to isolate **2** as its *N*-Boc derivative **3** via a standard Boc protection step without using DMAP, which promoted di-Boc derivatisation. A 50% overall yield for the four-step sequence to afford **3** could be achieved on scale-up.

Conversion of **3** to the tethered propargylated *N*-Boc derivatives **7** (Scheme 2) has been described previously[17] and was found to be optimal by propargylating in the final step. Thus, hydrogenolytic debenzylation of **3** to phenol **4** followed by alkylation with a monobenzyl-protected PEG bromide using sodium hydride in THF or potassium carbonate as base in acetonitrile at reflux gave the protected PEG-alkylated phenols **5a–c**. A second debenzylation followed by tosylation of the primary hydroxyl group gave tosylates **6a–c**. Finally, nucleophilic displacement by an excess of propargyloxide ion returned the best results overall for obtaining the final propargylated products **7b–d**, which were obtained as shown in Scheme 2, with the 4-PEG derivative considered to be well within striking distance of the substrate binding site. Each compound **7b–d** was exhaustively characterized[17] using ^1H, ^{13}C NMR spectroscopy as well as high resolution mass spectrometry (HRMS) and/or CHN combustion analysis. ^{13}C NMR spectroscopy was particularly useful for identifying the carbons of the PEG units to confirm that the correct length of tether was in place in each case, while ^1H NMR spectroscopy identified a characteristic fingerprint for the triad of aromatic signals with well-defined coupling relationships. Finally, the propargyl group could be easily discerned in both types of spectra. The exception in the library was the derivative without a PEG prepared by alkylation of phenol **4** with propargyl bromide, in which it was found that transformation into the



Scheme 2. Reagents and conditions: (i) H_2, Pd/C, EtOH, rt; (ii) 3-butynyl-1-tosylate, K_2CO_3, CH_3CN, 80 °C; (iii) $Bn(PEG)_nBr$ (n = 1, 2, 4), K_2CO_3, CH_3CN, 80 °C or NaH, DME, 80 °C; (iv) TsCl, Et_3N, DMAP, CH_2Cl_2, 0 °C–rt; (v) propargyl alcohol, NaH, THF, 70 °C.

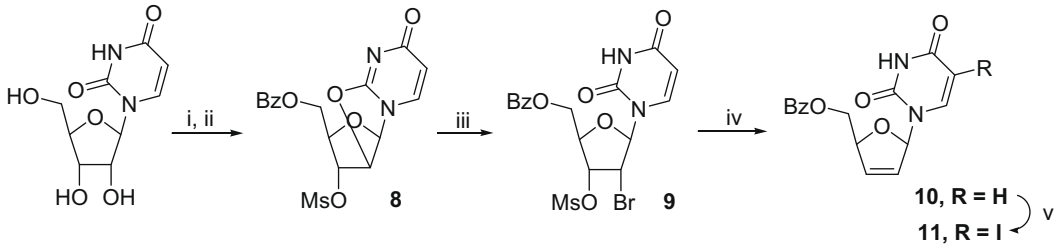
final double-drug via Pd(0) coupling and thiourea introduction resulted in an unstable product following debenzoylation, presumably as a result of having the two drugs in close proximity as well as the reactive nature of the propargylic linker. Fortunately, the stability of the final double-drug could be improved by extending the propargyl tether by one carbon atom using 3-butynyl-1-tosylate in the alkylation to afford intermediate compound **7a** as shown in Scheme 2.

For synthesis of the left-hand nucleoside partner, advantage was taken of the elegant Bristol-Myers Squibb process for converting 5-methyluridine into d4T[23] based on seminal work by Fox involving uridine.[24] This methodology could be carried out efficiently on a several-gram-scale with a single clean-up chromatography step at the end and was superior to other methodologies[25] such as that using acetyl bromide.[26] Thus, mesylation of the hydroxyl groups of uridine, followed by heating the product with sodium benzoate in acetamide furnished the 2′-anhydro nucleoside **8**.[24b] Subsequent opening using HBr (from AcBr in MeOH) to bromomesylate **9** followed by zinc-mediated elimination furnished the 5′-benzoate of d4U **10**[26a] as a crystalline solid. Interestingly, it was found that the zinc-elimination could be accelerated using 2% v/v concd HCl in the methanol solvent, presumably as a result of dissolution of surface oxide exposing metal surface, Scheme 3. Finally, iodination of **10** was accomplished using elemental iodine and ceric ammonium nitrate (CAN) in acetonitrile according to the procedure by Robins.[27] Running the reaction at around 40 °C for about five hours secured a high yield of the 5-iodo derivative **11** after chromatography (82%) with minimal by-product formation involving iodination of the dideoxyribose double bond.

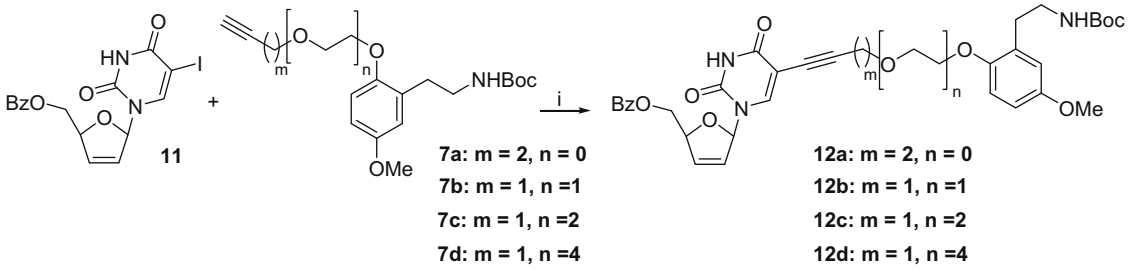
With the two coupling partners **7** and **11** in hand, attention was turned towards the key Sonogashira coupling. Literature precedent for such a reaction is well established following early work by Robins[28] and others,[29,30] and the topic for nucleosides has been recently reviewed.[31] Generally, the most encountered conditions use either $Pd(PPh_3)_4$ and $Pd(PPh_3)_2Cl_2$ as catalysts in varying mol % (around 20%) in deoxygenated DMF as solvent with triethylamine as base and with CuI in a 2:1 ratio to Pd catalyst. Excess alkyne ensures complete conversion of nucleoside in view of competing Glaser-type coupling of the alkyne, and one may form a furanopyrimidine cyclization by-product post Sonogashira coupling depending on the catalyst used.[32]

In the event, $Pd(PPh_3)_4$ was chosen as the Pd(0) catalyst. Generally, using $Pd(PPh_3)_4$ (10 mol %) with CuI (50%) at a higher ratio than normal with alkyne at 1.1 equiv in a deoxygenated DMF/THF (1:2) solvent medium furnished coupled products **12a–d** by tlc within 3–4 h at rt. Following the usual extractive work-up with aq disodium EDTA to remove metal salts, chromatography furnished the requisite coupled products in around a 70% isolated yield. Product integrity could be relatively easily discerned by observing representative resonances for each coupling partner in the ^1H or ^{13}C spectra. Importantly, the two NH signals could be discerned in the ^1H NMR spectra (in $CDCl_3$) at 8.50–9.00 for the pyrimidine signal and close to 5.00 ppm for the carbamate NH. Diagnostic peaks for key functional groups such as the triple bond could be identified in the IR spectrum, and HRMS using electrospray returned correct molecular ions in each case, Scheme 4.

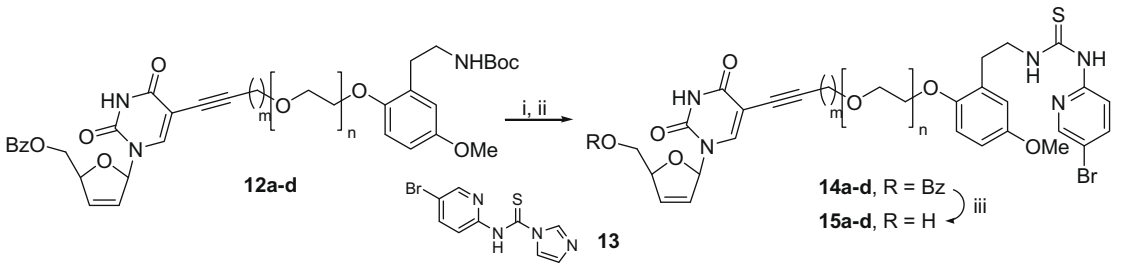
The end-game to the double-drug targets involved a three-step sequence involving Boc-deprotection, thiourea coupling and benzoate deprotection to the free nucleoside. Some concern was entertained regarding the two deprotection steps, particularly the first one involving trifluoroacetic acid as the standard reagent for Boc-deprotection. In the event, following some exhaustive optimization, it was established that exposing products **12a–d** to TFA at 0 °C in DCM resulted in deprotection in about 2 h to a more polar amine spot on TLC. In view of the anticipated water solubility of the amine, the final step was conducted without using an aqueous work-up. Thus, following addition of Hünig's base ($EtN(i\text{-}Pr)_2$) and the complete removal of all volatiles, the residue was redissolved in THF and the thiocarbonyl reagent **13** added according to the original procedure[33] described by the Eli Lilly group in their work on PETT NNRTIs. Reagent **13** could be readily prepared by reacting 2-amino-5-bromopyridine with 1,1′-thiocarbonyldiimidazole in acetonitrile at room temperature for 12 h to afford a precipitate that was used without purification. In our case, condensation between the amine and **13** could be realized in THF or DMF at room temperature overnight to afford the final double-drug targets **14** as their 5′-benzoate esters in about 60% over the two steps following chromatography. The lower temperature for condensation with **13** compared to the 100 °C (in DMF) used[33] by the Eli-Lilly group made a significant improvement to the overall yield. Thereafter, methoxide-catalysed benzoate deprotection in MeOH at 0 °C furnished the final targets **15**. Of crucial importance in this step in or-



Scheme 3. Reagents and conditions: (i) MsCl (3 equiv), pyr, 0 °C; (ii) NaBz (3 equiv), acetamide, 120 °C; (iii) CH_3COBr (5 equiv), EtOAc/MeOH (10:1), 70 °C; (iv) Zn, concd HCl (2% by vol), MeOH, rt; (v) CAN (0.6 eq), I_2 (0.6 eq), CH_3CN, 35 °C.



Scheme 4. Reagents and conditions: (i) $Pd(PPh_3)_4$ (10 mol %), CuI (50 mol %), Et_3N (2 equiv), DMF/THF (1:2), rt.



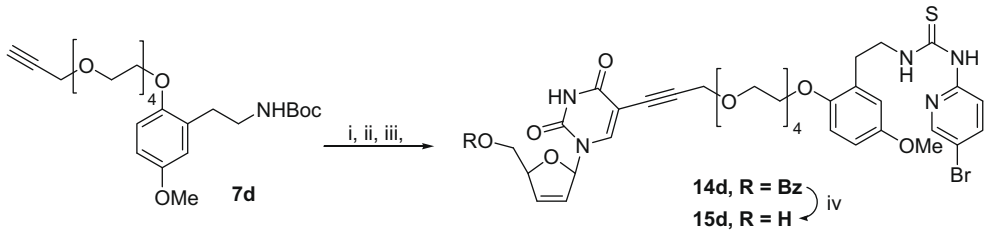
Scheme 5. Reagents and conditions: (i) TFA, CH_2Cl_2, 0 °C; (ii) $EtN(i\text{-}Pr)_2$ followed by **13**, THF, rt/overnight; (iii) NaOMe, MeOH, 0 °C.

der to avoid degradation was the mode of isolation, for which the best procedure turned out to be quenching with a minimal amount of glacial acetic acid followed by direct rapid flash-chromatography. In such a way one could minimise cleavage of the nucleoside moiety. Once isolated, though, the double-drugs were stable enough for biological testing and evaluation purposes. All final double-drug products were solids that could be exhaustively characterised by a full complement of spectroscopic techniques (see Section 5). However, their recrystallization tended to promote some ribose nucleoside cleavage, so HRMS (ES) was used to successfully provide an accurate molecular ion in each case, Scheme 5.

The one derivative that eluded realization was the 4-PEG derivative **15d**, as the product following TFA deprotection and condensation with **13** was found to lack the sugar ring by NMR spectroscopy. Since Sonogashira reactions are well known[34] to proceed in the presence of unprotected hydroxyl or amino groups, it was decided to revise the order of events in the sequence and deprotect the Boc-protecting group of alkyne **7d** first. Thereafter, following neutralization of TFA using K_2CO_3 in methanol and filtration of the salts the crude amine was subjected to the Sonogashira reaction and thiourea condensation steps to afford the 4-PEG double-drug **14d** as its 5′-benzoate in 40% yield over the three steps after chromatography. Deprotection with methoxide as usual furnished the final free nucleoside double-drug **15d** in a modest yield of 52%, Scheme 6.

The ^1H NMR and ^{13}C NMR spectra illustrated in Figure 5 for **15d** showing all of the required signals gave us great satisfaction.

As a final piece of synthesis, it was decided to prepare a pronucleotide of **15d** as the double-drug most likely to reach the DNA site in view of the 4-PEG spacer. The pronucleotide approach for enhancing nucleoside activity by bypassing the first rate-determining kinase-mediated phosphorylation step is well established with several variants on masked phosphate groupings. CycloSal[35] and phosphoramidate[36] functionalities provide two of the most frequently used options and we chose the latter in view of its proven ability to enhance the activity of d4T[37] as well as the likelihood of accessing it from the nucleoside double-drug **15d** in a single step. However, when **15d** was reacted with *p*-tolyl methoxyalaninyl phosphorochloridate prepared[38] according to a McGuigan published procedure with *N*-methylimidazole as a transfer base, no phosphoramidate could be isolated and only breakdown of the double-drug was observed by TLC. Given the relative robustness of the phosphoramidate grouping, it was thought that it could replace benzoate in the Sonogashira coupling. However, given that we doubted its stability towards TFA, we adopted the strategy developed for the 4-PEG double-drug **15d**. Thus, Boc-deprotection of *N*-Boc alkyne **7d** to the free amine as described before in Scheme 6 followed by Sonogashira coupling of the resultant amine with the phosphoramidate of 5-iodo-d4U **16** prepared from 5-iodo-d4U by a literature method,[39] and finally coupling of the amino group of the coupled product with **13** gave the target thiourea double-drug phosphoramidate **17** as a nearly 50:50 mixture of diastereomers at phosphorus in an overall yield of 20% for the three steps, Scheme 7. Given our experience in deriving full assignments



Scheme 6. Reagents and conditions: (i) TFA, CH_2Cl_2, 0 °C; (ii) **11**, $Pd(PPh_3)_4$ (10 mol %), CuI (50 mol %), Et_3N (2 equiv), DMF/THF (1:2), rt; (iii) **13**, THF, rt; (iv) NaOMe (cat), MeOH, 0 °C.

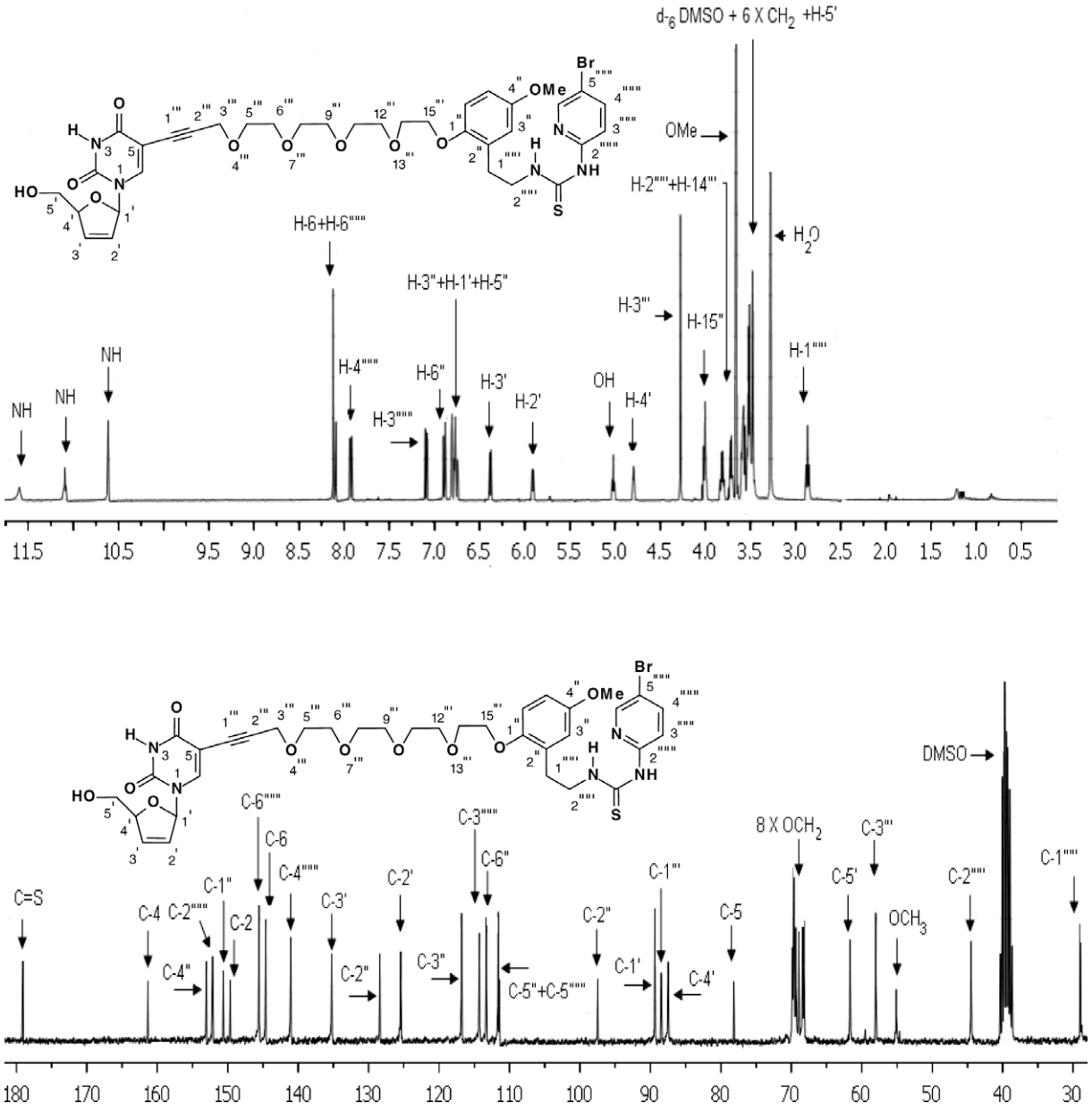
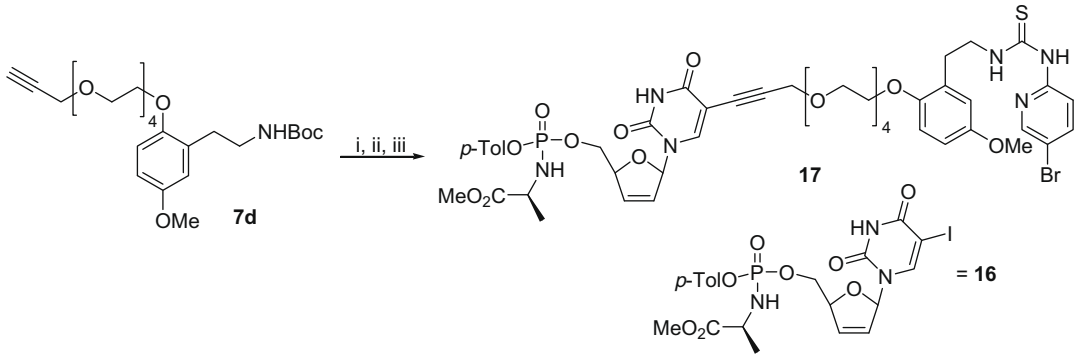


Figure 5. 400 MHz ^{1}H NMR and 75 MHz ^{13}C NMR spectra of **15d** in DMSO-d_6.

for the other double-drugs, **17** could be exhaustively characterized using a combination of both 1D and 2D ^{1}H, ^{13}C and ^{31}P NMR spectroscopic techniques in spite of the diastereomeric mixture.

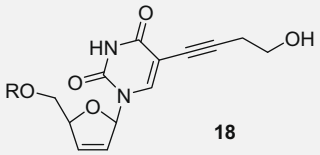
4. Biological results and discussion

The inhibitory activities of the bifunctional compounds **15a–d** and phosphoramidate **17** together with HI-236 and d4T as controls were measured against HIV-1 (IIIB) replication in MT-2 cell culture using an MTT assay.[40] The same compounds were also evaluated for their in vitro activity against RT directly in a steady-state RT inhibition assay using a D23/D36 primer/template in which the inhibition of incorporation of thymidine triphosphate (TTP) by each double-drug was measured as an IC_{50}. The results are shown in Table 1 expressed in μM units. [d4U]-Butyne-[HI-236] 15a showed a good inhibitory activity with an EC_{50} = 250 nM in the cell-culture assay as nine times more potent than d4T (EC_{50} = 2.3 μM) alone, and ca. six times less potent than HI-236 (EC_{50} = 0.042 μM) and thus closer in activity to the NNRTI component. The compound was ca. twofold less potent than HI-236 in the RT assay with IC_{50} values of HI-236 and **15a** (38 and 61 nM respectively) both improving relative to the cell-culture EC_{50} results. This was not unexpected in view of aspects of cell permeability and the greater possibility for degradation in the cell-culture experiment. Lengthening the spacer resulted in a steady reduction in activity (0.25, 1.3, 1.9 and 3.1 μM for **15a–d** respectively) in cell-culture. Notably, the 4-PEG derivative **15d** still retained appreciable activity (3.1 μM), remarkably so for such a large molecule. As with **15a**, the RT IC_{50} values for **15b–d** were similarly lower than their EC_{50} cell-culture values, with nanomolar activities for **15b** and **15c**. Disappointingly, the 4-PEG phosphoramidate **17** showed no significant improvement (3.1 to 2.9 μM) in cell culture compared to its unphosphorylated counterpart **15d**, effectively indicating that triphosphorylation of pro-drugs **15a–d** and **17** in cell-culture does not occur. The superior activity of the 4-PEG double-drug nucleoside **15d** in the RT assay compared with that of its phosphoramidate **17** (1.4 μM vs 2.3 μM) suggests binding of



Scheme 7. Reagents and conditions: (i) TFA, CH_2Cl_2, 0 °C; (ii) **16**, $Pd(PPh_3)_4$ (10 mol %), CuI (50 mol %), Et_3N (2 equiv), DMF/THF (1:2), rt; (iii) **13**, THF, rt.

Table 1
A comparison of cell-culture versus in vitro HIV-1 RT inhibition (μM) for double-drugs **15a–d** and **17**

Compound	Cell-culture EC_{50}[a] (μM)	Cell-culture CC_{50}[b] (μM)	RT assay[c] (μM)
HI-236	0.042	>1	0.038 ± 0.007
d4T	2.3	>100	9.6[d]
15a	0.25	17	0.061 ± 0.015
15b	1.3	38	0.575 ± 0.14
15c	1.9	43	0.850 ± 0.14
15d	3.1	18	1.4 ± 0.5
17	2.9	11	2.3 ± 0.8
 **18**	120[41]		N/A

[a] Effective concentration that inhibits viral-mediated T-cell death by 50%, determined by averaging samples of each concentration in triplicate.
[b] Concentration that kills 50% of the T-cells, also determined by averaging triplicate samples.
[c] Concentration of inhibitor that inhibits by 50% the steady-state thymidine incorporation into a D23/D36 primer-template as catalysed by RT.
[d] Based on pre-steady-state kinetic analysis.

the 5′-hydroxyl group of **15d**, which is considered to likely be taking place near to or at the substrate binding site.

Our results indicate that the activity of double-drugs **15a–d** and **17** is mainly due to the NNRTI component, with the size of the double-drugs suggesting that **15b–d** and **17** have the nucleoside drug outside of the NNRTI pocket. Although the data does not allow a firm conclusion to be made regarding the possibility or nature of NRTI binding possibilities, the relatively potent cell-culture EC_{50} values of 1.3 μM and 1.9 μM for 1-PEG and 2-PEG derivatives **15b,c** respectively (more active than d4T alone (2.3 μM)) strongly suggests the possibility of some cooperative binding outside of the pocket. If this is the case, the fact that Ladurée has shown the 5-alkynylated analogue **18**[41] (Table 1) to be completely inactive would support the view that the nucleoside part of the double-drugs **15b,c** may well forge cooperative interactions away from the substrate binding site, although derivatives **15d** and **17** containing the longer 4-PEG tether could well be interacting cooperatively near to the binding site based on anchoring from the NNRTI pocket. Such conclusions echo those of Monneret and coworkers based on the activities of their AZT-tether-HEPT bifunctionals in which activities also suggested out-of-pocket binding but without synergy between drug-sites, and to a lesser extent to those of Camarasa based on her nucleoside-spacer-TSAO double-drugs, since TSAO supposedly binds just outside of the conventional NNRTI pocket. The failure to achieve synergy in the double-drugs **15a–d** as well as the other systems mentioned may be mainly ascribed to the inability of cellular kinases to recognize the nucleoside portion of the double-drug.[39a] This conclusion unfortunately also applies to the monophosphate of **15d**, assuming that phosphoramidate **17** is hydrolysed to such a monophosphate within the cell-culture as a by-pass of the first rate-limiting phosphorylation. On a positive note, our results suggest that it might be worthwhile to consider designing extended NNRTI inhibitors containing a second binding agent out of but near to the front of the pocket. Given the plethora of X-ray structures of NNRTIs available,[4a] it should be possible to model certain residues for this purpose, for example, Glu138 on the outside of the pocket.[10] The original double-drug suggestion by Arnold[5] and Steitz[6] did not consider how the two drug-sites might communicate structurally in practice. Regarding the kinase recognition problem just mentioned, targeting protected triphosphates of double-drugs would present some serious challenges regarding their synthesis and stability. Moreover, communication between the two sites is made difficult if the intention is for the tether to exit the NNRTI binding pocket, since the front of the pocket points away from the substrate binding site. However, the possibility that the two drugs may communicate between the two sites by virtue of the tether exiting the NNRTI pocket from the back end close to Trp 229 provides a more direct connection. Results on this will be communicated in due course involving the diarylpyrimidine TMC-120 as the NNRTI component of such a double-drug system.

5. Experimental

5.1. General procedures for synthesis

Infrared (IR) absorptions were measured on a Perkin–Elmer Spectrum One FT-IR spectrometer. ^{1}H NMR spectra were recorded

on a Varian Mercury Spectrometer at 300 MHz or a Varian Unity Spectrometer at 400 MHz with Me_4Si as internal standard. ^{13}C NMR spectra were recorded at 75 MHz on a Varian Mercury Spectrometer or at 100 MHz on Varian Unity Spectrometer with Me_4Si as internal standard. High resolution mass spectra were recorded on a VG70 SEQ micromass spectrometer. Melting points were determined using a Reichert-Jung Thermovar hot-stage microscope and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on aluminium-backed Silica-Gel 60 F_{254} (70–230 mesh) plates. Column chromatography was performed with Merck Silica-Gel 60 (70–230 mesh).

Compounds **2**,[22] **5a**,[17] **6a**,[17] **6b**,[17] **7b**,[17] **7c**,[17] and **13**[33] have all been reported previously. 3-Butynyl 1-*p*-toluenesulfonate[42] was prepared from the alcohol by a standard tosylation procedure. The monobenzyl PEG-Br alkylating agents for formation of **5b** and **5c** were prepared by standard mono-benzylation and bromination (using PPh_3 and CBr_4) methods from the corresponding glycol.

5.2. Synthesis of intermediates for the coupling partners 7 and 11

5.2.1. *N*-[2-(2-Benzyloxy-5-methoxyphenyl)ethyl]-*tert*-butylcarbamate 3

Di-*tert*-butyldicarbonate (5.22 g, 23.93 mmol) in acetonitrile (6 mL) was added to a solution of crude amine **2** (4.10 g, 15.95 mmol) in acetonitrile (50 mL) and the reaction mixture was stirred at rt for 20 h. Aqueous NH_4Cl (100 mL) was added and the organic material extracted into EtOAc (3 × 100 mL). Following drying and evaporation of solvent the residue was subjected to column chromatography employing EtOAc/petroleum ether (15/85) to give carbamate **3** as colourless crystals (5.00 g, 88%); mp: 102–104 °C; IR ($CHCl_3$): ν_{max} 3449 (NH), 3007, 2935 (C–H), 1703 (C=O), 1501 (C=C), 1165 (C–N) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.45–7.34 (5H, m,), 6.85 (1H, d, J = 8.8 Hz,), 6.76 (1H, d, J = 2.8 Hz), 6.72 (1H, dd, J = 2.8, 8.8 Hz), 5.04 (2H, s,), 4.70 (1H, br s, NH), 3.78 (3H, s), 3.40 (2H, q, J = 6.4 Hz), 2.85 (2H, t, J = 6.4 Hz), 1.44 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.9 (C=O), 153.8, 150.9, 137.4, 129.2, 128.5, 127.8, 127.2, 116.8, 113.0, 112.0, 79.0, 70.8, 55.7, 40.8, 30.9, 28.4; HRMS (EI): *m/z* found 301.13383 [(M^+–*t*-butyl)+H]. $C_{17}H_{19}NO_4$ requires 301.13409 [(M^+–*t*-butyl)+H]; $C_{21}H_{27}NO_4$ requires C, 70.56; H, 7.61; N, 3.92. Found: C, 70.50; H, 7.62; N, 3.86.

5.2.2. *N*-[2-(2-Hydroxy-5-methoxyphenyl)ethyl]-*tert*-butylcarbamate 4

The carbamate **3** (4.50 g, 12.61 mmol) was added to 10% palladium-on-carbon (0.22 g, 1.26 mmol) in ethanol. Hydrogen gas was introduced to the reaction at rt for 5 h. The palladium was filtered from the solution through Celite and the precipitate was washed with ethanol (3 × 100 mL). The solvent was removed under reduced pressure and the residue was subjected to column chromatography using EtOAc/petroleum ether (4/6) to give **4** as a colourless solid (2.50 g, 74%); mp: 115–117 °C; IR ($CHCl_3$): ν_{max} 3457, 3326 (NH, OH), 1686 (C=O), 1508 (C=C), 1210, 1166 (C–N) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 6.99 (1H, br s, OH), 6.79 (1H, d, J = 8.4 Hz), 6.67 (1H, d, J = 2.8 Hz), 6.65 (1H, m), 5.00 (1H, br s, NH), 3.75 (3H, s), 3.33 (2H, q, J = 7.1 Hz), 2.81 (2H, t, J = 7.1 Hz), 1.46 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 157.0 (C=O), 153.2, 148.8, 126.0, 116.5, 116.2, 112.8, 80.0, 55.8, 41.0, 31.4, 28.4; HRMS (EI): *m/z* found 267.14329 (M^+). $C_{14}H_{21}NO_4$ (M^+) requires 267.14706; $C_{14}H_{21}NO_4$ requires C, 62.94; H, 7.92; N, 5.24. Found C, 62.82; H, 7.92; N, 5.16.

5.2.3. *N*-[2-(2-(5-Benzyloxy-3-oxapent-1-yloxy)-5-methoxyphenyl)ethyl]-*tert*-butylcarbamate 5b

2-(2-Benzyloxy)ethoxy-1-bromoethane (0.87 g, 3.36 mmol) in dry acetonitrile (10 mL) was added dropwise over 1 h to a refluxing and stirring mixture of phenol **4** (0.45 g, 1.68 mmol) and anhydrous potassium carbonate (0.93 g, 6.70 mmol) in dry acetonitrile (20 mL). After 20 h the mixture was filtered, the solvent evaporated and the residue subjected to silica-gel column chromatography using EtOAc/petroleum ether (1/9) to give **5b** as a colourless oil (0.61 g, 81%.); IR ($CHCl_3$): ν_{max} 3442, 3377 (NH, OH), 3007, 2928 (C–H), 1697 (C=O), 1501 (C=O), 1227 (C–N) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.31 (4H, m), 7.21 (1H, m), 6.76 (1H, d, J = 8.7 Hz), 6.70 (1H, d, J = 2.8 Hz), 6.68 (1H, dd, J = 2.8, 8.7 Hz), 4.83 (1H, br s, NH), 4.55 (2H, s), 4.06 (2H, t, J = 4.8 Hz), 3.82 (2H, t, J = 4.8 Hz), 3.72 (3H, s), 3.70 (2H, m), 3.62 (2H, m), 3.31 (2H, q, J = 6.5 Hz), 2.76 (2H, t, J = 6.5 Hz), 1.38 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.0 (C=O), 153.8, 151.0, 138.2, 129.4, 128.4, 127.7, 127.6, 116.6, 113.1, 112.0, 78.8, 73.3, 70.8, 69.9, 69.5, 68.5, 55.6, 40.7, 31.0, 28.4; HRMS (EI): *m/z* found 445.24814 (M^+). $C_{25}H_{35}NO_6$ (M^+) requires 445.24644.

5.2.4. *N*-[2-(2-(11-Benzyloxy-3,6.9-trioxaundec-1-yloxy)-5-methoxyphenyl)ethyl]-*tert*-butylcarbamate 5c

11-Benzyloxy-1-bromo-3,6.9-trioxaundecane (0.91 g, 2.62 mmol) in dry acetonitrile (10 mL) was added dropwise over 1 h to a refluxing and stirring mixture of phenol **4** (0.35 g, 1.31 mmol) and anhydrous potassium carbonate (0.72 g, 5.20 mmol) in dry acetonitrile (20 mL). After 20 h the mixture was filtered, the solvent evaporated and the residue subjected to silica-gel column chromatography using EtOAc/petroleum ether (1/9) to give **5c** as a colourless oil (0.56 g, 80%.); IR ($CHCl_3$): ν_{max} 3681, 3449 (N–H), 3021, 2913 (C–H), 1700 (C=O), 1501 (C=C) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.34 (4H, m), 7.21 (1H, m), 6.78 (1H, d, J = 8.8 Hz), 6.72 (1H, d, J = 3.0 Hz), 6.70 (1H, dd, J = 3.0 Hz, 8.8 Hz), 4.90 (1H, br s, NH), 4.57 (2H, s), 4.08 (2H, t, J = 4.9 Hz), 3.83 (2H, t, J = 4.9 Hz), 3.76 (3H, s), 3.72–3.68 (10H, m), 3.64 (2H, m), 3.35 (2H, q, J = 6.7 Hz), 2.80 (2H, *t*, J = 6.7 Hz), 1.43 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.9 (C=O), 153.7, 151.0, 138.3, 129.3, 128.3, 127.6, 127.5, 116.6, 113.0, 111.9, 78.7, 73.1, 78.7, 70.6, 70.6, 69.8, 69.4, 68.4, 55.6, 40.6, 31.0, 28.4; HRMS (ES): *m/z* found 534.3036 (M^++H). $C_{29}H_{44}NO_8$ requires (M^++H) 534.3067.

5.2.5. *N*-[2-(2-(11-*p*-Toluenesulphonyloxy-3,6.9-trioxaundec-1-yloxy)-5-methoxyphenyl)ethyl]-*tert*-butylcarbamate 6c

Triethylamine (0.18 mL, 1.36 mmol) was added to a stirring solution in dry CH_2Cl_2 (5 mL) at 0 °C of the alcohol (0.50 g, 1.13 mmol) obtained from hydrogenolysis of **5c**. *p*-Toluenesulfonyl chloride (0.26 g, 1.36 mmol) was added together with a catalytic amount of DMAP (20 mg). The reaction mixture was stirred at rt for 16 h, then diluted with CH_2Cl_2 (80 mL) and the organic layer washed with aq NH_4Cl (25 mL), water (30 mL), dried over $MgSO_4$ and the solvent removed under reduced pressure. Purification by column chromatography EtOAc in petroleum ether (8/2) gave **6c** as a colorless oil (0.58 g, 84%); IR ($CHCl_3$): ν_{max} 3377 (NH), 2928 (C–H), 1707 (C=O), 1501 (C=C), 1223 (C–N), 1176 (O–SO_2–) cm $^{-1}$; ^1H NMR (300 MHz, $CDCl_3$): δ 7.77 (2H, d, J = 8.4 Hz), 7.31 (2H, d, J = 8.4 Hz), 6.76 (1H, d, J = 9.2 Hz), 6.68 (2H, m), 4.85 (1H, br s, NH), 4.14 (2H, t, J = 4.8 Hz), 4.06 (2H, t, J = 4.8 Hz), 3.81 (2H, t, J = 4.8 Hz), 3.73 (3H, s), 3.69–3.57 (10H, m), 3.32 (2H, q, J = 6.5 Hz), 2.77 (2H, t, J = 6.5 Hz), 2.42 (3H, s), 1.40 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.9 (C=O), 153.8, 151.0, 144.7, 133.0, 129.7, 129.3, 127.9, 116.6, 113.0, 111.9, 78.8, 70.7 (3 × CH_2O), 70.5 (CH_2O), 69.8 (CH_2O), 69.2 (CH_2O), 68.6 (CH_2O), 68.4 (CH_2O), 55.6, 40.6, 31.0, 28.4, 21.5; HRMS (ES): *m/z* found 598.2673 (M^++H), $C_{29}H_{44}NO_{10}S$ requires (M^++H) 598.2686.

5.2.6. *N*-[2-(2-(3-Butynyl-1-oxy)-5-methoxyphenyl)ethyl]-*tert*-butylcarbamate 7a[16]

3-Butynyl-1-*p*-toluenesulfonate (1.34 g, 6.00 mmol), phenol **4** (0.80 g, 3.00 mmol) and anhydrous potassium carbonate (1.70 g,

12.0 mmol) were refluxed in dry acetonitrile (30 mL). After 20 h the mixture was filtered, the solvent evaporated and the residue subjected to silica-gel column chromatography using EtOAc/petroleum ether (1/9) to give to give **7a** as colourless needles (0.57 g, 61%); mp: 76–77 °C; IR ($CHCl_3$): ν_{max} 3455 (NH), 3309 (≡CH), 2413 (C≡C), 1707 (C=O), 1602 (C=C) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 6.75 (3H, m), 4.70 (1H, br s, NH), 4.05 (2H, t, J = 6.8 Hz), 3.75 (3H, s), 3.36 (2H, q, J = 6.6 Hz), 2.79 (2H, t, J = 6.6 Hz), 2.66 (2H, dt, J = 2.7, 6.8 Hz), 2.03 (1H, t, J = 2.7 Hz), 1.42 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.9 (C=O), 153.9, 150.6, 129.3, 116.8, 112.8, 112.0, 80.7, 78.9, 69.8, 66.8, 55.6, 40.7, 30.9, 28.4, 19.7; HRMS (EI): m/z found 319.17756 (M^+). $C_{18}H_{25}NO_4$ (M^+) requires 319.17836; $C_{18}H_{25}NO_4$ requires C, 67.89; H, 7.89; N, 4.39. Found: C, 67.10; H, 7.66; N, 3.67.

5.2.7. *N*-[2-(2-(11-Propargyloxy-3,6.9-trioxaundec-1-yloxy)-5-methoxyphenyl)ethyl]-*tert*-butylcarbamate 7d

To a stirred suspension of NaH (0.12 g, 60% in mineral oil, 3.00 mmol) in THF (10 mL) at 0 °C was added propargyl alcohol (252 mg, 4.50 mmol) dissolved in THF (2 mL) dropwise. The mixture was refluxed for 30 min and a solution of **6c** (435 mg, 0.73 mmol) in THF (10 mL) then added dropwise. After refluxing for 20 h, a solution of aq NH_4Cl (20 mL) was added and the organic material extracted into EtOAc (3 × 30 mL). The combined organic extracts were washed with water (2 × 20 mL), dried ($MgSO_4$) and the solvent removed to afford a residue, which was purified by column chromatography (15% EtOAc in petroleum ether) to afford **7d** (0.290 g, 83%) as a colourless oil; IR ($CHCl_3$): ν_{max} 3449 (NH), 3304 (≡CH), 3007, 2920 (C–H), 2116 (C≡C), 1703 (C=O), 1501 (C=C), 1227 (C–N) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 6.77 (1H, d, J = 8.8 Hz), 6.68 (2H, m), 4.88 (1H, br s, NH), 4.18 (2H, d, J = 2.1 Hz), 4.07 (2H, t, J = 4.8 Hz), 3.82 (2H, t, J = 4.8 Hz), 3.74 (3H, s), 3.72–3.64 (12H, m, 6 × CH_2O), 3.34 (2H, q, J = 6.5 Hz), 2.78 (2H, t, J = 6.5 Hz), 2.40 (1H, t, J = 2.1 Hz), 1.41 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.0 (C=O), 153.8, 151.0, 129.3, 116.6, 113.0, 112.0, 79.7, 78.8, 74.4, 70.8 (CH_2O), 70.7 (CH_2O), 70.6 (2 × CH_2O), 70.4 (CH_2O), 69.9 (CH_2O), 69.1 (CH_2O), 68.5 (CH_2O), 58.3, 55.6, 40.6, 31.0, 28.4; HRMS (ES): m/z found 482.2732 (M^++H). $C_{25}H_{40}NO_8$ requires (M^++H) 482.2754.

5.2.8. 5′-Benzoyl-5-iodo-d4U 11

To a solution of 5′-*O*-benzoyl-d4U **10** (0.88 g, 2.8 mmol)[26a] in CH_3CN (30 mL), were added cerium ammonium(IV) nitrate (0.92 g, 1.68 mmol) and iodine (0.43 g, 1.68 mmol). The mixture was stirred at 35 °C for 4 h before being diluted with a solution of sodium bisulfite (50 mL) and extracted with EtOAc (3 × 50 mL). The organic layer was dried over $MgSO_4$ and the solvent reduced under vacuum. Recrystallization from CH_2Cl_2/pet ether gave **11** as colourless needles (1.05 g, 85%): mp 168–169 °C (EtOAc, MeOH); ^1H NMR (300 MHz, $CDCl_3$): δ 11.78 (1H, br s, NH), 7.96 (2H, d, J = 7.9 Hz), 7.73 (1H, s), 7.67 (1H, m), 7.55 (2H, t, J = 7.9 Hz), 6.76 (1H, m), 6.54 (1H, dt, J = 1.7, 6.0 Hz), 6.12 (1H, dq, J = 1.5, 6.0 Hz), 5.18 (1H, m), 4.52 (2H, m); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.5, 160.2, 150.1, 144.0, 133.5, 133.3, 129.2, 129.0, 128.7, 126.5, 90.0, 84.0, 69.6, 65.3; HRMS (ES): m/z found 440.9930 (M^++H). $C_{16}H_{14}IN_2O_5$ requires (M^++H) 440.9942; $C_{16}H_{13}IN_2O_5$ requires C, 43.12; H, 2.97; N, 6.04. Found C, 43.66; H, 2.98; N, 6.36.

5.3. General procedure for Sonogashira coupling for compounds 7a–c to afford 12a–c

A solution of 5′-benzoyl-5-iodo-d4U **11** (0.5 mmol, 1 equiv) in dry DMF (2.5 mL) was added to a stirred solution of triethylamine (1.0 mmol, 2 equiv) and alkyne **7a–d** (0.6 mmol, 1.2 equiv) in THF (4 mL). The mixture was thoroughly degassed with nitrogen for 1 h. CuI (0.25 mmol, 0.5 equiv) and $Pd(PPh_3)_4$ (0.05 mmol, 0.1 equiv) were added to the degassed solution under a nitrogen atmosphere. The mixture was left stirring at rt for 4 h before adding 5% aq disodium EDTA (30 mL) and extracting with $CHCl_3$ (3 × 30 mL). Washing with water (15 mL), drying over $MgSO_4$, filtration and solvent evaporation under reduced pressure gave a residue that was purified by column chromatography employing EtOAc in petroleum ether mixtures to give the product as a yellow oil.

5.3.1. 5-{4-[2-(2-*tert*-Butoxycarbonylaminoethyl)-4-methoxyphenoxy]but-1-ynyl}-5′-*O*-benzoyl-2′,3′-didehydro-2′,3′-dideoxyuridine 12a

Using **11** (70 mg, 0.20 mmol) with alkyne **7a** (0.10 g, 0.30 mmol) gave **12a** (95 mg, 94%): $[\alpha]_D$ −14.4 (*c* 1.60, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3692, 3606, 3451 (NH), 3011, 2934 (C–H), 2243 (C≡C), 1707 (C=O), 1502 (C=C) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 8.79 (1H, br s), 8.00 (2H, d, J = 7.7 Hz), 7.61 (1H, s), 7.53 (1H, t, J = 7.7 Hz), 7.42 (2H, t, J = 7.7 Hz), 6.91 (1H, m), 6.70 (3H, m), 6.39 (1H, dt, J = 1.7, 5.8 Hz), 5.99 (1H, dq, J = 1.4, 5.8 Hz), 5.20 (1H, m), 4.83 (1H, br s), 4.64 (1H, dd, J = 4.3, 12.5 Hz), 4.50 (1H, dd, J = 3.0, 12.5 Hz), 3.94 (2H, t, J = 6.8 Hz), 3.74 (3H, s), 3.32 (2H, m), 2.77 (2H, t, J = 6.9 Hz), 2.68 (2H, t, J = 6.8 Hz), 1.40 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.2, 161.4, 153.9, 153.9, 150.5, 149.4, 141.6, 133.5, 133.3, 132.0, 129.7, 129.2, 128.6, 127.0, 116.7, 112.9, 112.0, 100.7, 91.3, 90.7, 85.0, 78.8, 72.4, 66.7, 65.1, 55.6, 40.6, 31.0, 28.4, 20.8; FAB HRMS: m/z found 654.24300 [M+Na]$^+$. $C_{34}H_{37}N_3O_9Na$ [M+Na]$^+$ requires 654.24274.

5.3.2. 5-{6-[2-(2-*tert*-Butoxycarbonylaminoethyl)-4-methoxyphenoxy]hexa-4-oxa-1-ynyl}-5′-O-benzoyl-2′, 3′-didehydro-2′, 3′-dideoxyuridine 12b

Using **11** (0.30 g, 0.68 mmol) with alkyne **7b** (0.26 g, 0.75 mmol) gave **12b** (0.34 g, 76%): $[\alpha]_D$ +16.2 (c 1.0, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3449, 3384 (NH), 3007, 2928 (C–H), 2246 (C≡C), 1711 (C=O), 1501 (C=C), 1169 (C-N) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 8.42 (1H, br s), 8.01 (2H, d, J = 7.5 Hz), 7.68 (1H, s), 7.56 (1H, m), 7.45 (2H, t, J = 7.5 Hz), 6.92 (1H, m), 6.76 (1H, d, J = 8.7 Hz), 6.68 (2H, m), 6.40 (1H, dt, J = 1.7, 6.0 Hz), 6.00 (1H, dq, J = 1.3, 6.0 Hz), 5.21 (1H, m), 4.94 (1H, br s), 4.69 (1H, dd, J = 4.2, 12.5 Hz), 4.50 (1H, dd, J = 3.0, 12.5 Hz), 4.27 (2H, s), 4.07 (2H, t, J = 4.5 Hz), 3.85 (2H, m), 3.74 (3H, s), 3.33 (2H, m), 2.78 (2H, t, J = 6.9 Hz), 1.42 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.2, 161.3, 156.0, 153.8, 151.0, 149.4, 142.2, 133.6, 133.5, 129.7, 129.3, 129.2, 128.6, 127.0, 116.5, 113.0, 111.9, 99.9, 90.8, 90.0, 85.1, 78.8, 77.2, 68.4, 68.2, 65.0, 59.0, 55.6, 40.6, 30.8, 28.4; HRMS (ES): m/z found 662.2698 (M^++H), $C_{35}H_{40}N_3O_{10}$ requires (M^++H) 662.2714.

5.3.3. 5-{9-[2-(2-*tert*-Butoxycarbonylaminoethyl)-4-methoxyphenoxy]nona-4,7-dioxa-1-ynyl}-5′-O-benzoyl-2′, 3′-didehydro-2′, 3′-dideoxyuridine 12c

Using **11** (0.20 g, 0.46 mmol) with alkyne **7c** (0.20 g, 0.51 mmol) gave **12c** (0.23 g, 72%): $[\alpha]_D$ +32.1 (c 1.0, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3674, 3377 (NH), 3014 (C–H), 2225 (C≡C), 1711 (C=O), 1501 (C=C), 1212 (C–N) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 8.82 (1H, br s), 8.02 (2H, d, J = 7.6 Hz), 7.68 (1H, s), 7.56 (1H, m), 7.46 (2H, t, J = 7.6 Hz), 6.92 (1H, m), 6.77 (1H, d, J = 8.8 Hz), 6.69 (2H, m), 6.39 (1H, dt, J = 1.5, 6.0 Hz), 5.99 (1H, dq, J = 1.3, 6.0 Hz), 5.21 (1H, m), 4.97 (1H, br s), 4.70 (1H, dd, J = 4.2, 12.4 Hz), 4.51 (1H, dd, J = 2.9, 12.4 Hz), 4.25 (2H, s), 4.08 (2H, t, J = 4.9 Hz), 3.84 (2H, t, J = 4.9 Hz), 3.75 (3H, m), 3.73 (4H, m), 3.35 (2H, m), 2.80 (2H, t, J = 6.9 Hz), 1.43 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.2, 161.1, 156.0, 153.7, 151.0, 149.4, 142.2, 133.5, 133.4, 129.7, 129.3, 129.2, 128.6, 127.0, 116.5, 113.0, 112.0, 99.9, 90.7, 90.1, 85.1, 78.9, 77.2, 70.5, 69.8, 69.1, 68.4, 65.0, 58.9, 55.6, 40.6, 31.0, 28.4; HRMS (ES): m/z found 706.2958 (M^++H). $C_{37}H_{44}N_3O_{11}$ requires (M^++H) 706.2976.

5.4. General procedure for the synthesis of thioureas 14a–d

Trifluoroacetic acid (0.20 mL) was added to a solution of compounds **12a–c** (0.2 mmol) in CH_2Cl_2 (3 mL) at 0 °C, and the solution stirred for 2 h. Diisopropylethylamine (0.30 mL) was added, the solvent evaporated in vacuo and the crude amine dried under vacuum for 1 h, after which it was dissolved in THF (5 mL), thiourea **13** (0.24 mmol, 1.2 equiv) added and the reaction mixture stirred at room temperature for 20 h. Following evaporation of the solvent, the residue was subjected directly to column chromatography using (EtOAc in pet ether = 7:3) to give the desired products **14a–c** as pale-yellow solids.

5.4.1. 5-{4-[2-(2-(5-Bromo-2-pyridinyl)aminothiocarbonylamino)-ethyl)-4-methoxyphenoxy]but-1-ynyl}-5′-*O*-benzoyl-2′,3′-didehydro-2′,3′-dideoxyuridine 14a

Using **12a** (100 mg, 0.16 mmol) with **13** (54 mg, 0.19 mmol) gave **14a** (72 mg, 60%); mp 95–97 °C; $[\alpha]_D$ −9.9 (c 0.8, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3934, 3681 (NH), 2297 (C≡C), 1714 (C=O), 1602 (C=C), 1418 (C=S), 1212 (C–N) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 10.92 (1H, t, J = 2.7 Hz), 9.75 (1H, br s), 9.13 (1H, s), 8.03 (2H, m), 7.91 (1H, d, J = 2.3 Hz), 7.61 (1H, s), 7.56 (1H, m), 7.45 (2H, m), 7.28 (1H, dd, J = 2.3, 8.8 Hz), 6.96 (1H, m), 6.80 (1H, m), 6.76 (2H, m), 6.62 (1H, d, J = 8.8 Hz), 6.37 (1H, dt, J = 1.6, 6.0 Hz), 6.09 (1H, m), 5.19 (1H, m), 4.65 (1H, dd, J = 4.2, 12.3 Hz), 4.53 (1H, dd, J = 3.8, 12.3 Hz), 3.97 (4H, m), 3.79 (3H, s), 3.03 (1H, m), 2.88 (1H, m), 2.71 (2H, t, J = 6.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.1 (C=S), 164.7, 162.2, 153.6, 151.6, 150.9, 149.3, 146.3, 141.8, 140.6, 133.3, 133.1, 129.7, 129.3, 128.8, 128.6, 127.7, 118.3, 113.3, 112.0, 111.6, 111.2, 100.6, 91.3, 90.7, 84.9, 72.6, 66.5, 65.3, 55.5, 45.4, 30.3, 20.9; HRMS (ES): *m/z* found 746.1262 (M^++H). $C_{35}H_{33}N_5O_7BrS$ requires (M^++H) 746.1284.

5.4.2. 5-{6-[2-(2-(5-Bromo-2-pyridinylaminothiocarbonylamino)-ethyl)-4-methoxyphenoxy]hexa-4-oxa-1-ynyl}-5′-O-benzoyl-2′,3′-didehydro-2′,3′-dideoxyuridine 14b

Using **12b** (0.20 g, 0.30 mmol) with **13** (0.11 g, 0.39 mmol) gave **14b** (0.13 g, 60%); mp: 73–76 °C; $[\alpha]_D$ −20.2 (c 1.0, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3601, 3391 (NH), 2957 (C–H), 2254 (–C≡C–), 1718 (C=O), 1501 (C=C), 1462 (C=S) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 10.82 (1H, m), 9.00 (1H, br s), 8.84 (1H, br s), 8.03 (1H, d, J = 2.4 Hz), 8.00 (2H, m), 7.70 (1H, s), 7.65 (1H, dd, J = 2.4, 8.8 Hz), 7.56 (1H,, m), 7.45 (2H, m), 6.93 (1H, m), 6.87 (1H, d, J = 8.8 Hz), 6.79 (1H, d, J = 2.8 Hz), 6.77 (1H, d, J = 8.8 Hz), 6.73 (1H, dd, J = 2.8, 8.8 Hz), 6.40 (1H, dt, J = 1.7, 6.0 Hz), 6.02 (1H, dq, J = 1.4, 6.0 Hz), 5.21 (1H, m), 4.68 (1H, dd, J = 4.3, 12.5 Hz), 4.54 (1H, dd, J = 2.9, 12.5 Hz), 4.25 (2H, s), 4.00 (4H, m), 3.83 (2H, m), 3.75 (3H, s), 2.98 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.2 (C=S), 166.3, 161.6, 153.7, 151.8, 151.2, 149.4, 146.6, 142.6, 141.0, 133.6, 133.4, 29.7, 129.2, 128.9, 128.7, 127.0, 117.5, 113.6, 112.8, 112.5, 111.6, 100.0, 90.9, 90.0, 85.2, 77.2, 68.5, 68.3, 65.1, 59.2, 55.6, 45.7, 29.9; HRMS (ES): *m/z* found 776.1379 (M^++H). $C_{36}H_{35}N_5O_8SBr$ requires (M^++H) 776.1390.

5.4.3. 5-{9-[2-(2-(5-Bromo-2-pyridinylaminothiocarbonylamino)-ethyl)-4-methoxyphenoxy]nona-4,7-dioxa-1-ynyl}-5′-O-benzoyl-2′,3′-didehydro-2′,3′-dideoxyuridine 14c

Using **12c** (0.15 g, 0.21 mmol) with **13** (76 mg, 0.27 mmol) gave **14c** (0.10 g, 63%); mp 88–91 °C; $[\alpha]_D$ −18.5 (c 1.0, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3406 (NH), 3029, 2949 (C–H), 2152 (–C≡C–), 1718, 1671 (C=O), 1501 (C=C), 1469 (C=S), 1126 (C–N) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 11.01 (1H, t, J = 4.8 Hz), 9.29 (1H, br s), 8.94 (1H, br s), 8.04 (1H, d, J = 2.4 Hz), 8.00 (2H, d, J = 7.4 Hz), 7.67 (1H, s), 7.65 (1H, dd, J = 2.4, 8.8 Hz), 7.56 (1H, m), 7.43 (2H, t, J = 7.4 Hz), 6.91 (1H, m), 6.80 (1H, d, J = 9.2 Hz), 6.79 (1H, d, J = 2.9 Hz), 6.77 (1H, d, J = 8.8 Hz), 6.73 (1H, dd, J = 2.9, 9.2 Hz), 6.38 (1H, dt, J = 1.6, 6.0 Hz), 5.98 (1H, m), 5.19 (1H, m), 4.67 (1H, dd, J = 4.3, 12.5 Hz), 4.50 (1H, dd, J = 3.0, 12.5 Hz), 4.21 (2H, s), 4.03 (2H, t, J = 4.7 Hz), 3.98 (2H, q, J = 6.6 Hz), 3.82 (2H, t, J = 4.7 Hz), 3.74 (3H, s), 3.71–3.64 (4H, m), 2.97 (2H, t, J = 6.6 Hz); ^{13}C (75 MHz, $CDCl_3$): δ 179.2 (C=S), 166.2, 161.4, 153.6, 151.7, 151.2, 149.5, 146.6, 142.5, 141.0, 133.5, 133.4, 129.7, 129.2, 128.8, 128.6, 127.0, 117.5, 113.5, 112.7, 112.5, 111.5, 100.0, 90.8, 90.1, 85.1, 77.0, 70.6, 69.8, 69.2, 68.4, 65.1, 59.0, 55.5, 45.6, 29.8; HRMS (ES): *m/z* found 820.1638 (M^++H). $C_{38}H_{39}N_5O_9SBr$ requires (M^++H) 820.1652.

5.4.4. 5-{15-[2-(2-(5-Bromo-2-pyridinylaminothiocarbonylamino)-ethyl)-4-methoxyphenoxy]pentadeca-4,7,10,13-tetraoxa-1-ynyl}-5′-O-benzoyl-2′,3′-didehydro-2′,3′-dideoxyuridine 14d

Trifluoroacetic acid (0.20 mL) was added to a solution of alkyne **7d** (0.12 g, 0.25 mmol) in CH_2Cl_2 (3 mL) at 0 °C, and the solution stirred for 2 h. Anhydrous K_2CO_3 (0.10 g, 0.75 mmol) was added, the mixture stirred for a further 15 min then filtered through Celite. Solvent evaporation in vacuo and drying of the residue under vacuum for 1 h gave the crude amine, which was dissolved in dry THF (4 mL) with DMF (2 mL) and **11** (0.10 g, 0.23 mmol) and triethylamine (0.06 mL, 0.46 mmol) were added. The mixture was thoroughly degassed with nitrogen for 1 h. CuI (23 mg, 0.12 mmol) and $Pd(PPh_3)_4$ (27 mg, 0.02 mmol) were added to the degassed solution under a nitrogen atmosphere and the mixture was left stirring at rt for 4 h. The reaction mixture was then dissolved in MeOH/$CHCl_3$ (1:4) (30 mL), which was washed with portions (15 mL) of 5% aq disodium EDTA, water (10 mL) and dried over $MgSO_4$. Filtration, solvent removal under reduced pressure and column chromatography of the residue employing EtOAc/MeOH/Et_3N (5/4/1) gave the coupled amine product, which was reacted with thiourea **13** (85 mg, 0.30 mmol) in dry THF (5 mL) at room temperature for 20 h. Following evaporation of solvent, the residue was subjected directly to column chromatography using EtOAc/pet ether (9/1) to afford the double-drug **14d** as a solid (76 mg, 40% over the three steps); mp 112–115 °C; $[\alpha]_D$ −24.1 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3377, 3217 (NH), 3007, 2920 (C–H), 2239 (–C≡C–), 1722, 1675 (C=O), 1501 (C=C), 1465 (C=S), 1227 (C–N) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 11.09 (1H, t, J = 5.1 Hz), 8.88 (1H, br s), 8.76 (1H, br s), 8.05 (1H, d, J = 2.4 Hz), 8.02 (2H, d, J = 7.6 Hz), 7.68 (1H, s), 7.68 (1H, dd, J = 2.4, 8.8 Hz), 7.58 (1H, m), 7.46 (2H, t, J = 7.6 Hz), 6.93 (1H, m) 6.81 (1H, d, J = 2.8 Hz), 6.78 (1H, d, J = 8.8 Hz), 6.75 (1H, dd, J = 2.8, 8.8 Hz), 6.74 (1H, d, J = 8.8 Hz), 6.41 (1H, dt, J = 1.7, 6.0 Hz), 6.00 (1H, dq, J = 1.2, 6.0 Hz), 5.22 (1H, m,), 4.70 (1H, dd, J = 4.2, 12.5 Hz), 4.52 (1H, dd, J = 3.0, 12.5 Hz), 4.21 (2H, s), 4.02 (4H, m), 3.83 (2H, t, J = 5.8 Hz), 3.77 (3H, s), 3.71–3.64 (12H, m), 2.99 (2H, t, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 179.1 (C=S), 166.2, 161.3, 153.5, 151.8, 151.2, 149.5, 146.4, 142.3, 141.0, 133.5, 133.4, 129.6, 129.1, 128.8, 128.6, 126.9, 117.5, 113.6, 112.7, 112.4, 111.5, 100.0, 90.7, 90.1, 85.1, 76.9, 70.8, 70.6, 70.5, 70.5, 70.3, 69.8, 69.1, 68.4, 65.0, 58.9, 55.5, 45.7, 29.8; HRMS (ES): *m/z* found 908.2178 (M^++H). $C_{42}H_{47}N_5O_{11}SBr$ requires (M^++H) 908.2176.

5.5. General procedure for benzoate deprotection of 14a–d to afford 15a–d

A solution of NaOMe in methanol (0.03 mL, 2 M, 0.06 mmol, 0.6 equiv) was added to a solution of the nucleoside (0.1 mmol) in MeOH (2 mL) at 0 °C. The mixture was stirred for 30 min, acetic acid (0.05 mL) was added, and the crude mixture was diluted with CH_2Cl_2 (1 mL) and subjected directly to column chromatography employing MeOH/CH_2Cl_2 (2/8) to give the desired product as a pale-yellow solid.

5.5.1. 5-{4-[2-(2-(5-Bromo-2-pyridinylaminothiocarbonylamino)-ethyl)-4-methoxyphenoxy]but-1-ynyl}-2′,3′-didehydro-2′,3′-dideoxyuridine 15a[16]

Compound **14a** (83 mg, 0.11 mmol) gave **15a** (43 mg, 60%); mp 121–122 °C; $[\alpha]_D$ +22.4 (c 1.10, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3934, 3688 (NH), 2304 (–C≡C), 1696 (C=O), 1606 (C=C), 1425 (C=S) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 11.05 (1H, t, J = 4.2 Hz), 8.70 (1H, br s), 7.99 (1H, d, J = 2.6 Hz), 7.97 (1H, s), 7.56 (1H, dd, J = 2.6, 8.8 Hz), 6.97 (1H, m), 6.80 (1H, m), 6.77 (2H, m), 6.66 (1H, d, J = 8.8 Hz), 6.37 (1H, dt, J = 1.5, 5.8 Hz), 5.86 (1H, m), 4.93 (1H, m), 4.05 (2H, t, J = 5.9 Hz), 3.95 (2H, m), 3.80 (2H, m), 3.78 (3H, s), 2.98 (2H, m), 2.84 (2H, t, J = 5.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.9, 162.2, 153.4, 151.5, 150.9, 149.1, 146.6, 143.6, 140.8, 135.6, 129.3, 128.9, 118.0, 113.4, 112.8, 111.4, 110.8, 100.3, 91.3, 90.4, 87.7, 72.5, 66.8, 62.9, 55.6, 45.7, 30.2, 21.0; HRMS (ES): *m/z* found 642.1008 (M^++H). $C_{28}H_{29}N_5O_6BrS$ requires (M^++H) 642.1022.

5.5.2. 5-{6-[2-(2-(5-Bromo-2-pyridinylaminothiocarbonylamino)-ethyl)-4-methoxyphenoxy]hexa-4-oxa-1-ynyl}-2′,3′-didehydro-2′,3′-dideoxyuridine 15b

Compound **14b** (100 mg, 0.14 mmol) gave **15b** (59 mg, 69%); mp 76–79 °C; $[\alpha]_D$ +49.1 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3681, 3594, 3391 (NH, OH), 3022, 2928 (C–H), 2239 (–C≡C–), 1715, 1697 (C=O), 1501 (C=C), 1465 (C=S), 1216 (C–N) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 11.09 (1H, t, J = 5.1 Hz), 9.44 (1H, br s), 9.09 (1H, br s), 8.14 (1H, s), 8.00 (1H, d, J = 2.4 Hz), 7.65 (1H, dd, J = 2.4, 8.7 Hz), 6.97 (1H, m), 6.83 (1H, d, J = 8.7 Hz), 6.75 (3H, m), 6.35 (1H, dt, J = 1.7, 6.0 Hz), 5.85 (1H, m), 4.93 (1H, m), 4.38 (2H, s), 4.06–3.78 (8H, m), 3.75 (3H, s), 3.36 (1H, br s), 2.97 (2H, t, J = 6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.8 (C=S), 161.8, 153.7, 151.8, 151.1, 149.9, 146.4, 144.7, 141.0, 135.1, 128.9, 126.0, 117.5, 113.8, 112.9, 112.6, 111.6, 99.3, 90.3, 89.4, 87.7, 77.5, 68.4, 68.4, 62.8, 59.3, 55.6, 45.8, 30.0; HRMS (ES): *m/z* found 672.1139 (M^++H). $C_{29}H_{31}N_5O_7SBr$ requires (M^++H) 672.1128.

5.5.3. 5-{9-[2-(2-(5-Bromo-2-pyridinylaminothiocarbonylamino)-ethyl)-4-methoxyphenoxy]nona-4,7-dioxa-1-ynyl}-2′,3′-didehydro-2′,3′-dideoxyuridine 15c

Compound **14c** (80 mg, 0.11 mmol) gave **15c** (37 mg, 54%); mp 130–134 °C; $[\alpha]_D$ +41.1 (*c* 1.0, DMSO); IR (DMSO): ν_{max} 3442, 3260 (NH, OH), 2928 (C–H), 2246, 2123 (–C≡C–), 1700 (C=O), 1462 (C=S), 1223 (C–N) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 11.50 (1H, br s),1.05 (1H, t, J = 5.0 Hz),10.52 (1H, br s), 8.11 (1H, d, J = 2.6 Hz), 8.10 (1H, s), 7.93 (1H, dd, J = 2.6, 8.7 Hz), 7.12 (1H, d, J = 8.7 Hz), 6.92 (1H, d, J = 8.7 Hz), 6.82 (1H, d, J = 3.0 Hz), 6.80 (1H, m), 6.77 (1H, dd, J = 3.0, 8.7 Hz), 6.40 (1H, dt, J = 1.7, 6.0 Hz), 5.93 (1H, dq, J = 1.4 Hz, 6.0 Hz), 4.94 (1H, t, J = 5.0 Hz), 4.81 (1H, m), 4.31 (2H, s), 4.03 (2H, t, J = 4.9 Hz), 3.84 (2H, q, J = 6.6 Hz), 3.74 (2H, t, J = 4.9 Hz), 3.68 (3H, s), 3.66–3.58 (6H, m, H-5′), 2.90 (2H, t, J = 6.6 Hz); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.1 (C=S), 161.3, 153.1, 152.1, 150.6, 149.5, 145.6, 144.5, 141.0, 135.2, 128.4, 127.4, 116.8, 114.3, 113.4, 111.6, 111.4, 97.5, 89.4, 88.5, 87.5, 78.1, 69.5, 68.9, 68.5, 68.2, 61.7, 58.0, 55.1, 44.5, 28.9; HRMS (ES): *m/z* found 716.1389 (M^++H), $C_{31}H_{35}N_5O_8SBr$ requires (M^++H) 716.1390.

5.5.4. 5-{15-[2-(2-(5-Bromo-2-pyridinylamino thiocarbonylamino)ethyl)-4-methoxyphenoxy]pentadeca-4,7,10,13-tetraoxa-1-ynyl}-2′,3′-didehydro-2′,3′-dideoxyuridine 15d

Compound **14d** (70 mg, 0.08 mmol) gave **15d** (32 mg, 52%); mp 63–65 °C; $[\alpha]_D$ + 53.2 (c 1.0, DMSO); IR (DMSO): ν_{max} 3442, 3283 (NH, OH), 2246, 2123 (C≡C), 1660 (C=O), 1469 (C=S), 1226 (C–N) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 11.60 (1H, br s) 11.09 (1H, t, J = 4.8 Hz), 10.62 (1H, br s), 8.12 (1H, s), 8.09 (1H, d, J = 2.4 Hz), 7.93 (1H, dd, J = 2.4, 8.8 Hz), 7.09 (1H, d, J = 8.8 Hz), 6.89 (1H, d, J = 8.8 Hz), 6.81 (1H, d, J = 3.2 Hz), 6.78 (1H, m), 6.75 (1H, dd, J = 3.2, 8.8 Hz), 6.38 (1H, dt, J = 1.7, 6.0 Hz), 5.91 (1H, dq, J = 1.4, 6.0 Hz), 5.02 (1H, t, J = 5.2 Hz), 4.79 (1H, m), 4.28 (2H, s), 4.00 (2H, t, J = 4.9 Hz), 3.81 (2H, q, J = 6.6 Hz), 3.71 (2H, t, J = 4.9 Hz), 3.66 (3H, s), 3.60–3.47 (14H, m), 2.87 (2H, t, J = 6.6 Hz); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.1 (C=S), 161.4, 153.0, 152.2, 150.6, 149.6, 145.6, 144.6, 141.1, 135.2, 128.4, 125.5, 116.8, 114.3, 113.3, 111.6, 111.5, 97.5, 89.4, 88.5, 87.5, 78.2, 69.8, 69.7, 69.6, 69.6, 69.4, 68.9, 68.4, 68.2, 61.7, 58.0, 55.1, 44.5, 29.0; HRMS (ES): *m/z* found 804.1932 (M++H). $C_{35}H_{43}N_5O_{10}SBr$ requires (M++H) 804.1914.

5.6. 2′,3′-Didehydro-2′,3′-dideoxy-5-iodouridine (5-iodo-d4U)

To a solution of **11** (0.80 g, 1.82 mmol) in dry MeOH (15 mL), was added a solution of NaOMe in methanol (0.90 mL, 2 M, 1.80 mmol) at 0 °C. The mixture was stirred at rt for 2 h before being diluted with aq NH_4Cl (25 mL) and then extracted using $CHCl_3$/MeOH (4:1) (3 × 40 mL). Drying ($MgSO_4$), evaporation of solvent and purification of the residue on silica-gel chromatography using EtOAc as eluent gave 2′,3′-didehydro-2′,3′-dideoxy-5-iodouridine as a colourless solid (0.45 g, 74%); mp 176–177 °C; ^1H NMR (300 MHz, DMSO-d_6): δ 11.58 (1H, br s), 8.23 (1H, s), 6.78 (1H, m), 6.40 (1H, dt, J = 1.7, 6.0 Hz), 5.94 (1H, dq, J = 1.4, 6.0 Hz), 5.01 (1H, t, J = 4.9 Hz), 4.83 (1H, m), 3.62 (2H, m); ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.3, 150.3, 145.7, 135.2, 125.7, 89.1, 87.4, 68.6, 61.5.

5.7. 2′,3′-Didehydro-2′,3′-dideoxy-5-iodouridine-5′-[*p*-tolylmethoxyalaninyl phosphate] 16

p-Tolyl methoxylalaninyl phosphorochloridate[38] (1.77 g, 6.42 mmol) and 2′,3′-didehydro-2′,3′-dideoxy-5-iodouridine (0.72 g, 2.14 mmol) were dissolved in THF (25 mL) and *N*-methylimidazole (1.02 mL, 12.84 mmol) was added with vigorous stirring. After 24 h at rt the solvent was removed under vacuum. The residue was dissolved in $CHCl_3$ (100 mL) and washed with hydrochloric acid solution (1 M, 2 × 30 mL), aq $NaHCO_3$ (2 × 30 mL), and then water (3 × 30 mL). The organic layer was dried over $MgSO_4$ and the solvent evaporated under vacuum. Purification of the residue by chromatography on silica-gel eluting with 3% MeOH in $CHCl_3$ gave **16** (0.45 g, 35%) as a 1:1 mixture of diastereoisomers and as a colourless solid; mp 40–44 °C; IR ($CHCl_3$): ν_{max} 3384 (NH), 3007 (C–H), 1743, 1704 (C=O), 1505 (C=C), 1245 (P=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (some peaks are split due to diastereoisomers at P) 8.91 (1H, br s), 7.92, 7.90 (1H, s), 7.11 (4H, m), 6.94, 6.92 (1H, m), 6.38, 6.32 (1H, dt, J = 1.7, 6.0 Hz), 5.94, 5.86 (1H, dq, J = 1.3, 6.0 Hz), 5.05 (1H, m), 4.46–3.82 (4H, m), 3.72, 3.71 (3H, s), 2.31 (3H, s), 1.41, 1.36 (3H, d, J = 7.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ (values bearing an asterisk are given as an average of peaks, split due to diastereoisomers and/or C–P coupling) 173.9*, 159.9*, 150.3, 148.2*, 144.6*, 134.6, 133.7*, 133.0*, 126.9*, 120.0*, 90.2*, 85.3*, 69.0*, 66.6*, 52.4*, 50.0*, 21.0*, 20.8*.

^{31}P NMR ($CDCl_3$): δ 3.7, 3.5 (1:1); HRMS (ES): *m/z* found 592.0356 (M^++H). $C_{20}H_{24}N_3O_8PI$ requires (M^++H) 592.0346.

5.8. 5-{15-[2-(2-(5-Bromo-2-pyridinylaminothiocarbonylamino)-ethyl)-4-methoxyphenoxy]pentadeca-4,7,10,13-tetraoxa-1-ynyl}-2′,3-didehydro-2′,3′-dideoxy-5-iodouridine-5′-5′-(*p*-methylphenyl methoxyalaninyl phosphate)-2′,3′-didehydro-2′,3′-dideoxyuridine 17

Trifluoroacetic acid (0.20 mL) was added to a solution of alkyne **7d** (92 mg, 0.19 mmol) in CH_2Cl_2 (2 mL) at 0 °C, and the solution stirred for 2 h. Anhydrous K_2CO_3 (80 mg, 0.57 mmol) was added, the mixture was stirred for a further 15 min and then filtered through Celite, The solvent was then evaporated in vacuo, the

crude amine dried under vacuum for 1 h, dissolved in dry THF (3 mL) with DMF (2 mL), and 5-iodouridine-5′-[*p*-methylphenyl methoxyalaninyl phosphate] **16** (0.10 g, 0.17 mmol), followed by triethylamine (0.05 mL, 0.34 mmol) were added, The mixture was thoroughly degassed with nitrogen for 1 h. CuI (17 mg, 0.09 mmol) and $Pd(PPh_3)_4$ (20 mg, 0.02 mmol) were then added to the degassed solution under a nitrogen atmosphere. The mixture was left stirring at rt for 2 h, after which a mixture of MeOH/$CHCl_3$ (1:4, 30 mL) was added and washed with portions (2 × 10 mL) of 5% aq disodium EDTA, water (10 mL) and then dried over $MgSO_4$. Filtration and solvent evaporation under reduced pressure gave a crude product, which was flashed through a silica-gel column employing EtOAc/MeOH/Et_3N (5/4/1) as eluent. The coupled amine product was dissolved in dry THF (3 mL), thiourea **13** (65 mg, 0.23 mmol) was added and the mixture stirred at room temperature for 20 h. Following evaporation of solvent, the residue was subjected directly to column chromatography using EtOAc/MeOH (9/1) to give 17 as a pale-yellow solid (36 mg, 20% over the three steps); mp 51–54 °C; $[\alpha]_D$ +4.4 (c 1.0, $CHCl_3$); IR ($CHCl_3$): ν_{max} 3391 (NH), 3007, 2928 (C–H), 1707 (C═O), 1505 (C═C), 1462 (C═S), 1259 (P═O), 1223 (C–N) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ (some peaks are split due to diastereoisomers at P) 11.11 (1H, t, J = 4.9 Hz, NH), 9.11 (1H, br s, NH), 9.05, 9.00 (1H, s, NH), 8.03 (1H, d, J = 2.4 Hz), 7.79, 7.78 (1H, s), 7.66, 7.63 (1H, dd, J = 2.4, 8.8 Hz), 7.08 (4H, s), 6.96, 6.94 (1H, m), 6.80 (1H, d, J = 3.0 Hz), 6.78 (1H, d, J = 8.8 Hz), 6.74 (1H, d, J = 8.8 Hz), 6.72 (1H, dd, J = 3.0, 8.8 Hz), 6.37, 6.27 (1H, dt, J = 1.7, 6.0 Hz), 5.91, 5.81 (1H, dq, J = 1.4, 6.0 Hz), 5.02 (1H, m), 4.41–3.97 (10H, m), 3.81 (2H, t, J = 4.9 Hz), 3.75 (3H, s), 3.68, 3.67 (3H, s), 3.70–3.57 (12H, m), 2.97 (2H, t, J = 6.6 Hz), 2.28 (3H, s), 1.37, 1.33 (3H, d, J = 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ (values bearing an asterisk are given as an average of peaks, split due to diastereoisomers and/or C-P coupling) 179.2 (C═S), 173.9*, 161.2, 153.6, 151.9, 151.3, 149.6, 148.3*, 146.5, 143.1*, 141.0, 134.7, 133.8, 130.1*, 128.8, 126.7*, 120.0*, 117.5, 113.6, 112.7, 112.4, 111.5, 99.9, 91.3*, 90.2, 85.4*, 77.2, 70.8, 70.6, 70.5 (2 × OCH_2), 70.2*, 69.9, 69.2*, 68.4, 66.7 (d, J_{cp} = 4.5 Hz, C-5′), 58.9, 55.6, 52.5, 50.3*, 45.8, 29.8, 20.9*, 20.7; ^{31}P NMR ($CDCl_3$): δ 3.63, 3.56 (1:1); HRMS (ES): *m/z* found 1059.2548 (M^++H). $C_{46}H_{57}N_6O_{14}SBrP$ requires (M^++H) 1059.2574.

5.9. Anti-HIV evaluation

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: MT-2 cells and HTLV-III_B/H9 from Dr. Robert Gallo.

Antiviral activity and cellular toxicity were determined using the MTT colorimetric method[40] in the following way. MT-2 cells[43] at a concentration of 1 × 10^5 cells per milliliter were infected with wild type HIV-IIIB[44] at a multiplicity of infection (MOI) of 0.1. Infected and mock-infected cells were incubated in growth medium (RPMI 1640, 10% dFBS, kanamycin) for five days with varying concentrations of each compound being tested in triplicate in a 96-well plate. MTT, a cell-permeable tetrazolium dye was then added to each well. After 5 h, acidified isopropanol was added to lyse the cells and stop the reaction. The plates were gently shaken overnight, and the absorbance measured at 595 nm on a plate reader. The average of these triplicate samples were then plotted versus inhibitor concentration to generate dose–response curves. The 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) of the compounds were defined as the concentrations required to inhibit viral replication and to reduce the number of viable cells by 50%, respectively.

5.9.1. Steady-state IC_{50} determination

6 nM RT (active sites based on pre-steady-state active site determination) was pre-incubated for at least 15 min with 1 μM 5′-radiolabeled primer/template prior to mixing with appropriate concentrations of inhibitor and allowed to incubate for a minimum of 15 additional minutes on ice. DMSO concentrations were kept constant at less than 2%. DMSO alone was added as a no inhibitor control for each set of experiments. Reactions were initiated by the addition of 5 μM dTTP and 10 mM $MgCl_2$ and were quenched after 15 min at 37 °C with 0.3 M EDTA. All concentrations represent final concentrations after mixing. Reaction products were subjected to 20% denaturing polyacrylamide gel-electrophoresis and quantitated on a Bio-Rad Molecular Imager FX. Product formation was plotted as a function of inhibitor concentration and fitted to a hyperbola to generate IC_{50} curves. IC_{50} values are defined as the concentration of inhibitor that inhibits steady-state single nucleotide incorporation by 50%.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.025.

References and notes

1. Souza, T. M. L.; Rodrigues, D. Q.; Ferreira, V. F.; Marques, I. P.; da Costa Santos, F. C.; Cunha, A. C.; Vieira de Souza, M. C. B.; de Palmer Paixao Frugulhetti, I. C.; Bou-Habib, D. C.; Fontes, C. F. L. *Curr. HIV Res.* **2009**, *7*, 327.
2. Marsden, M. D.; Zack, J. A. *J. Antimicrob. Chemother.* **2009**, *63*, 7.
3. Mehellou, Y.; De Clercq, E. *J. Med. Chem.* **2010**, *53*, 521.
4. (a) Zhan, P.; Liu, X.; Li, Z.; Pannecouque, C.; De Clercq, E. *Curr. Med. Chem.* **2009**, *16*, 3903; (b) Basavapathruni, A.; Anderson, K. S. *Curr. Pharm. Des.* **2006**, *12*, 1857.
5. Nanni, R. G.; Ding, J.; Jacobo-Molina, A.; Hughes, S. H.; Arnold, E. *Perspect. Drug Discovery Des.* **1993**, *1*, 129.
6. Smerdon, S. J.; Jäger, J.; Wang, J.; Kohlstaedt, L. A.; Chirino, A. J.; Friedman, J. M.; Rice, P. A.; Steitz, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3911.
7. Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4046.
8. (a) Spence, R.; Kati, W.; Anderson, K. S.; Johnson, K. A. *Science* **1995**, *267*, 988; (b) Rittinger, K.; Divita, G.; Goody, R. S. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8046; (c) Gu, Z.; Quan, Y.; Li, Z.; Arts, E. J.; Wainberg, M. A. *J. Biol. Chem.* **1995**, *270*, 31046.
9. Velázquez, S.; Alvarez, R.; San-Félix, A.; Jimeno, M. L.; De Clercq, E.; Balzarini, J.; Camarasa, M.-J. *J. Med. Chem.* **1995**, *38*, 1641–1649.
10. Tomassi, C.; Van Nhien, A. N.; Marco-Contelles, J.; Balzarini, J.; Pannecouque, C.; De Clercq, E.; Soriano, E.; Postel, D. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2277–2281.
11. Velázquez, S.; Tuñón, V.; Jimeno, M. L.; Chamorro, C.; De Clercq, E.; Balzarini, J.; Camarasa, M.-J. *J. Med. Chem.* **1999**, *42*, 5188.
12. (a) Fossey, C.; Vu, A.-H.; Vidu, A.; Zarafu, I.; Laduree, D.; Schmidt, S.; Laumond, G.; Aubertin, A.-M. *J. Enzyme Inhib. Med. Chem.* **2007**, *22*, 591; (b) Pedersen, L.; Jørgensen, P. T.; Nielsen, S.; Hansen, T. H.; Nielsen, J.; Pedersen, E. B. *J. Med. Chem.* **2005**, *48*, 1211; (c) Velázquez, S.; Lobatón, E.; De Clercq, E.; Koontz, D. L.; Mellors, J. W.; Balzarini, J.; Camarasa, M. J. *J. Med. Chem.* **2004**, *47*, 3418; (d) Sugeac, E.; Fossey, C.; Ladurée, D.; Schmidt, S.; Laumond, G.; Aubertin, A.-M. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 175.
13. (a) Gavriliu, D.; Fossey, C.; Ciurea, A.; Delbederi, Z.; Sugeac, E.; Ladurée, D.; Schmidt, S.; Laumond, G.; Aubertin, A. M. *Nucleosides Nucleotides Nucleic Acids* **2002**, *21*, 505; (b) Pontikis, R.; Dollé, V.; Guillaumel, J.; Dechaux, E.; Note, R.; Nguyen, C. H.; Legraverend, M.; Bisagni, E.; Aubertin, A. M.; Grierson, D. S.; Monneret, C. *J. Med. Chem.* **2000**, *43*, 1927; (c) Renoud-Grappin, M.; Fossey, C.; Fontaine, G.; Ladurée, D.; Aubertin, A. M.; Kirn, A. *Antiviral Chem. Chemother.* **1998**, *9*, 205.
14. Muhanji, C. I.; Hunter, R. *Curr. Med. Chem.* **2007**, *14*, 127.
15. An interesting variation based on targeting both IN and RT in the form of a non-cleavable *portmanteau inhibitor* is: Wang, Z.; Bennett, E. M.; Wilson, D. J.; Salomon, C.; Vince, R. *J. Med. Chem.* **2007**, *50*, 3416–3419.
16. Hunter, R.; Muhanji, C. I.; Hale, I.; Bailey, C. M.; Basavapathruni, A.; Anderson, K. S. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2614.
17. Hunter, R.; Younis, Y.; Muhanji, C.-I.; Curtin, T.-L.; Naidoo, K. J.; Petersen, M.; Bailey, C. M.; Basavapathruni, A.; Anderson, K. S. *Bioorg. Med. Chem.* **2008**, *16*, 10270.

18. (a) Mao, C.; Sudbeck, E. A.; Venkatachalam, T. K.; Uckun, F. M. *Biochem. Pharmacol.* **2000**, *60*, 1251; (b) Mao, C.; Sudbeck, E. A.; Venkatachalam, T. K.; Uckun, F. M. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1593; (c) Vig, R.; Mao, C.; Venkatachalam, T. K.; Tuel-Ahlgren, L.; Sudbeck, E. A.; Uckun, F. M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1461.
19. (a) Ruth, J. L.; Cheng, Y. C. *J. Biol. Chem.* **1982**, 10261; (b) Ruth, J. L.; Cheng, Y. C. *Mol. Pharmacol.* **1981**, *20*, 415.
20. Chinchilla, R.; Najera, C. *Chem. Rev.* **2007**, *107*, 874.
21. Pauwels, R. *Curr. Opin. Pharmacol.* **2004**, *4*, 437.
22. Glennon, R. A.; Liebowitz, S. M.; Leming-Doot, D.; Rosecrans, J. A. *J. Med. Chem.* **1980**, *23*, 990.
23. (a) Chen, B. C.; Quinlan, S. L.; Ried, J. G.; Spector, R. H. *Tetrahedron Lett.* **1998**, *39*, 729; (b) Chen, B. C.; Quinlan, S. L.; Stark, D. R.; Reid, G.; Audia, V. H.; George, J. G.; Eisenreich, E.; Brundidge, S. P.; Racha, S.; Spector, R. H. *Tetrahedron Lett.* **1995**, *36*, 7957.
24. (a) Codington, J. F.; Doerr, I. L.; Fox, J. J. *J. Org. Chem.* **1964**, *29*, 558; (b) Codington, J. F.; Fecher, R.; Fox, J. J. *J. Am. Chem. Soc.* **1960**, *82*, 2794.
25. Sheng, J.; Hassan, A. E. A.; Huang, Z. *J. Org. Chem.* **2008**, *73*, 3725.
26. (a) Starrett, J. E., Jr.; Tortolani, D. R.; Baker, D. C.; Omar, M. T.; Hebbler, A. K.; Wos, J. A.; Martin, J. C.; Mansuri, M. M. *Nucleosides Nucleotides* **1990**, *9*, 885; (b) Mansuri, M. M.; Starrett, J. E., Jr.; Wos, J. A.; Tortolani, D. R.; Brodfuehrer, P. R.; Howell, H. G.; Martin, J. C. *J. Org. Chem.* **1989**, *54*, 4780.
27. Asakura, J.; Robins, M. J. *J. Org. Chem.* **1990**, *55*, 4928–4933.
28. Robins, M. J.; Barr, P. J. *J. Org. Chem.* **1983**, *48*, 1854.
29. Hobbs, F. W., Jr. *J. Org. Chem.* **1989**, *54*, 3420.
30. Crisp, G.; Flynn, B. L. *J. Org. Chem.* **1993**, *58*, 6614.
31. Agrofolio, L. A.; Gillaizeau, I.; Saito, Y. *Chem. Rev.* **2003**, *103*, 1875.
32. Kelleher, M. R.; McGuigan, C.; Bidet, O.; Carangio, A.; Weldon, H.; Andrei, G.; Snoeck, R.; De Clercq, E.; Balzarini, J. *Nucleosides Nucleotides Nucleic Acids* **2005**, *24*, 643.
33. Bell, F. W.; Cantrell, A. S.; Högberg, M.; Jaskunas, S. R.; Johansson, N. G.; Jordan, C. L.; Kinnick, M. D.; Lind, P.; Morin, J. M., Jr.; Noréen, R.; Öberg, B.; Palkowitz, J. A.; Parrish, C. A.; Pranc, P.; Sahlberg, C.; Ternansky, R. J.; Vasileff, R. T.; Vrang, L.; West, S. J.; Zhang, H.; Zhou, X.-X. *J. Med. Chem.* **1995**, *38*, 4929.
34. (a) Robins, M. J.; Vinayak, R. S.; Wood, S. G. *Tetrahedron Lett.* **1990**, *31*, 3731; (b) Olivi, N.; Spruyt, P.; Peyrat, J.-F.; Alami, M.; Brion, J.-D. *Tetrahedron Lett.* **2004**, *45*, 2607.
35. Meier, C. *Eur. J. Org. Chem.* **2006**, 1081.
36. Balzarini, J.; Karlsson, A.; Aquaro, S.; Perno, C.-F.; Cahard, D.; Naesens, L.; De Clercq, E.; McGuigan, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7295.
37. Siddiqui, A. Q.; Ballatore, C.; McGuigan, C.; De Clercq, E.; Balzarini, J. *J. Med. Chem.* **1999**, *42*, 393.
38. McGuigan, C.; Pathirana, R.; Balzarini, J.; De Clercq, E. *J. Med. Chem.* **1993**, *36*, 1048.
39. (a) Mehellou, Y.; Balzarini, J.; McGuigan, C. *Org. Biomol. Chem.* **2009**, *7*, 2548; (b) Mehellou, Y.; McGuigan, C.; Brancale, A.; Balzarini, J. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3666; (c) McGuigan, C.; Tsang, H. W.; Cahard, D.; Turner, K.; Velazquez, S.; Salgado, A.; Bidois, L.; Naesens, L.; De Clercq, E.; Balzarini, J. *Antiviral Res.* **1997**, *35*, 195.
40. Pannecouque, C.; Daelemans, D.; De Clercq, E. *Nat. Protocols* **2008**, *3*, 427.
41. Ciurea, A.; Fossey, C.; Gavriliu, D.; Delbederi, Z.; Sugeac, E.; Ladurée, D.; Schmidt, S.; Laumond, G.; Aubertin, A. M. *J. Enzyme Inhib.* **2004**, *19*, 511.
42. Deng, B.-L.; Hartman, T. L.; Buckheit, R. W., Jr.; Pannecouque, C.; De Clercq, E.; Fanwick, P. E.; Cushman, M. *J. Med. Chem.* **2005**, *48*, 6140.
43. (a) Haertle, T.; Carrera, C. J.; Wasson, D. B.; Sowers, L. C.; Richmann, D. D.; Carson, D. A. *J. Biol. Chem.* **1988**, *263*, 5870; (b) Harada, S.; Koyanagi, Y.; Yamamoto, N. *Science* **1985**, *229*, 563.
44. (a) Popovic, M.; Read-Connole, E.; Gallo, R. *Lancet* **1984**, *2*, 1472; (b) Popovic, M.; Sarngadharan, M. G.; Read, E.; Gallo, R. C. *Science* **1984**, *224*, 497; (c) Ratner, L.; Haseltine, W.; Patarca, R.; Livak, K. J.; Starcich, B.; Josephs, S. F.; Doran, E. R.; Rafalski, J. A.; Whitehorn, E. A.; Baumeister, K.; Ivanoff, L.; Petteway, S. R., Jr.; Pearson, M. L.; Lautenberger, J. A.; Papas, T. S.; Ghrayab, J.; Chang, N. T.; Gallo, R. C.; Wong-Stall, F. *Nature* **1985**, *313*, 277.