Post-print of (paper) Nucleosides, Nucleotides and Nucleic Acids, 2008, 27, 351-367

(DOI: 10.1080/15257770801943990 122, 27-35).

*NOVEL N-3 SUBSTITUTED TSAO-T DERIVATIVES: SYNTHESIS AND ANTI-HIV-EVALUATION*

Received 8 May 2007; accepted 15 November 2007.

NOVEL N-3 SUBSTITUTED TSAO-T DERIVATIVES: SYNTHESIS AND ANTI-HIV-EVALUATION

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Abstract

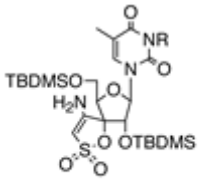
Novel derivatives of the anti-HIV-1 agent, TSAO-T, bearing at the N-3 position alkylating groups or photoaffinity labels were prepared and evaluated for their anti-HIV activity. All of these compounds demonstrated pronounced anti-HIV-1 activity and inhibited HIV-1 RT; however, we were unable to detect stable covalent linkages between inhibitor and enzyme. In addition, compounds with an alcohol functional group connected to the N-3 position through a cis or trans double bond have been prepared. These compounds have been useful to study how the conformational restriction of the linker affects in the interaction between the N-3 substituent and the HIV-1 RT enzyme.

Keywords AIDS; HIV-1 reverse transcriptase; TSAO-T

## Introduction

Human immunodeficiency virus (HIV) reverse transcriptase (RT) is an attractive target for the development of anti-AIDS drugs.1-3 This enzyme plays a multifunctional role in the conversion of the ssRNA viral genome to dsDNA. HIV-1 RT is an asymmetric heterodimer which contains two subunits of 66 and 51 kDa designated as p66 and p51, respectively.4-6 The larger subunit (p66) contains both polymerase and RNase H activities, while the smaller subunit (p51) lacks these funtions.4-6 The catalytic activity of the RT is only observed when it is in its (hetero)dimeric form.7-9

TSAO-T (Figure 1) is the prototype of a unique class of non-nucleoside reverse transcriptase inhibitors (NNRTIs).10-13 TSAO derivatives are highly functionalised nucleosides, which have a structure and a mechanism of action quite distinct from conventional NNRTIs.12 Experimental data on TSAO derivatives strongly suggest a specific interaction of the amino group of the 3'-spiro moiety with the carboxylic acid group of a glutamic acid residue at position 138 of the p51 subunit of HIV-1 RT.14-17 This residue is located at the □7-□8 loop of the p51 subunit.18,19 Moreover, other amino acids of the p66 subunit are also needed for an optimal interaction of TSAO derivatives with HIV-1 RT.20 So, TSAO compounds are among the very few RT inhibitors for which amino acids at both HIV-1 RT subunits (p51 and p66) are needed for optimal interaction with the enzyme.




**TSAO-T;** R = H
**TSAO-hp$^3$T;** R = $(CH_2)_3OH$

Fig. 1. Structure of TSAO-T and TSAO-hp3T

Since the first TSAO derivatives were synthesized many efforts to co-crystallize these molecules with their target enzyme (HIV-1 RT) were pursued. Unfortunately, they have been unsuccessful so far. In 2001, computational docking studies led us to propose a putative binding mode of the N-3 methyl derivative of TSAO-T (TSAO-m3T) with HIV-1 RT.21

The molecular model was in agreement with known structure-activity and mutagenesis data for this unique class of inhibitors, and also with biochemical evidence indicating that TSAO molecules can affect enzyme dimerization.22 In fact, the model provided strong evidence that TSAO-m3T straddles between both subunits at the p66/p51 interface but in a manner that does not make use of the NNRTI binding pocket.

For the purpose of getting more information about the binding site of TSAO molecules we report herein the synthesis and antiviral evaluation of novel TSAO derivatives with alkylating groups or photoaffinity labels that were designed to form covalent interactions with HIV-1 RT. As the anchoring point of these moieties we chose the N-3 position of thymine since, as shown by our previous biological results, several compounds modified at this position showed anti HIV-1 activity comparable or even higher than those found for TSAO-T.13,23 On the other hand, our docking model indicates that the N-3 substituent at the thymine base of TSAO-m3T was found running parallel to the subunit interface and that the presence of functional groups at this position might provide additional interactions with amino acids at or near the p51/p66 interface.23

As some of the amino acids found at the dimer interface near the TSAO binding site have side chains with nucleophilic functional groups (Glu-A169, Thr-A165, Lys-B49), we have first prepared TSAO derivatives bearing at the N-3 position of thymine strong electrophilic moieties such as an epoxide or a nitrogen mustard with a reactive N,N-bis-(2-chloroethyl)amine group.The alkylating properties of these groups might lead to the formation of a covalent bond between the novel TSAO derivatives and the amino acids of the HIV-1 RT that might persistently disturb the enzyme and facilitate the location of the inhibitor binding site.

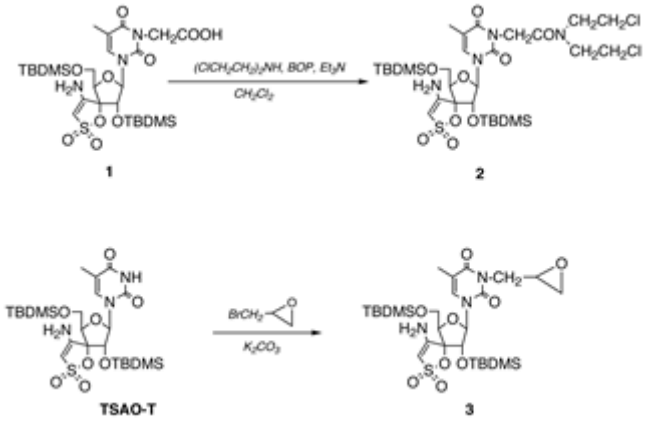
Second, we prepared photoaffinity labeled TSAO derivatives. These TSAO molecules retain the structural motifs necessary for binding with the RT and incorporate at the N-3 position a photoreactive moiety, such as aryl azide and benzophenone. These compounds would first selectively interact with the HIV-1 RT and then, upon irradiation with an appropriate light source, the photoactive group might react with the enzyme, via different intermediates, forming a covalent linkage with the amino acids proximal to the TSAO binding site. In both of these series of compounds, polymethylene linkers were used as spacers in order to obtain quite flexible molecules able to adopt the optimum conformation to interact with the enzyme.

We were also interested in novel TSAO derivatives that may destabilize the RT by disrupting crucial interface interactions. We have previously seen that attachment of a hydroxypropyl substituent at the N-3 position of TSAO-T resulted in a two-fold better inhibition (TSAO-hp3T) than the prototype compound TSAO-T (Figure 1).21,23 This result is consistent with the prediction that an additional hydrogen bond could be formed with the protein backbone (Pro-B140). However, the improvement in activity was lower than expected and we reasoned that the putative extra hydrogen bond may be partially offset by the inherent flexibility of the methylene linker.21 With the aim to enhance the interaction of the N-3 substituent with HIV-1 RT we prepared a third series of compounds in which an unsaturated bond in the linker connecting the distal functional group (alcohol) with the N-3 position of the thymine has been introduced. The nature of the unsaturation may play an essential role in the relative orientation of the TSAO molecule and the terminal functional group. Therefore, compounds containing a cis- or trans- double bond into the linker were prepared.

## Results and discussion

### Chemical results

In order to interact covalently with the amino acids of the HIV-1 RT close to the TSAO binding site, derivatives 2 and 3 bearing a bis-alkylating and epoxide moiety, respectively at the N-3 position (Scheme 1) have been prepared. Compound 2 was obtained in a 68% yield by coupling the N-3 carboxylic acid TSAO derivative 123 with N,N-bis(2-chloro-ethyl)amine in the presence of BOP and triethylamine (Scheme 1). The N-3 epoxi derivative 3 could be readily prepared in one step following our previously described method for the selective N-3 alkylation of TSAO derivatives).13,23 Thus, treatment of TSAO-T (Scheme 1) with epibromohydrin in the presence of anhydrous potassium carbonate afforded the N-3-derivative 3 in 60% yield.

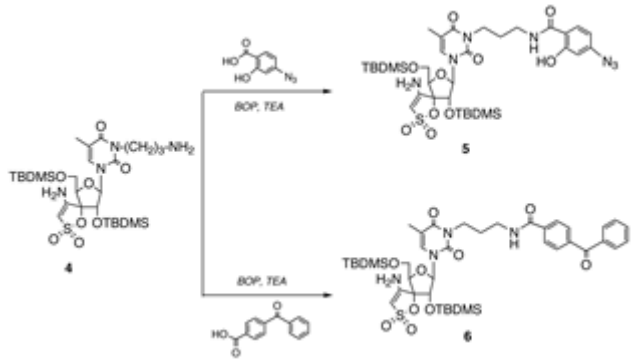



Scheme 1. Synthesis of N-3 substituted TSAO derivatives 2 and 3

In addition, a second series of compounds was prepared in which a photoactive group was attached at the N-3 position of TSAO-T (Scheme 2). Two different photoactive moieties, arylazide and benzophenone were chosen as they have been described as being among the most successful for labeling receptors and enzymes.24-26 Upon irradiation, these photoactivable groups produce extremely reactive species namely, a diradical, or a (singlet) nitrene for the benzophenone and arylazide, respectively24 that bind covalently to receptors and enzymes. Because it is essentially imposible to predict which group will be most readily reactive with the receptor, the use of different groups increases the likelihood of a successful covalent incorporation.

As shown in Scheme 2, compound 5 which possesses an arylazide photophore, was synthesized in 85% yield by a method that consisted of coupling the previously described N-3 aminopropyl TSAO derivative 423 with 4-azidosalicylic acid27 in the presence of BOP and trietylamine. In a similar way, coupling of 4 with commercially available 4-benzoylbenzoic acid afforded 6 in 76 % yield.

Finally, compounds with rigid side chains connecting a distal functional group (alcohol) with the N-3 position of TSAO-T by incorporating E or Z double bonds into the linker were prepared as follows.



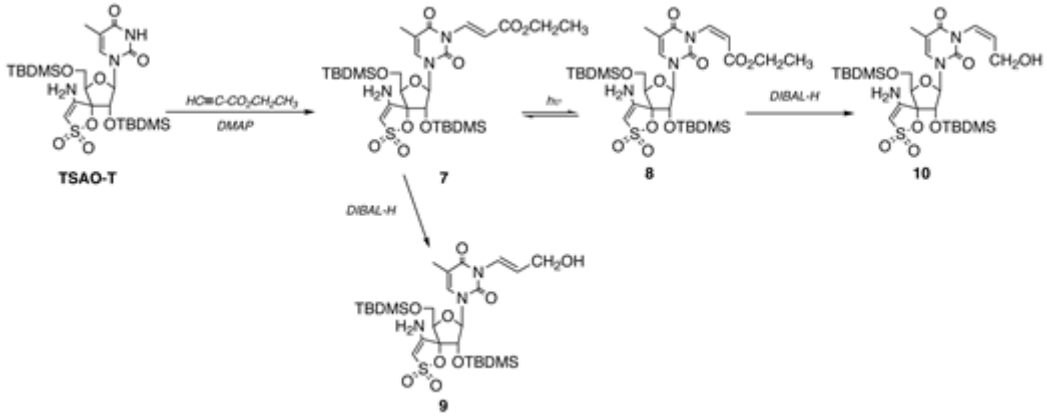

Scheme 2. Synthesis of N-3 substituted TSAO derivatives 5 and 6

Synthesis of the ethoxycarbonylvinyl E isomer 7 was carried out following the procedure developed by Villarrasa et al28 that consisted on addition of the NH of a pyrimidine ring to the triple bond of an activated alkyne. Thus, when TSAO-T was treated with

ethyl propiolate and DMAP, in acetonitrile at room temperature, a quick reaction occurred to afford 7 in very good yield (82 %) (Scheme 3). Only one isomer, of E configuration, was obtained, in the light of the 1H spectrum (olefinic protons at  6.99 and 8.26 ppm, with JCH=CH 14.7 Hz).28-30

For comparative purposes we were also interested in the synthesis of the corresponding Z isomer. Photochemical isomerization of an alkene of E configuration is a specially convenient route for the synthesis of the thermodynamically less stable Z isomer.31-33 This method has been applied with success for the trans-cis isomerization of an ethoxycarbonylethyl moiety attached at the N-3 position of a thymine.34 Based on these precedents we decided to carry out the photochemical isomerization of the E-isomer 7 to the corresponding Z-isomer 8. Due to the presence of other light-absorbing groups in the TSAO molecule, the reaction conditions were carefully adjusted to give a preponderance of the Z isomer without affecting to the other light-sensitive groups.

Thus, by performing the UV difference spectra of TSAO-T and compound 7 one absorption maximum at 289 nm was detected that should correspond to the ethoxycarbonyl vinyl moiety (data not shown). This was the wavelength used for the UV irradiation in order to ensure that only the vinyl moiety was affected and not the rest of the molecule. Thus, we irradiated with a medium-pressure mercury lamp a methanolic solution of the E isomer 7, and monitored the progress of the reaction by HPLC. After 30 min of irradiation with 290 nm light an equilibrium (1:1.5) mixture of E and Z isomers was observed. Prolonged reaction times (6h) did not affect the rate of isomerisation. It is interesting to note that no degradation and/or side reactions occurred in this photochemical trans-cis isomerisation.




Scheme 3. Synthesis of N-3 substituted TSAO derivatives 7 - 10

The 1HNMR spectrum of the mayor component of the mixture (compound 8) revealed that the olefinic protons appeared at  6.12 and 6.70 ppm, with a vecinal coupling constant of JCH=CH 9.0 Hz as corresponds to a Z isomer.29,30 In addition, the stereochemistry of the double bond of 8 was unequivocally established as Z by NOE difference experiments. Thus, irradiation of one of the vinylic protons ( 6.12 ppm) caused the enhancement of the signal for the other vinylic proton ( 6.70 ppm) indicating that was the Z isomer.

Finally, DIBAL-H (1M in THF) reduction of the carboxylic acid ester of 7 afforded the corresponding hydroxy methyl vinyl derivative 9 in 61 % yield (Scheme 3). A similar reaction with compound 8 gave 10 in 52% yield.

Structures of the novel N-3 TSAO derivatives were assigned on the basis of their analytical and spectroscopic data.

Biological results

All the synthesized compounds showed pronounced inhibitory activity, in cell culture, against HIV-1 replication (EC50 = 0.04-0.75 M) while being invariably inactive against HIV-2 (Table 1). There is a good agreement with the anti-HIV activities obtained in both MT-4 and CEM cell cultures.

TSAO derivative 3 contained an epoxide moiety attached to the N-3 position showed, in cell culture, an anti-HIV-1 activity comparable to that of compound 2 with a bis-N-chloroethyl moiety at this position (Table 1). These groups could be introduced without apreciable loss of antiviral activity against the HIV-1 RT but did not improve activity, suggesting that although they were well-tolerated, they were not able to interact covalently with the enzyme.

The photoaffinity-labeled TSAO derivatives 5 and 6 were 3- to 10-fold less effective in comparison with TSAO-T (Table 1). However, they were at least 20-fold less cytostatic in cell culture than TSAO-T. Interestingly, the compounds showed marked activity against purified HIV-1 RT (IC50 ~ 2-fold lower than TSAO-T), (Table 1) which suggests that the loss of antiviral activity in cell culture may be due to pharmacokinetic considerations (uptake and/or metabolism). The potent inhibitory activity of 5 and 6 against recombinant HIV-1 RT indicated that these compounds might be promising candidates for photoaffinity labeling experiments.

The E-hydroxymethylvinyl derivative 9 was 10-fold more active in cell culture than the corresponding Z-isomer 10, and almost equally active than the prototype TSAO-hp3T (Table 1). With respect to the recombinant HIV-1 RT (Table 1), the E-isomer 9 showed an IC50 value (IC50 = 0.5 □M) similar to the IC50 found for TSAO-hp3T (IC50 = 0.7 □M). Compound 9 proved to be three-fold more active against HIV-1 RT than the Z-isomer 10 (IC50 = 1.6 □M). These data are in agreement with what was observed in cell culture. Taken together, these data point to the impact of the relative orientation of the TSAO molecule and the terminal hydroxy group for an optimal interaction with the enzyme. Thus, the propyl linker, present in TSAO-hp3T, as well as the E-methylvinyl linker (present in 9) gave the most potent inhibitors while compound 10 with the Z-methylvinyl linker was 3-fold less active. These results indicated that the restricted Z-conformation may not allow the best interaction of the terminal OH with the HIV-1 RT.

Table 1. Inhibitory effects of test compounds on HIV-1 and HIV-2 replication in MT-4 and CEM cell culture and recombinant HIV-1 RT

| Comp. | $EC_{50}$ (μM)[a] | | | | | $CC_{50}$ (μM)[b] | $IC_{50}$[c] (μM) |
|---|---|---|---|---|---|---|---|
| | MT-4 | | CEM | | | CEM | |
| | HIV-1 | HIV-2 | HIV-1 | HIV-2 | HIV1/138K | | HIV-1 RT |
| **2** | 0.08 ± 0.02 | >125 | 0.04 ± 0.02 | ≥125 | > 25 | 86 ± 30 | 8.2 ± 1.3 |
| **3** | 0.11 ± 0.11 | >50 | 0.10 ± 0.02 | >50 | > 10 | 47 ± 16 | 2.4 ± 0.5 |
| **5** | 0.68± 0.33 | >50 | 0.37 ± 0.25 | >50 | > 50 | ≥250 | 0.4 ± 0.1 |
| **6** | 0.29± 0.12 | >50 | 0.16 ± 0.09 | >50 | > 50 | ≥250 | 0.5 ± 0.1 |
| **9** | 0.04 ± 0.0 | >2 | 0.05 ± 0.03 | >2 | > 2 | 3.4± 1.9 | 0.5 ± 0.1 |
| **10** | 0.32 ± 0.02 | >25 | 0.12 ± 0.0 | >25 | > 5 | 44 ± 7.8 | 1.6 ± 0.2 |
| **TSAO-T**[d] | 0.06± 0.03 | >20 | 0.06 ± 0.01 | >20 | > 10 | 12 ± 3 | 1.0 ± 0.2 |
| **TSAO-hp$^3$T**[e] | 0.03± 0.03 | >2 | 0.01± 0.01 | >2 | > 10 | 3.9 ± 0.29 | 0.7 ± 0.1 |

a 50% effective concentration, or the compound concentration required to inhibit HIV-induced cytopathicity by 50%.
b 50% cytostatic concentration, or the compound concentration required to inhibit cell proliferation (CEM) or to reduce cell viability (MT-4) by 50%.
c 50% inhibitory concentration, or the compound concentration required to inhibit recombinant HIV-1 RT activity by 50%.
d Data taken from ref 13.
e Data taken from ref 23.

Finally, the novel compounds were also evaluated for their inhibitory activity against a mutant HIV-1 strain that contains the E138K mutation in its RT which is known to afford pronounced resistance to TSAO-T.15 None of the compounds showed inhibitory activity against such mutant HIV-1/138K strain in CEM cell culture (Table 1) pointing to a similar mechanism of interaction of the compounds with HIV-1 RT as the prototype TSAO-T.

Photochemical Kinetics

Before attempting to use the arylazide 5 and benzophenone 6 TSAO derivatives for photoaffinity labeling, the effect of UV irradiation on the UV-vis spectrum was examined for both compounds. This was done both to demonstrate that the target

compounds could be photolyzed and to establish the optimal conditions (wavelength, power, irradiation time) required for biological testing. As shown in Figure 2, compound 5 (Figure 2A) has two absorption maxima at 268 and 309 nm while compound 6 (Figure 2B) has only one absorption maximum at 260 nm. Photodecomposition of 5 and 6 was examined by monitoring the UV change of absorbance upon irradiation at different times.

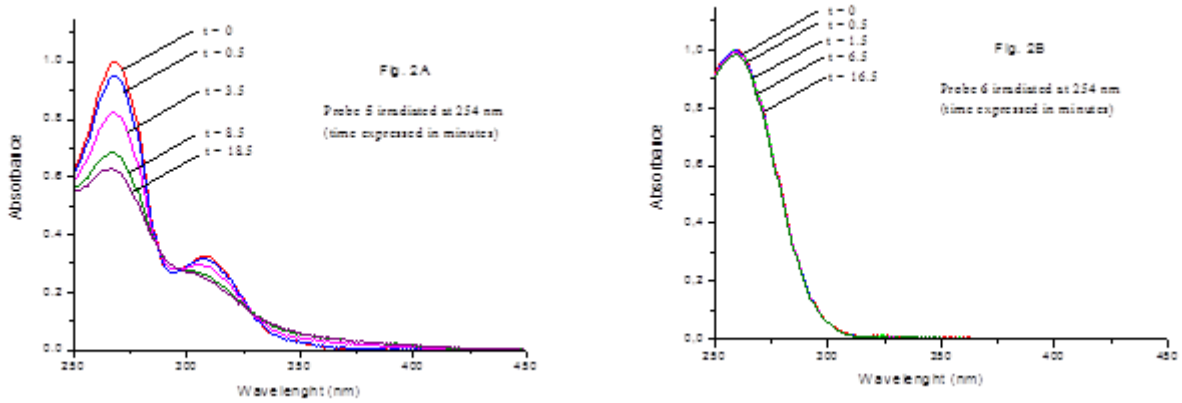



Fig. 2. Photodecomposition of arylazide derivative 5 (A) and the benzophenone derivative 6 (B). The spectra were taken at time 0 (top line), and at the times indicated after photolysis at 254 nm.

Figure 2A showed the spectral changes that occurred upon UV irradiation of a 25 □M methanol solution of 5 at 254 nm wavelength using a higher power hand lamp for fixed periods of time (0.5-10 min). Large changes in the spectrum were seen over the whole spectral region for this compound. Irradiation of a solution of 6 in methanol (50 □M) under the power and wavelength conditions previously mentioned (Figure 2B) resulted in only small spectral changes even after 10 minutes of irradiation.

Results from Figure 3 show the spectral changes that occurred upon UV irradiation of 5 (Figure 3A) and 6 (Figure 3B) at 366 nm wavelenth using a lower power hand lamp for fixed periods of time. These spectral changes overall were similar to those that were observed for the photolysis at 254 nm, however, they were relatively smaller.

It is well known that when irradiating at wavelengths of less that 280 nm, not only makes the reactive species unmasked, but photoinduced chemistry mediated by the protein itself starts to occur. This undesired effect, dictated by aromatic residues in the protein, can lead to damage of the protein and obscure results. For this reason although a great sensitivity to photolysis was observed at 254 nm, irradiation using wavelengths longer than 300 nm should be preferred in order to prevent the previously mentioned problem.35

Taken together, these results indicated that 5 can be photolytically activated by UV ligth more efficiently than 6. Although a short wavelength UV-light (254 nm) allow the optimal photoactivation of 5 and 6 a long wavelength (>254 nm) should be preferable in order to prevent the damage of the protein.

Effect of UV irradiation on the interaction of arylazide 5 and benzophenone 6 derivatives with HIV-1 RT

A preliminary study of the interaction of the photoaffinity analogues 5 and 6 with HIV-1 RT in the presence of UV light was carried out. Wild-type HIV-1 RT (200 nM) was incubated at room temperature with varying concentrations of 5 and 6 and then exposed to UV irradiation (312 nm) for varying times (30 s to 30 min). Following irradiation, RT was separated from unbound drug by passage through a Sephadex G-25 Quick Spin Protein Column (Roche). Thereafter, the RNA-dependent DNA polymerase activity of the enzyme was assessed and compared with the control reactions. These included a no drug control that was exposed to all experimental steps including irradiation, and a drug control which did not undergo UV exposure. Initial experiments demonstrated a marked loss of RT activity (5-10%) after 4 min. Complete loss of RT activity was observed at prolonged exposure times (20-30 min), however this was attributable to UV damage and not to cross-linking of the TSAO derivative to RT. Analysis of the cross-linking experiments that were carried out for shorter time periods demonstrated no discernable difference with the control reactions, which suggested that 5 and 6 were not effectively cross-linked with HIV-1 RT. Consistent with this result, the UV and CD spectra of enzymes that were subjected to UV irradiation were essentially identical to the control enzymes, confirming that compounds 5 and 6 did not cross-link to RT.

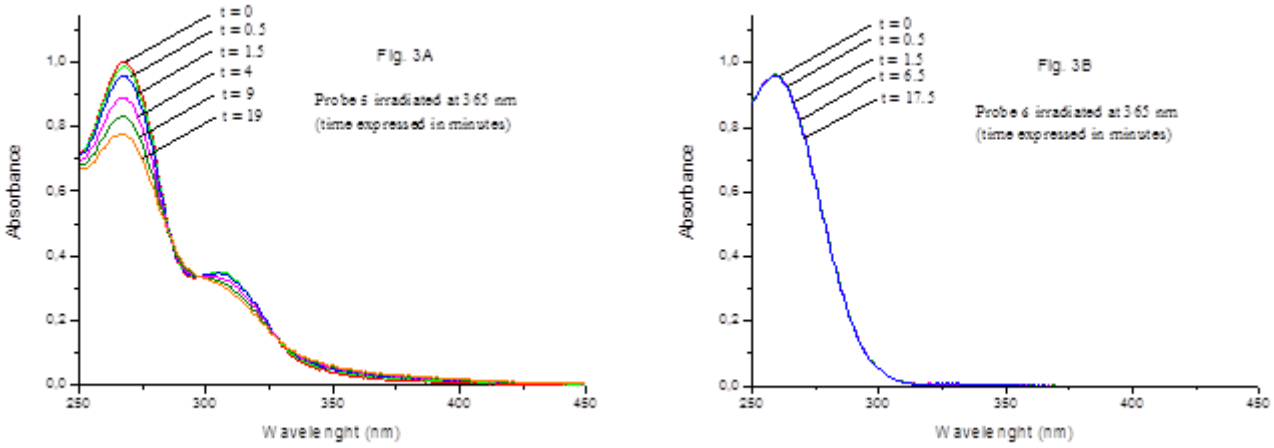



Fig. 3. Photodecomposition of arylazide derivative 5 (A) and the benzophenone derivative 6 (B). The spectra were taken at time 0 (top line), and at the times indicated after photolysis at 366 nm

Experimental

Synthesis

Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. Electrospray mass spectra were measured on a quadropole mass spectrometer equippped with an electrospray source (Hewlett Packard, LC/MS HP 1100). 1H NMR spectra were recorded with a Varian Gemini, a Varian XL-300 and a Bruker AM-200 spectrometer operating at 300 and 200 MHz with Me4Si as internal standard. 13C NMR spectra were recorded with a Varian XL-300 and a Bruker AM-200 spectrometer operating at 75 MHz and at 50 MHz with Me4Si as internal standard. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 (Merck). Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a ChromatotronR (Kiesegel 60 PF254 gipshaltig; Merck), layer thickness (1 mm), flow rate (5 mL min-1). Preparative layer chromatography was performed on 20 x 20 cm glass plates coated with a 2 mm layer of silica gel PF254 (Merck). Analytical HPLC was carried out on a Waters 484 System using a Bondapak C18 (3.9 x 300mm: 10mm). Isocratic conditions were used: Mobile phase CH3CN/H2O (0.05%TFA). Flow rate: 1 ml/min. Detection: UV (254 nm). All retention times are quoted in minutes. Irradiations were carried out in a Pyrex cell (inmersion well) with light of 290 nm wavelenth using a medium-pressure mercury lamp. IR spectra were recorded with a Schimadzu IR-435 spectrometer.UV spectra were recorder with a Perkin Elmer Lambda 16 Spectrophotometer.

[1-[2',5'-Bis-O-(tert-butyldimethylsilyl)- -D-ribofuranosyl]-3-N-[[N,N-bis(2-chloroethyl)carbamoyl]methyl]thymine]-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide) (2)

To a cooled (-20ºC) solution of compound 123 (0.1 g, 0.16 mmol) and triethylamine (TEA) (0.04 mL, 0.32 mmol) in dry dichloromethane (4 mL) was added N,N-bis(2-chloro-ethyl)amine (0.03 g, 0.16 mmol). After 15 min, BOP (0.07 g, 0.16 mmol) was added. The mixture was stirred at room temperature overnight and then evaporated to dryness. The residue was dissolved in ethyl acetate (2 mL) and washed successively with 10 % citric acid (5 mL), 10 % NaHCO3 (5 mL) and brine (5 ml). The organic phase was dried (Na2SO4), filtered and evaporated to dryness. The residue was purified by CCTLC on the chromatotron (hexane: ethyl acetate, 2:1) to give 2 (0.07 g, 68%) as a white foam; (Found: C, 46.36; H, 6.28; N, 7.51. Calcd. for C30H52Cl2N4O9SSi2: C, 46.68; H, 6.79; N, 7.26%); H [300 MHz; (CD3)2CO; Me4Si] 0.78, 0.93 (18H, 2s, 2t-Bu), 1.95 (3H, s, CH3-5), 3.71 (4H, m, CH2Cl), 3.94 (4H, m, CH2N), 4.05 (1H, dd, H-5'a, J4',5'a 3.8 Hz, J5'a,5'b 13.2 Hz), 4.13 (1H, dd, H-5'b, J4',5'b 3.5 Hz ), 4.34 (1H, t, H-4'), 4.80 (1H, d, H-2'), 4.89 (2H, d, CH2N, J 10.7 Hz), 5.74 (1H, s, H-3''), 6.07 (1H, d, H-1', J1',2' 8.0 Hz), 6.50 (2H, bs, NH2-4''), 7.63 (1H, s, H-6); C [75 MHz; (CD3)2CO; Me4Si] 13.6 (CH3-5), 18.3, 18.9 [(CH3)3-C-Si], 25.5, 26.6 [(CH3)3-C-Si], 44.2 (2 CH2Cl), 45.6 (NCH2), 49.5 (2 CH2N), 47.0 (CH2N), 49.1 (CH), 62.9 (C-5'), 75.3 (C-2'), 83.6 (C-4'), 88.7 (C-3''), 93.4 (C-

3’), 92.7 (C-1’), 111.1 (C-5), 134.9 (C-6), 151.5 (C-4’’), 151.9 (C-2), 163.1 (C-4), 163.9 (CON); m/z (ESI; positive ion mode) 771.4 (M+H+. C30H52Cl2N4O9SSi2 requires 770.24).

[1-[2',5'-Bis-O-(tert-butyldimethylsilyl)-□-D-ribofuranosyl]-3-N-[2.3-epoxypropyl]thymine]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) (3)

To a solution of TSAO-T13 (0.10 g, 0.17 mmol) in dry acetone (5 mL) was added dried and powdered K2CO3 (0.026 g, 0.19 mmol) and epibromohydrin (0.032 mL, 0.51 mmol). The reaction mixture was refluxed for 8 h and then concentrated to dryness. The residue was dissolved in ethyl acetate (20 mL), washed with brine (2 x 20 mL), dried (Na2SO4), filtered, evaporated to dryness and purified by CCTLC on the chromatotron (dichloromethane/ethyl acetate, 20:1) to give 3 (0.075 g, 60%) as a white, amorphous solid ; (Found: C, 50.08; H, 7.49; N, 6.10. Calcd. for C27H47N3O9SSi2: C, 50.21; H, 7.33; N, 6.51%); □H [300 MHz; (CD3)2CO; Me4Si] 0.81, 0.96 (18H, 2s, 2t-Bu), 1.95 (3H, s, CH3-5), 2.60 (1H, m, OCHa), 2.69 (1H, m, OCHb), 3.12 (1H, m, CH), 3.81 (1H, m, NCHa), 4.06 (1H, dd, H-5’a, J4’,5’a 4.1 Hz, J5’a,5’b 12.0 Hz), 4.11 (1H, dd, H-5’b, J4’,5’b 6.7 Hz), 4.26 (1H, m, NCHb), 4.35 (1H, t, H-4’), 4.69 (1H, d, H-2’), 5.78 (1H, s, H-3’’), 6.11 (1H, d, H-1’, J1’,2’ 6.4 Hz), 6.57 (1H, bs, NH2-4’’), 7.56 (1H, s, H-6); □C [75 MHz; (CD3)2CO; Me4Si] 13.3 (CH3-5), 18.2, 18.8 [(CH3)3-C-Si], 25.6, 26.4 [(CH3)3-C-Si], 43.5 (OCH2), 47.0 (CH2N), 49.1 (CH), 62.5 (C-5’), 75.2 (C-2’), 83.9 (C-4’), 88.5 (C-3’’), 93.5 (C-3’), 92.5 (C-1’), 111.5 (C-5), 134.2 (C-6), 151.6 (C-4’’), 151.7 (C-2), 163.4 (C-4); m/z (ESI; positive ion mode) 646.2 (M+H+. C27H47N3O9SSi2 requires 645.26).

[1-[2',5'-Bis-O-(tert-butyldimethylsilyl)-□-D-ribofuranosyl]-3-N-[3-N’-(4-azidosalicylyl)aminopropyl]thymine]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) (5)

To a solution of compound 423 (0.10 g, 0.16 mmol) in dry dichloromethane (10 mL) 4-azido salicylic acid (Husain et al., 1983) (0.034g, 0.19 mmol) and BOP (0.070 g, 0.16 mmol) was added. After 15 min, triethylamine (TEA) (0.032 mL, 0.24 mmol) was added. The mixture was stirred at room temperature overnight and then evaporated to dryness. The residue was dissolved in ethyl acetate (2 mL) and washed successively with 10 % citric acid (10 mL), 10 % NaHCO3 (10 mL) and brine (10 ml). The organic phase was dried (Na2SO4), filtered and evaporated to dryness. The residue was purified by CCTLC on the chromatotron (dichloromethane: methanol, 50:1) to give 5 (0.11 g, 85%) as a white foam; (Found: C, 50.26; H, 6.48; N, 12.45. Calcd. for C34H53N7O10SSi2: C, 50.54; H, 6.61; N, 12.13%); □□□□ (MeOH)/nm 268 (□/dm3 mol-1 cm-1 60600) and 309 (22000); □□□□(KBr)/cm-1 2100 (N3); □H [300 MHz; (CD3)2CO; Me4Si] 0.79, 0.97 (18H, 2s, 2t-Bu), 1.92 (2H, m, CH2CH2N), 1.94 (3H, s, CH3-5), 3.42 (2H, m, CH2NHCO), 4.04 (2H, m, H-5’a, CH2N), 4.10 (1H, dd, H-5’b, J4’,5’b 3.6 Hz, J5’a,5’b 12.3 Hz), 4.33 (1H, t, H-4’), 4.64 (1H, d, H-2’), 5.76 (1H, s, H-3’’), 6.10 (1H, d, H-1’, J1’,2’ 8.1 Hz), 6.43 (1H, bs, NH2-4’’), 6.54 (1H, s, Ph), 6.60 (1H, d, Ph), 7.51 (1H, s, H-6), 7.77 (1H, d, Ph), 8.20 (1H, t, NHCO);□□C [75 MHz; (CD3)2CO; Me4Si] 12.8 (CH3-5), 18.0, 18.6 [(CH3)3-C-Si], 25.3, 26.0 [(CH3)3-C-Si], 28.9 (CH2), 37.0 (CH2NHCO), 39.4 (CH2N), 62.8 (C-5’), 75.1 (C-2’), 84.5 (C-4’), 87.1 (C-3’’), 92.2 (C-3’), 92.3 (C-1’), 108.0 (CH-Ph), 110.1 (CH-Ph), 111.2 (C-5), 128.7 (CH-Ph), 134.2 (C-6), 145.9 (C-N3), 151.6 (C-4’’), 151.6 (C-2), 163.3 (C-4), 163.3 (CONH); m/z (ESI; positive ion mode) 808.3 (M+H+. C34H53N7O10SSi2 requires 807.31).

[1-[2',5'-Bis-O-(tert-butyldimethylsilyl)-□-D-ribofuranosyl]-3-N-[3-N’[(4-(benzoyl)-carbonylphenyl]aminopropyl]thymine]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) (6)

Following the previously described method, a solution of 423 (0.1 g. O.15 mmol), BOP (0.066 g, 0.15 mmol), 4-benzoylbenzoic acid (0.040 g, 0.18 mmol) and TEA (0.030 mL, 0.23 mmol) in dry dichloromethane was stirred at room temperature overnight and then evaporated to dryness. The residue was purified by CCTLC on the chromatotron (hexane:ethyl acetate, 1:1) to give 6 (0.097 g, 76%) as a white foam; (Found: C, 57.28; H, 6.47; N, 6.14. Calcd. for C41H58N4O10SSi2: C, 57.58; H, 6.84; N, 6.55%). □□□□ (MeOH)/nm 260 (□/dm3 mol-1 cm-1 28000); □H [300 MHz; (CD3)2CO; Me4Si] □: 0.80, 0.96 (18H, 2s, 2t-Bu), 1.93 (2H, m, CH2CH2N), 1.95 (3H, s, CH3-5), 3.46 (2H, m, CH2NHCO), 4.10 (4H, m, H-5’a, CH2N), 4.34 (1H, t, H-4’), 4.66 (1H, d, H-2’), 5.77 (1H, s, H-3’’), 6.12 (1H, d, H-1’, J1’,2’ 8.0 Hz), 6.47 (1H, bs, NH2-4’’), 7.51 (9H, m, Ph), H-6), 8.22 (1H, t, NHCO);□□C [75 MHz; (CD3)2CO; Me4Si] 12.8 (CH3-5), 18.1, 18.6 [(CH3)3-C-Si], 25.0, 26.3 [(CH3)3-C-Si], 29.1 (CH2), 37.4 (CH2NHCO), 39.5 (CH2N), 62.8 (C-5’), 75.1 (C-2’), 84.9 (C-4’), 87.3 (C-3’’), 92.1 (C-1’), 92.3 (C-3’), 112.5 (C-5), 130.0, 130.2, 129.0, 127.6 (Ph), 134.3 (C-6), 137.9, 138.9, 140.3 (C-Ph), 151.7 (C-4’’), 151.8 (C-2), 163.3 (C-4), 166.0 (CONH), 195.8 (PhCOPh). m/z (ESI; positive ion mode) 855.5 (M+H+ C41H58N4O10SSi2 requires 854.34).

[1-[2',5'-Bis-O-(tert-butyldimethylsilyl)-□-D-ribofuranosyl]-3-N-[(E)-2-ethoxycarbonylvinyl]thymine]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) (7)

A mixture of TSAO-T13 (0.4 g, 0.68 mmol), ethyl propiolate (0.082 mL, 0.84 mmol) and 4-(dimethylamino)pyridine (DMAP) (0.102 g, 0.84 mmol) was stirred at room temperature for 1.5 h. Solvent was evaporated to dryness and the residue was dissolved in ethyl acetate (20 mL) and washed successively with HCl 0.1 N (2 □ 15 mL), 10 % NaHCO3 (2 □ 15 mL), and brine (2 □ 15 mL). The organic phase was dried (Na2SO4), filtered, evaporated to dryness and purified by CCTLC on the chromatotron (hexane:ethyl acetate, 2:1) to give 7 (0.39 g, 82%) as a white foam; (Found: C, 50.30; H, 7.45; N, 6.85. Calcd. for C29H49N3O10SSi2: C, 50.63; H, 7.18; N, 6.11%); HPLC (CH3CN:H2O, 70:30) retention time: 10.58 min; □H [300 MHz; (CD3)2CO; Me4Si] 0.80, 0.97 (18H, 2s, 2t-Bu), 1.27 (3H, t, OCH2CH3, J 7.0 Hz), 1.98 (3H, s, CH3-5), 4.06 (1H, dd, H-5'a, J4',5'a 3.4 Hz, J5'a,5'b 12.2 Hz), 4.12 (1H, dd, H-5'b, J4',5'b 3.6 Hz), 4.20 (2H, q, OCH2CH3), 4.39 (1H, t, H-4'), 4.65 (1H, d, H-2'), 5.78 (1H, s, H-3''), 6.13 (H-1', d, 1H, J1',2' 8.1 Hz), 6.45 (2H, bs, NH2-4''), 6.99 (1H, d, NCH=CH), 7.56 (1H, s, H-6), 8.26 (1H, d, NCH=CH, J 14.7 Hz). □C [75 MHz; (CD3)2CO; Me4Si] 13.2 (CH3-5), 14.5 (OCH2CH3), 18.1, 18.6 [(CH3)3-C-Si], 25.0, 26.3 [(CH3)3-C-Si], 61.0 (OCH2), 63.1 (C-5'), 75.5 (C-2'), 85.5 (C-4'), 87.6 (C-3''), 92.7 (C-1', C-3'), 111.4 (C-5), 113.8 (NCH=CH), 134.8 (C-6), 135.3 (NCH=CH), 151.7 (C-4''), 150.9 (C-2), 162.3 (C-4), 167.4 (CO). m/z (ESI; positive ion mode) 688.4 (M+H+ C29H49N3O10SSi2 requires 687.29).

[1-[2',5'-Bis-O-(tert-butyldimethylsilyl)-□-D-ribofuranosyl]-3-N-[(Z)-2-ethoxycarbonylvinyl]thymine]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) (8)

A solution of the E isomer 7 (0.100 g, 0.14 mmol) in methanol (100 mL) in a Pyrex cell (inmersion well) was irradiated under argon atmosphere at room temperature with light of 290 nm wavelenth using a medium-pressure mercury lamp. The progress of the reaction was monitored by HPLC. After 30 min of irradiation an equilibrium (1:1.5) mixture of E and Z isomers were obtained. Prolonged reaction times (6h) did not affect to the composition of the mixture. Solvent was evaporated under reduced pressure to give a residue which were purified by preparative thin layer chromatography using as eluent (dichloromethane:ethyl acetate, 40:1). After several developments two different moving bands were separated.

From the fastest moving band E isomer 7 (0.040 g, 40%) was isolated.

From the slowest moving band Z isomer 8 (0.060 g, 60%) was isolated; (Found: C, 50.30; H, 7.45; N, 6.85. Calcd. for C29H49N3O10 SSi2: C, 50.63; H, 7.18; N, 6.11%). HPLC (CH3CN:H2O, 70:30) retention time : 5.46 min. □H [300 MHz; (CD3)2CO; Me4Si] □: 0.83, 0.96 (18H, 2s, 2t-Bu), 1.17 (3H, t, OCH2CH3, J 5.2 Hz), 1.93 (s, 3H, CH3-5), 4.05 (1H, dd, H-5'a, J4',5'a 4.1 Hz, J5'a,5'b 11.4 Hz), 4.09 (q, 2H, OCH2CH3), 4.10 (1H, dd, H-5'b, J4',5'b 3.5 Hz ), 4.31 (t, 1H, H-4'), 4.70 (1H, d, H-2'), 5.75 (1H, s, H-3''), 6.01 (1H, d, H-1', J1',2' 7.9 Hz), 6.12 (d, 1H, NCH=CH), 6.47 (bs, 1H, NH2-4''), 6.70 (d, 1H, NCH=CH, J 9.0 Hz), 7.55 (s, 1H, H-6). (ESI; positive ion mode) 688.4 (M+H+ C29H49N3O10SSi2 requires 687.29).

[1-[2',5'-Bis-O-(tert-butyldimethylsilyl)-□-D-ribofuranosyl]-3-N-[(E)-3-hydroxy-1-propenyl]thymine]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) (9)

DIBAL-H (1 M solution in THF, 0.28 mL, 0.28 mmol) was added dropwise into a solution of 7 (0.2 g, 0.29 mmol) in dry THF (0.5 mL) at 0ºC over 10 min with stirring under argon. After the reaction mixture was allowed to reach room temperature, stirring was continued for 3 h. Saturated aqueous NH4Cl (0.5 mL) was then added, and the gel-like solid was filtered off using a Celite bed. Evaporation of the solvent gave a residue that was purified by CCTLC on the chromatotron using hexane/ethyl acetate (2:1) to give 9 (0.11 g, 61%) as a white foam; (Found: C, 50.50; H, 7.85; N, 6.75. Calcd. for C27H47N3O9SSi2: C, 50.21; H, 7.33; N, 6.51%). □H [300 MHz; (CD3)2CO; Me4Si] 0.82, 0.98 (18H, 2s, 2t-Bu), 1.96 (3H, s, CH3-5), 4.05 (1H, dd, H-5'a, J4',5'a 3.7 Hz, J5'a,5'b 12.2 Hz), 4.13 (1H, dd, H-5'b, J4',5'b 3.5 Hz ), 4.16 (1H, t, OH, J 5.8 Hz), 4.23 (2H, m, CH2OH), 4.36 (1H, t, H-4'), 4.65 (1H, d, H-2'), 5.78 (1H, s, H-3''), 6.12 (1H, d, H-1', J1',2' 8.2 Hz), 6.50 (1H, bs, NH2-4''), 6.56 (1H, m, NCH=CH), 6.82 (1H, m, NCH=CH, J 14.4 Hz), 7.52 (s, 1H, H-6). □C [75 MHz; (CD3)2CO; Me4Si] 13.8 (CH3-5), 18.1, 18.6 [(CH3)3-C-Si], 25.0, 26.3 [(CH3)3-C-Si], 61.7 (CH2OH), 63.5 (C-5'), 75.1 (C-2'), 85.9 (C-4'), 87.2 (C-3''), 92.8 (C-1', C-3'), 111.6 (C-5), 123.4 (CH=CH), 130.3 (NCH=CH), 134.6 (C-6), 151.4 (C-4''), 150.6 (C-2), 162.5 (C-4). (ESI; positive ion mode) 646.6 (M+H+ C27H47N3O9SSi2 requires 645.26).

[1-[2',5'-Bis-O-(tert-butyldimethylsilyl)-□-D-ribofuranosyl]-3-N-[(Z)-3-hydroxy-1-propenyl]thymine]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) (10)

Following the previosly described method for the synthesis of 9, a solution of 8 (0.050 g, 0.07 mmol) in dry THF (0.25 mL) was treated with DIBAL-H (1 M solution in THF, 0.14 mL, 0.14 mmol). Purification of the residue by CCTLC on the chromatotron using hexane/ethyl acetate (2:1) afforded 10 (0.058 g, 52%) as a white foam; (Found: C, 50.50; H, 7.85; N, 6.75. Calcd. for

C27H47N3O9SSi2: C, 50.21; H, 7.33; N, 6.51%). □H [300 MHz; (CD3)2CO; Me4Si] 0.82, 0.96 (18H, 2s, 2t-Bu), 1.94 (3H, s, CH3-5), 3.64 (1H, t, OH, J = 5.8 Hz), 3.92 (2H, t, CH2OH), 4.04 (1H, dd, H-5'a, J4',5'a 3.8 Hz, J5'a,5'b 12.3 Hz), 4.12 (1H, dd, H-5'b, J4',5'b 3.4 Hz ), 4.32 (1H, t, H-4'), 4.69 (1H, d, H-2'), 5.74 (1H, s, H-3''), 5.89 (1H, m, NCH=CH), 6.05 (1H, d, H-1', J1',2' 8.0 Hz), 6.09 (m, 1H, NCH=CH, J 9.5 Hz), 6.45 (bs, 1H, NH2-4''), 7.55 (s, 1H, H-6). (ESI; positive ion mode) 646.6 (M+H+ C27H47N3O9SSi2 requires 645.26).

Biological Methods

Cells and Viruses

Human immunodeficiency virus type 1 [HIV-1 (IIIB)] was obtained from Dr. R.C. Gallo (when at the National Cancer Institute, Bethesda, MD). HIV-2 (ROD) was provided by Dr. L. Montagnier (when at the Pasteur Institute, Paris, France).

Activity Assay of Test Compounds against HIV-1 and HIV-2 in CEM and MT-4 Cell Cultures

A total number of 4 x 106 CEM or 3 x 105 MT-4 cells per milliliter were infected with HIV-1 (IIIB ) or HIV-2 (ROD) or HIV-1/138K (a virus strain that was selected in the presence of TSAO-m3T and that contained the E138K mutation in its RT) at ~ 100 CCID50 (50% cell culture infective dose) per milliliter of cell suspension. Then an amount of 100 µL of the infected cell suspension was transferred to microtiter plate wells and mixed with 100 µL of the appropriate dilutions of the test compounds. Syncytia formation (CEM) or HIV-induced cytopathicity (MT-4) was recorded microscopically (CEM) or by trypan blue dye exclusion (MT-4) in the HIV-infected cell cultures after 4 days (CEM) or 5 days (MT-4). The 50% effective concentration (EC50) of the test compounds was defined as the compound concentration required to inhibit virus-induced cytopathicity (CEM) or to reduce cell viability (MT-4) by 50%. The 50% cytostatic or cytotoxic concentration (CC50) was defined as the compound concentration required to inhibit CEM cell proliferation by 50% or to reduce the number of viable MT-4 cells in mock-infected cell cultures by 50%.

Inhibitory Effect of Test Compounds against Recombinant HIV-1 RT

HIV-1 RT was purified as described previously).36,37 Inhibition of the RNA-dependent DNA polymerase activity of HIV-1 RT was assessed using a fixed time scintillation proximity assay. Briefly, reactions were carried out in 50mM Tris-HCl pH 7.8 (37°C), 50mM KCl, 1mM MgCl2 and contained 700nM of poly(rA)-oligo(dT)18 (the oligo(dT)18 was synthesized with a biotin moiety on the 5'-end), 5□M [3H]TTP, and variable concentrations of ligand dissolved in DMSO (3% final concentration). Reactions were initiated by the addition of 20 nM of wild-type or E138K HIV-1 RT and incubated for 20 min at 37 °C. Reactions were quenched by addition of 0.5 mg/ml scintillant impregnated streptavidin coated polyvinyltoluene beads (Amersham) in a quench solution containing 100 mM EDTA. Plates were allowed to rest for 2 hrs prior to counting radioactivity using a Hewlett-Packard plate reader. The 50% inhibitory concentration (IC50) was defined as the concentration of the compound required to inhibit enzyme activity by 50%.

Photoaffinity cross-linking experiments

200 nM wild-type HIV-1 RT was incubated at room temperature with compounds 5 and 6 (concentrations ranged from 1 □M to 25 □M) in 50 Tris-HCl pH 7.8, 50 mM KCl, and 1mM MgCl2. The reaction mixtures were then irradiated with a hand-held UV lamp (Fisher Biotech) at 312 nm wavelength with an intensity of 1.15 mW/cm2 for varying times (30 s to 30 min).

Following irradiation, RT was separated from unbound drug by passage through a Sephadex G-25 Quick Spin Protein Column (Roche). Thereafter, the RNA-dependent DNA polymerase activity of the enzyme was assessed as described above. Control reactions included no inhibitor (and in this case they were subjected to UV irradiation) or inhibitor (without exposure to UV irradiation).

In addition to the activity assays described above, the RT that was subjected to UV irradiation was also assessed by ultraviolet (UV) absorption and circular dichroism (CD) spectroscopy. The UV absorption spectroscopy was carried out using a Beckman Coulter DU530 UV/VIS spectrophotometer. CD spectra were collected at ambient room temperature (24 oC) using an Aviv Instruments CD Spectrometer (Model 202).

Conclusions

As shown above, all the compounds showed pronounced activity against HIV-1 replication in cell culture. The most antivirally active compounds were the E-hydroxymethylvinyl 9 and the bis-N-chloroethyl 2 derivatives.

The alkylating moieties (epoxide and nitrogen mustard) introduced at the N-3 position of TSAO-T were tolerated without loss of activity but they did not improve activity.

On the other hand, presence of an alcohol functional group linked to the N-3 position of thymine has a significant impact on the activity provided that this moiety was linked to this position through a flexible polymethylene linker or a trans double bond.

Benzophenone and arylazide containing TSAO analogues derivatives were synthetically accesible. Before attempting to use these analogues for photoaffinity labeling, we have first established that the presence of the photophore in the side chain is well tolerated without a significant loss of activity. The arylazide analogue 5 was more efficiently photolyzed than the benzophenone derivative 6. A preliminary study of the interaction of the photoaffinity analogs 5 and 6 with HIV-1 RT in the presence of UV light was carried out, however successful cross-linking to the target protein was not obtained. Although we were unable to cross-link the current compounds to HIV-1 RT, our studies demonstrated that the cross-linking approach is feasible in that photoaffinity labels can be introduced on the N-3 of TSAO without apreciable loss of inhibitory activity. The future challenge is to alter the orientation and/or length of the spacer or to include different moieties at the molecule to identify a TSAO derivative that can cross-link to RT.

Acknowledgements

We thank Dr. Francisco Amat for his help and advise with the photochemical experiments. We thank the Ministery of Education of Spain for a grant to María Cruz Bonache. The Spanish MEC (project SAF2006-12713-C02) is also acknowledged for financial support. NSC was supported by grants GM068406 and R21 AI060393 from the National Institutes of Health (USA).

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