Hybrids of [TSAO-T]−[Foscarnet]: The First Conjugate of Foscarnet with a Non-nucleoside Reverse Transcriptase Inhibitor through a Labile Covalent Ester Bond†

Sonsoles Velázquez,*,‡ Esther Lobatón,‡ Erik De Clercq,§ Dianna L. Koontz,|| John W. Mellors,|| Jan Balzarini,§ and María-José Camarasa‡

Instituto de Química Médica (C.S.I.C.), Juan de la Cierva 3, E−28006 Madrid, Spain, and Rega Institute for Medical Research, K. U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium, and University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

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This paper describes the first example of combination of non-nucleoside reverse transcriptase inhibitors such as TSAO derivatives and foscarnet (PFA) in a single molecule through a labile covalent ester bond. The essential criteria in the design of these hybrids [TSAO-T]−[PFA] was to explore if the conjugation of foscarnet with the highly lipophilic TSAO derivative may facilitate the penetration of the conjugates through the cell membrane and if the hybrids escape extracellular hydrolysis and regenerate the parent inhibitors intracellulary. Several [TSAO-T]−[PFA] conjugates proved markedly inhibitory to HIV-1. Some of them also showed potent activity against PFA-resistant HIV-1 strains but fewer had detectable inhibitory activity against TSAO-resistant HIV-1 strains. These results indicated a pivotal role of the TSAO component of the hybrid but not the PFA component in the activity of the conjugates. Moreover, stability studies of the [TSAO-T]−[PFA] conjugates demonstrated that the compounds were stable in PBS whereas some of the conjugates regenerated the parent inhibitors in extracts from CEM cells.

Introduction

Emergence of HIV drug resistance and the need for long-term antiretroviral treatment are currently the main causes for the failure of antiretroviral therapy. Combination of different anti-HIV agents has become the standard clinical practice to keep the viral load at undetectable levels and to prevent emergence of virus drug resistance.[1,2]

Among the human immunodeficiency virus (HIV) reverse transcriptase (RT) inhibitors, the so-called non-nucleoside RT inhibitors (NNRTIs) have gained a primary role in the treatment of HIV infection in combination with nucleoside analogue RT inhibitors (NRTIs) and HIV protease inhibitors (PIs).[3,4] The virus can be markedly suppressed for a relatively long period of time when exposed to multiple drug combination therapy (highly active antiretroviral therapy, HAART).

The [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-*β*-d-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) nucleosides (TSAO) are a rather unique class of NNRTIs[5,6] that seem to interact at the interface between the p51 and p66 subunits of HIV-1 RT.[7] The prototype compound of this family is the thymine derivative designated as TSAO-T (**1**) and the most selective compound is its 3-*N*-methyl substituted derivative TSAO-m^{3}T (**2**) (Figure 1). Biochemical studies have revealed that both TSAO-T and its 3-*N*-ethyl derivative

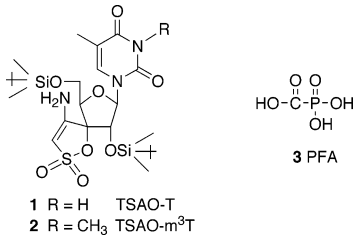

Figure 1. Structures of TSAO-T (**1**), TSAO-m^{3}T (**2**), and phosphonoformic acid (PFA, **3**).

are able to destabilize the p66/p51 RT heterodimer in a concentration-dependent manner resulting in a loss of its ability to bind DNA.[8,9] This suggests a new and different mechanism of inhibition of HIV-1 RT with regard to the other known NNRTIs. HIV-1 resistance to NNRTIs is primarily associated with substitution of amino acids at the lipophilic NNRTIs binding pocket in the p66 subunit.[4] However, TSAO compounds select a single mutation (Glu-138-Lys) in the p51 subunit of HIV-1 RT.[10]

Phosphonoformate (PFA, foscarnet, **3**) (Figure 1) is an effective antiviral agent approved for intravenous treatment of human cytomegalovirus (HCMV) retinitis in patients with AIDS.[11] Although primarily used in the treatment of AIDS-related HCMV infection, PFA is also effective against HIV replication.[12] PFA is proposed to inhibit HIV RT by blocking the pyrophosphate binding site. Although PFA is a potent inhibitor of HIV RT, its highly ionic nature at physiological pH is an impediment to its cellular uptake.[13]

Numerous combination experiments have been performed between different classes of RT inhibitors.[1,2]

* To whom correspondence should be addressed. Telephone: +34-91 5622900. Fax: +34-91 5644853. E-mail: iqmsv29@iqm.csic.es.

† Dedicated to Prof. Dr. Jan Balzarini with best wishes on the occasion of his birthday.

‡ Instituto de Química Médica (C.S.I.C.).
§ Rega Institute for Medical Research.
|| University of Pittsburgh School of Medicine.

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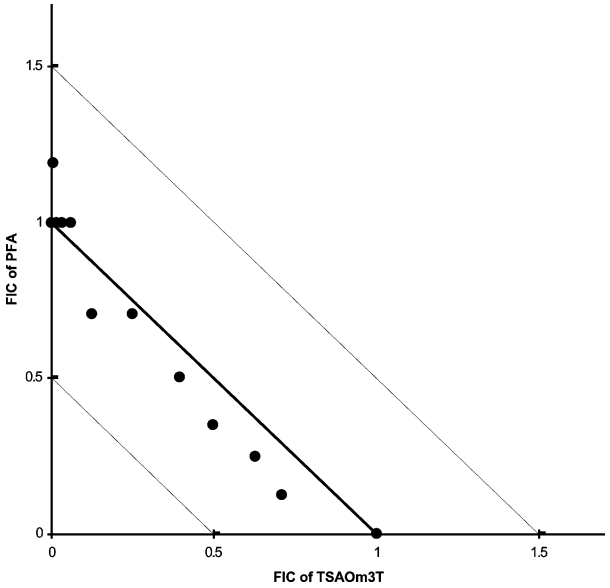


Figure 2. Isobologram of the anti-HIV-1(III_B) activity of combinations of PFA and TSAO-m^3T in CEM cell cultures.

However, combinations of PFA with NRTIs other than AZT, or with NNRTIs, are yet to be reported. Different combinations using TSAO derivatives with either NRTIs or NNRTIs have been explored.[14,15] Interestingly, in preliminary experiments we found that PFA was a potent inhibitor of TSAO-characteristic HIV-1 RT/138 Lys mutant strain. The activity against the 138K mutant was 1 order of magnitude higher than that against virus wild type [IC_{50} (μg/mL) = 0.34 vs 2.59, respectively]. This observation prompted us to combine both inhibitors in an attempt to prevent or delay the emergence of TSAO-resistant virus. A variety of concentrations of TSAO-m^3T and PFA have been combined and added to CEM cell cultures infected with a low multiplicity of HIV-1 infection. It would be interesting to reveal whether combinations of TSAO-m^3T and PFA at a variety of concentrations are synergistic, additive or antagonistic in displaying their anti-HIV activity. Analysis of the antiviral activity of TSAO-m^3T and PFA combinations through construction of an isobologram revealed additivity of the antiviral activity of both drugs (Figure 2).

These results led us to combine TSAO derivatives and foscarnet in a single molecule through a labile covalent ester bond (**I**, Figure 3), as an alternative approach to combination therapy, taking into consideration the highly lipophilic nature of the TSAO molecule and the hydrophilic nature of PFA. The major consideration in the design of these hybrids was to explore whether the lipophilic TSAO molecule may serve as driving-force for PFA into the cells thus resulting in improvement in the cell membrane permeability of PFA. Regardless of whether the PFA was linked to the TSAO molecule through the $PO(OH)_2$ or the COOH group, a decreased negative charge on this molecule (conjugate) relative to PFA ought to facilitate movement (permeability) across the cell membrane. The [TSAO-T]−[PFA] conjugate may also escape extracellular hydrolysis and once inside the cell would liberate the parent compounds. Therefore, we prepared [TSAO-T]−[PFA] conjugates containing the parent drugs linked via ester bonds to permit hydrolytic

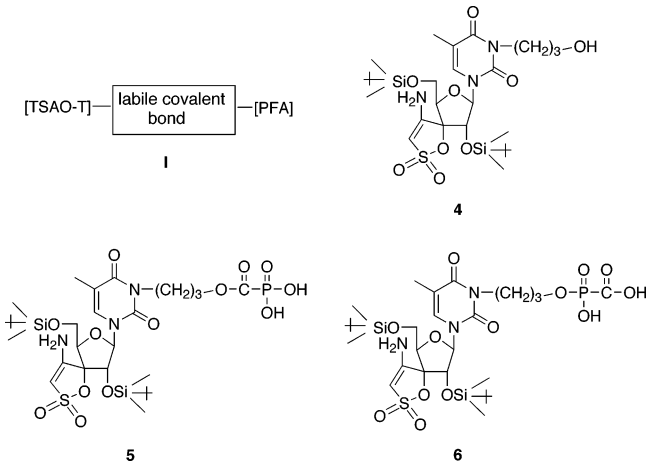


Figure 3. Hybrids [TSAO-T]−[PFA] of general formula **I**. Structures of TSAO derivative **4** and [TSAO-T]−[PFA] conjugates **5** and **6**.

cleavage at physiological pH, or enzyme-mediated catalysis.

Covalent conjugate combination of TSAO-T and PFA would differ substantially from simple noncovalent combination of these two drugs in that the conjugate, transported initially as such into the cells, could have a different antiviral profile. For example, an enzyme-labile conjugate could gradually release the anti-HIV parent compounds upon hydrolysis resulting in different kinetics of uptake/release/availability to the target than that afforded by the TSAO-T and PFA mixture.

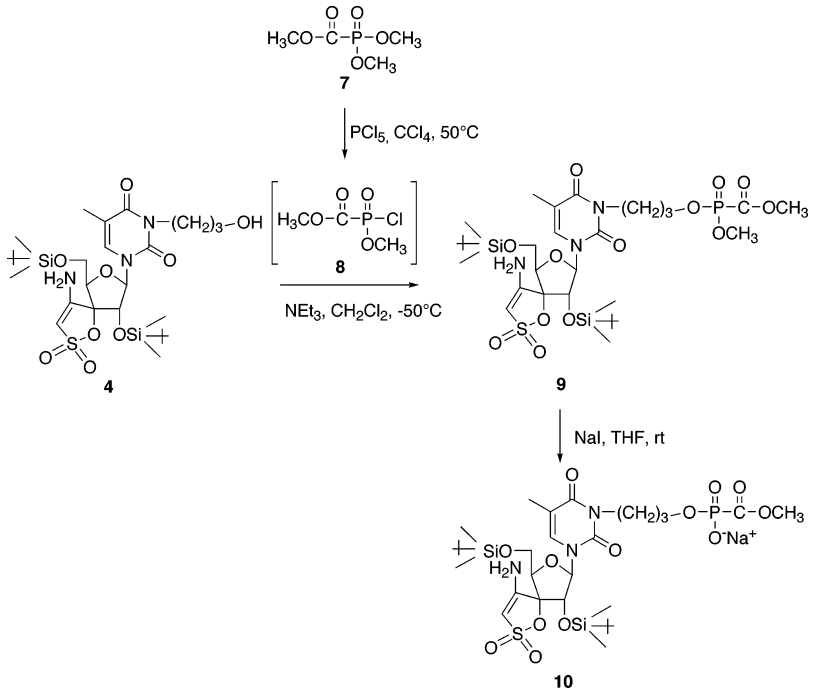
As TSAO molecule the *N*-3 hydroxypropyl derivative **4** (Figure 3) was chosen because attachment of the $(CH_2)_3OH$ substituent to the N3 atom of the thymine of the prototype TSAO-T resulted in a 2-fold better inhibitor.[7] Moreover, from a chemical point of view, compound **4** allows ester formation between PFA and the OH functionality of the N3 thymine substituent. Two types of [TSAO-T]−[PFA] adducts are possible, depending on whether attachment of PFA to the hydroxyl group of the TSAO derivative is via the carboxyl (**5**) or the phosphonyl moieties (**6**) (Figure 3).

Although PFA ester derivatives in which the carboxyl or phosphonyl group was linked to a nucleoside[16] or, in particular, to anti-HIV nucleosides have been previously described,[17−19] [TSAO-T]−[PFA] conjugates are, to the best of our knowledge, the first example of conjugates between PFA and NNRTIs. This paper describes the synthesis of [TSAO-T]−[PFA] conjugates and their in vitro anti-HIV activity against wild-type HIV-1 virus and TSAO-resistant and PFA-resistant HIV-1 variants. Stability studies of the [TSAO-T]−[PFA] conjugates in cell extracts have also been carried out.

Results

Synthesis. Linkage by a carboxylic ester bond to prepare the target [TSAO-T]−[PFA] conjugates **5** was first attempted by condensation of TSAO derivative **4** with (diethylphosphono)formic acid chloride in the presence of NEt_3 and DMAP according to the procedures described by Charvet et al.[18a] Alternatively, nucleoside **4** was first reacted with triphosgene in pyridine followed by addition of triethyl phosphite.[18a] However, all these acylation attempts were unsuccessful. In these reactions

Scheme 1

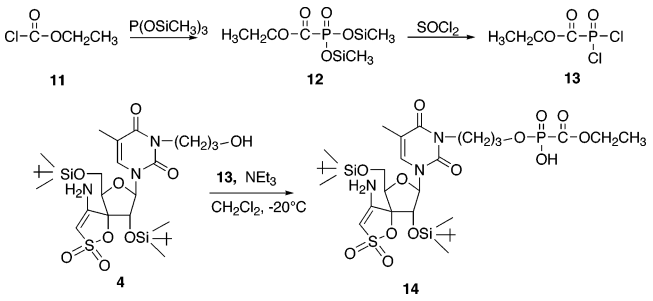


only unreacted material (**4**) together with deprotected TSAO derivatives were isolated.

Linkage by a phosphoric ester bond was achieved following the synthetic strategies summarized in Schemes 1−4. We first addressed our attention to the synthesis of PFA diester intermediate **10** (Scheme 1) and **14** (Scheme 2) which after deprotection would give the target conjugate **6** with the free carboxylic acid. Two methods were used to prepare these [TSAO-T]−[PFA] conjugate intermediates. In the first (Scheme 1), we used the described method for the synthesis of PFA diesters[17b,18b] which involves formation of a PFA triester by reaction of phosphonochloridate and the appropriated alcohol, in the presence of triethylamine, followed by deesterification with NaI. Thus, as shown in Scheme 1, when trimethyl phosphonoformate **7** was treated with PCl_5 in CCl_4 at 50 °C, and the crude reaction product **8**, after removal of volatile materials, was reacted with the *N*-3 hydroxypropyl TSAO derivative **4** in dry CH_2Cl_2, the PFA triester **9** was obtained in 91% yield. Compound **9** was subsequently treated with NaI in dry THF, under argon, to give selective *O*-methyl cleavage on phosphorus[20] yielding the PFA diester **10** as a sodium salt in 80% yield. The PFA diester **14** was prepared by coupling the TSAO derivative **4** with a dichlorophosphonylformate (**13**)[18a] (Scheme 2) as follows. Tris(trimethylsilyl) phosphite was treated with ethyl chloroformate **11** to yield ethyl [bis(trimethylsilyl)phosphono]formate **12** (Scheme 2) in 75% yield. Treatment of compound **12** with $SOCl_2$, according to the procedure described by Vaghefi et al.,[16a] gave the ethyl (dichlorophosphonyl)formate **13**. Finally, coupling between the TSAO derivative **4** and **13** in CH_2Cl_2 in the presence of NEt_3 at −20 °C yielded the PFA diester **14** in 31% yield.

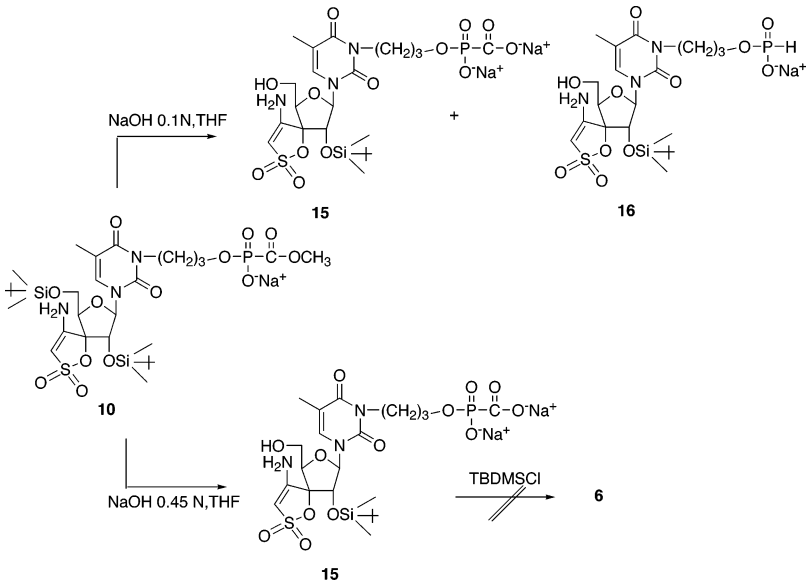
To prepare the final [TSAO-T]−[PFA] conjugate **6**, the PFA diester **10** (obtained in higher yield) was saponified with aqueous sodium hydroxide in THF. Initial attempts of base-catalyzed hydrolysis of **10** with 0.1 N NaOH in

Scheme 2

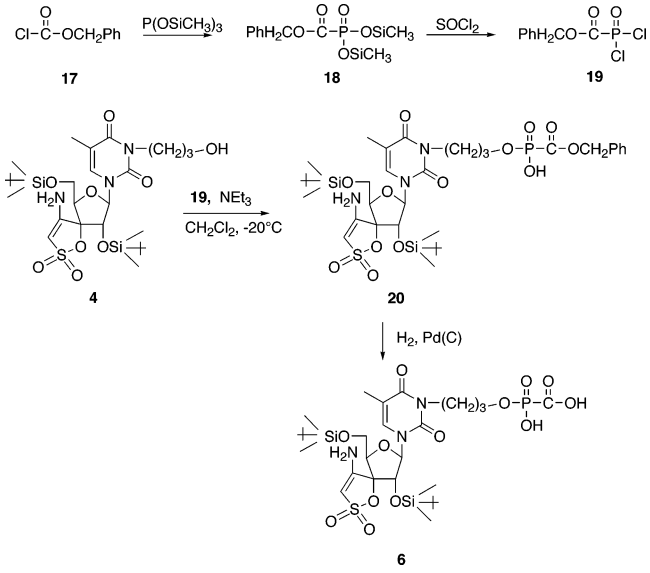


THF to avoid deprotection of the labile 5′-*tert*-butyldimethylsilyl group (TBDMS) (Scheme 3) failed to give the final conjugate **6**. Instead, mixtures of the 5′-deprotected hydrolysis product **15** and the decarboxylated derivative **16** (obtained via P−C bond cleavage) were obtained. The products were isolated as the disodium (**15**) and monosodium (**16**) salts in 53% and 43% yield, respectively. Several previous studies have highlighted the overall hydrolytic instability of phosphonoformate triesters,[21] and the significant problem of hydrolitic P−C bond cleavage which results in the destruction of the parent drug structure. In the previous studies the rate and product distribution are dependent on pH and ester leaving group abalities.[21a] Interestingly, when the saponification of PFA diester **10** was attempted with 0.45 N NaOH, only the hydrolysis product **15** was isolated (Scheme 3). However, although hydrolysis can be diverted away from abortive P−C bond cleavage, deprotection of the 5′-TBDMS group of the sugar moiety could not be avoided in the hydrolysis step. Similar results were observed with [TSAO-T]−[PFA] conjugate **14** when submitted to the same saponification procedures. All the silylation attempts of the 5′-deprotected [TSAO-T]−[PFA] conjugate **15** to give the desired target compound **6** (Scheme 3) failed whatever the experimental standard conditions (TBDMSCl/pyridine, DMAP or imidazole) were used. The starting material

Scheme 3



Scheme 4



was recovered unchanged and/or P−C bond cleavage was also observed in some of the experiments.

These results promted us to try a different strategy for the synthesis of the disilylated target conjugate **6**. The approach involved the use of a benzyl as protecting group for the carboxylic moiety of PFA (Scheme 4), which would be removed in the last step under mild conditions by catalytic hydrogenation, to prevent the desilylation unwanted side-reaction. The PFA diester benzyl derivative **20** was prepared by reaction of the TSAO derivative **4** and benzyl (dichlorophosphonyl)-formate **19**, following a similar procedure to that described for the synthesis of PFA diester **14**. First, it was necessary to prepare the phosphorylating reagent **19**. Thus, reaction of tris(trimethylsilyl) phosphite with benzyl chloroformate (**17**) gave benzyl [bis(trimethylsilyl)-phosphono]formate **18**. Treatment of **18** with $SOCl_2$ gave the phosphorylating reagent **19** in moderate yield. Reaction of TSAO compound **4** with **19**, in the presence of NEt_3, led to the formation of the PFA diester **20** in 54% yield. Finally, the target [TSAO-T]−[PFA] conjugate **6** was obtained in 60% yield, by catalytic hydrogenation (H_2/Pd/C) of the benzyl intermediate [TSAO-T]−[PFA] conjugate **20**.

Biological Evaluation. Several TSAO−PFA conjugates were evaluated for their inhibitory activity against HIV-1 and HIV-2 in MT-4 and CEM cell cultures (Table 1). Foscarnet and the parent TSAO compound **4** were included as controls. Foscarnet was equally active against both HIV-1 and HIV-2 with EC_{50} values ranging between 105 and 140 μM. Foscarnet proved cytotoxic at a CC_{50} of 230 μM (MT-4 cells). The TSAO derivative **4** was highly inhibitory against HIV-1 in both cell lines (EC_{50}: 0.01−0.03 μM) but was inactive against HIV-2 and cytotoxic at ∼ 3.9 μM [selectivity index (ratio CC_{50}/EC_{50}) ∼ 100−380). Compound **9**, representing the dimethyl triester TSAO−PFA conjugate was ∼8−9-fold less potent against HIV-1 but also 5-fold less cytotoxic. It still lacked antiviral activity against HIV-2 at non-toxic concentrations (10 μM). The benzyl derivative and also the ethyl and methyl ester derivatives lost ∼100- to 200-fold antiviral potency compared with the parent compound **4**. Interestingly, the unsubstituted (deprotected) TSAO−PFA conjugate **6** was markedly active (EC_{50}: 0.47−0.82 μM) (∼25- to 50-fold less active than **4**) but was also less cytotoxic (CC_{50}: 111−178 μM), resulting in a selectivity comparable with the parental TSAO derivative **4**. It is important to note that the deprotected conjugate **6** still lacked any measurable activity against HIV-2.

When evaluated against a TSAO-m^{3}T resistant HIV-1 strain containing the E138K mutation in its reverse transcriptase (Table 1), only **14**, **20**, and **6** showed inhibitory activity (EC_{50}: 20, 25 and 50 μM, respectively). PFA was active against the mutant virus strain with an EC_{50} of 87 μM (comparable to wild-type virus). In contrast to PFA (IC_{50} for HIV-1 RT and HIV-1/138K RT of 24 and 56 μM, respectively), all TSAO derivatives lost potency against purified mutant RT enzyme (IC_{50} wild-type HIV-1 RT: 3.7−11 μM; IC_{50} mutant HIV-1/138K RT: 293 → 500 μM) (Table 2). These findings indicate that the protected alkyl- and benzyl-substituted derivatives as well as the deprotected TSAO−PFA conjugate

Table 1. Inhibitory Activity of [TSAO-T]−[PFA] Conjugates against HIV-1 (III_B), HIV-2 and TSAO-Resistant Mutant HIV-1_{E138k} in MT-4 and CEM Cell Cultures

	EC_{50}[a] (μM)					CC_{50}[b] (μM)		
	MT-4		CEM		MT-4	CEM		
compound	HIV-1	HIV-2	HIV-1	HIV-2	HIV-1_{E138K}			log *P*[c]
4	0.03 ± 0.01	>2	0.01 ± 0.01	105 ± 31	>2	3.86 ± 0.29	3.86 ± 0.29	2.26
PFA	133 ± 40	140 ± 35	121 ± 26	>10	87	230 ± 7.3	-	−0.56
9	0.282 ± 0.005	>10	0.08 ± 0.0	>50	>10	18.4 ± 1.9	20.9± 0.85	7.12
10	20.1 ± 3.0	>50	4.5 ± 0.7	>50	>50	55.5 ± 26.7	146 ± 41.7	4.01
14	4.23 ± 0.57	>50	2.13 ± 1.62	>50	25 ± 7.1	84.3 ± 12.2	135 ± 1.4	4.01
20	1.26 ± 0.37	>50	3.0 ± 0.0	>50	20	84.0 ± 37.4	138 ± 7.8	5.02
6	0.82 ± 0.05	>50	0.47 ± 0.46	>50	≥50	111 ± 2.1	178 ± 62.4	0.63

[a] 50% Effective concentration, or compound concentration required to inhibit HIV-induced cytopathicity in cell culture by 50%. [b] 50% Cytostatic concentration, or compound concentration required to inhibit CEM cell proliferation by 50% or to reduce MT-4 cell viability in mock-infected cell cultures by 50%. [c] Log *P*: Interactive prediction analysis using E-state atom indices with neural network algorithms. Algorithm available at www.logp.com.

Table 2. Inhibitory Activity of Test Compounds against HIV-1 RT Wild-Type and Mutant HIV-1/138Lys RT

	IC_{50}[a] (μM)	
compound	HIV-1 WT RT	HIV-1/138Lys RT
TSAO-m^3T	3.1 ± 1.2	>100
PFA	24	56 ± 15
4	8.1 ± 4.6	>100
9	11 ± 4.0	>500
10	4.7 ± 0.2	394 ± 6.0
14	6.3 ± 3.1	293 ± 14
20	3.8 ± 4.0	312 ± 20
6	3.7 ± 0.3	437 ± 66

[a] 50% Inhibitory concentration or drug concentration required to inhibit the RT-catalyzed reaction using poly rC.dG and [^{3}H]dGTP as the template/primer and radiolabeled substrate, respectively.

Table 3. Anti-HIV Activity of TSAO Derivative **4**, PFA, and [TSAO-T]−[PFA] Conjugate **6** against the PFA-Resistant Mutant HIV_{W88G} in P4/R5 Cells

	EC_{50}[a] (μM)		
virus	**4**[b]	PFA	**6**
HIV-1(LAI)	0.043 ± 0.024	44 ± 21	0.30 ± 0.25
HIV-1_{W88G}	0.027 ± 0.006	> 300 ± 99	0.16 ± 0.06
Fold-R[c]	0.63	> 6.9	0.53

[a] 50% Effective concentration (mean ± standard deviation). Results are the average of five independent experiments. [b] In this particular case, results are the average of three experiments. [c] Calculated by taking the average EC_{50} for mutant HIV-1_{W88G} divided by the average EC_{50} for HIV-1(LAI).

inhibit HIV-1 RT predominantly by the TSAO part of the molecule and not by the PFA part. Otherwise, a more pronounced inhibitory activity of the conjugates against TSAO-resistant mutant HIV-1 RT should have been observed.

The TSAO derivative **4**, the TSAO−PFA conjugate **6**, and PFA were evaluated for their inhibitory activity in P4/R5 against a mutant PFA-resistant HIV-1 encoding the W88G mutation in RT (Table 3). Whereas PFA was >7-fold less active against the HIV-1_{W88G} strain, both TSAO and TSAO−PFA conjugate **6** did not lose potency against HIV-1_{W88G} compared with wild-type HIV-1 (Table 3). Thus, the PFA-resistant virus did not show cross-resistance with the TSAO−PFA conjugate **6**, again highlighting the key role of the TSAO portion of the molecule and not the PFA part in the antiviral activity of TSAO−PFA conjugate.

The log *P* values were calculated for each of the compounds.[22] There was no correlation between lipohilicity of the test compounds and their antiviral activity. The most lipophilic compound (**9**) proved more active

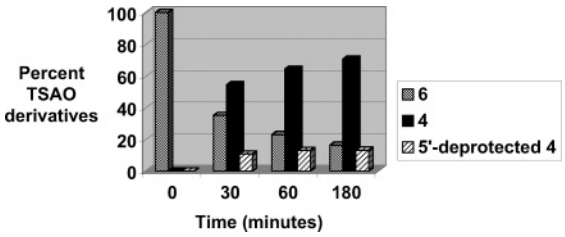


Figure 4. Stability of [TSAO-T]−[PFA] conjugate **6** in extracts from CEM cells.

than the more hydrophilic diester **10**, **14**, and **20** analogues, but the deprotected conjugate **6**, being the most hydrophilic derivative among all tested compounds, was more inhibitory against HIV-1 than **10** and **20** but less inhibitory than **9** and **4**. Therefore, we conclude that differences in efficiency of cellular uptake (due to lipophilicity of the drugs) is probably not the only contributing factor to the antiviral activity of the compounds.

When the stability of the compounds was evaluated in phosphate-buffered saline (PBS, pH 7.4), all the conjugates proved fully stable within 3 h of incubation. No traces of any degradation products could be detected within this time period. Interestingly, when concentrated CEM cell extracts were exposed to the test compounds, only the deprotected TSAO−PFA conjugate **6** released the parent TSAO compound **4**. In fact, ≥ 80% of drug was converted to the parent compound within 3 h of incubation, reflecting an intracellular half-life of ∼30 min in the concentrated cell extract (Figure 4). Some 5′-deprotected **4** was also observed when exposed to CEM cell extracts (data not shown). These findings suggest that in the deprotected TSAO−PFA conjugate the P−O bond can be intracellularly cleaved regenerating the parent TSAO and the intact PFA molecule at equimolar concentrations. It should be kept in mind, however, that the cell extracts that were used to examine the stability of the conjugates are derived from highly concentrated CEM cell suspensions, whose cell number markedly exceed the number of CEM cells used in the virus-infected cell cultures.

Given the pronounced antiviral activity of **4**, the cell extract study, the rather weak activity of PFA against HIV-1, and the fact that compound **6** has a comparable anti-RT activity as compound **4**, we may conclude that the activity of the TSAO−PFA conjugate **6** seen in cell culture could be probably due either to the conjugate as such and, to a minor extent, to the release of the

parent drug **4** if the conjugate would have been efficiently taken up by the cells. However, in case of poor uptake of the TSAO–PFA conjugate **6**, it is possible that the antiviral activity seen is mainly due to either the intact conjugate or to the released parental TSAO molecule **4** after intracellular hydrolysis of the conjugate. The antiviral efficacy of conjugated compounds depends on many factors, such as enzyme inhibition, cell membrane permeability, extracellular stability, intracellullar hydrolysis, and the interactions between them which are very complex. Additional research is required to reveal the exact mechanism of antiviral action of the deprotected TSAO–PFA conjugate derivative.

Experimental Section

Chemical Procedures. Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. IR spectra were obtained on a Perkin-Elmer spectrum one spectrophotometer. ^{1}H NMR spectra were recorded with a Varian Gemini, a Varian XL-300, and a Bruker AM-200 spectrometer operating at 300 and at 200 MHz with Me_4Si as internal standard. ^{13}C NMR spectra were recorded with a Varian XL-300 and a Bruker AM-200 spectrometer operating at 75 MHz and at 50 MHz with Me_4Si as internal standard. ^{31}P spectra were recorded on a Varian INOVA 400 spectrometer operating at 161.89 MHz, using acetone-d_6 or CD_3OD as solvent at 30 °C with phosphoric acid as external standard. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck). Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a chromatotron (Kiesegel 60 PF_{254} gipshaltig, Merck) layer thickness 1 mm or 2 mm, flow rate 5 mL/min. Preparative reverse phase purification was carried out using reverse phase SPE cartridges.

Triethylamine, dichloromethane and toluene were dried by refluxing over calcium hydride. Tetrahydrofuran was dried by refluxing over sodium/benzophenone.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-[[[(methoxycarbonyl)methoxyphosphonyl]-oxy]propyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (9). PCl_5 (0.102 g, 0.496 mmol) was added to a solution of trimethylphosphonoformate **7** (72 μL, 0.54 mmol) in dry CCl_4 (4.5 mL), and the suspension was warmed to 50 °C for 1.5 h. The reaction mixture was evaporated to dryness under reduced pressure. The residue **8** was cooled to −50 °C under an argon atmosphere, and a solution of **4** (0.1 g, 0.15 mmol) and Et_3N (75 μL, 2.56 mmol) in dry CH_2Cl_2 (1.1 mL), also precooled to −50 °C, was added. Once the reaction was completed, the solvent was evaporated to dryness and the residue was purified by CCTLC on the chromatotron (hexane: ethyl acetate, 1:1) to give compound **9** (0.12 g, 91%) as a white amorphous solid. IR (KBr) 1710 cm^{-1}. ^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ: 0.83, 0.98 (2s, 18H, 2*t*-Bu), 1.95 (d, 3H, J = 1.2 Hz, CH_3-5), 2.01 (m, 2H, CH_2), 3.87 (m, 3H, OCH_3), 4.10 (m, 5H, CH_2, OCH_3), 4.30 (m, 2H, H-5′), 4.28 (m, 2H, CH_2O), 4.35 (m, 1H, H-4′), 4.67 (d, 1H, $J_{1',2'}$ = 8.2 Hz, H-2′), 5.78 (s, 1H, H-3″), 6.11 (d, 1H, H-1′), 6.50 (bs, 2H, NH_2), 7.53 (d, 1H, H-6). ^{13}C NMR [50 MHz, $CDCl_3$] δ: 12.87 (CH_3-5), 18.34, 19.01 [$(CH_3)_3CSi$], 25.69, 26.22 [$(CH_3)_3CSi$], 30.38 (d, J_{P,CH_2} = 6.7 Hz, CH_2), 38.20 (NCH_2), 51.72 (d, J_{P,CH_3} = 3.8 Hz, CO_2CH_3), 52.6 (d, J_{P,CH_3} = 6.2 Hz, OCH_3), 62.91 (C-5′), 65.6 (d, J_{P,CH_2} = 6.1 Hz, CH_2O), 76.22 (C-2′), 85.68, 87.54 (C-4′, C-1′), 91.15 (C-1′), 93.16 (C-3′), 111.99 (C-5), 134.31 (C-6), 152.42, 153.25 (C-2, C-4″), 163.85 (C-4), 174.20 (d, $J_{P,C}$ = 248 Hz, CO_2CH_3). ^{31}P NMR [161.88 MHz, $(CD_3)_2CO$] δ: −2.53. Anal. ($C_{30}H_{54}N_3O_{13}PSSi_2$) C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-[[[(methoxycarbonyl)hydroxyphosphonyl]oxy]-propyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide), Sodium Salt (10). NaI (0.017 g, 0.11 mmol) was added to a stirred solution of **9** (0.1 g, 0.13 mmol) in dry THF (1.5 mL), and the solution was left to stir under argon at room temperature. After 6 h, the reaction mixture was evaporated to dryness and the residue was purified by CCTLC on the chromatotron (ethyl acetate:methanol, 5:1) to give compound **10** (0.079 g, 80%) as a white amorphous solid. IR (KBr) = 1710 cm^{-1}. ^{1}H NMR [400 MHz, CD_3OD] δ: 1.00, 1.16 (2s, 18H, 2*t*-Bu), 2.12 (m, 2H, CH_2), 2.17 (d, 3H, J = 0.9 Hz, CH_3-5), 3.94 (s, 3H, CO_2CH_3), 4.19 (m, 6H, $NCH_2CH_2CH_2O$, 2H-5′), 4.53 (t, 1H, J = 3.8 Hz, H-4′), 4.74 (d, 1H, $J_{1',2'}$ = 8.2 Hz, H-2′), 5.83 (s, 1H, H-3″), 6.27 (d, 1H, H-1′), 7.75 (d, 1H, H-6). ^{13}C NMR [100 MHz, CD_3OD] δ: 9.30 (CH_2), 13.16 (CH_3-5), 18.73, 19.18 [$(CH_3)_3CSi$], 25.95, 26.53 [$(CH_3)_3CSi$], 29.94 (d, J_{P,CH_2} = 6.9 Hz, CH_2), 40.16 (NCH_2), 51.73 (d, J_{P,CH_2} = 3.8 Hz, CO_2CH_3), 63.33 (C-5′), 65.33 (d, J_{P,CH_2} = 6.1 Hz, CH_2-OP), 76.10 (C-2′), 85.79 (C-4′), 87.54 (C-1′), 91.16 (C-3″), 93.54 (C-3′), 112.22 (C-5), 135.26 (C-6), 150.48 (C-2),153.17 (C-4″), 164.55 (C-4), 174.61 (d, $J_{P,C}$ = 248 Hz, CO_2CH_3). ^{31}P NMR [161.88 MHz, CD_3OD] δ: −7.41. Anal. ($C_{29}H_{51}N_3NaO_{13}PSSi_2$) C, H, N, S.

Ethyl [Bis(trimethylsilyl)phosphono]formate (12).[18a] Tris(trimethylsilyl) phosphite (25 mL, 67 mmol) was added dropwise to ethyl chloroformate **11** (7.2 mL, 67 mmol) in an ice bath under an argon atmosphere. The mixture was left stirring overnight at room temperature and distilled under 10 mmHg. Trimethylsilyl chloride distilled first, then bis(trimethylsilyl)phosphorous acid, and finally ethyl [bis(trimethylsilyl)phosphono]formate **12** (13 g, bp_{10} 100–102 °C, bp_{10} 68–70 °C[18a]) was obtained in 75% yield. ^{1}H NMR [200 MHz, $(CD_3)_2CO$] δ: 0.28 (m, 27H, $9CH_3$), 1.29 (t, 3H, J = 7.2 Hz, $CO_2CH_2CH_3$), 4.23 (q, 2H, J = 7.1 Hz, $CO_2CH_2CH_3$).

Ethyl (Dichlorophosphonyl)formate (13).[18a] Ethyl [bis-(trimethylsilyl)phosphono]formate **12** (5 g, 18 mmol) was dissolved in 20 mL of dry toluene, under an argon atmosphere. Thionyl chloride (3.2 mL, 53 mmol) was added, and the mixture was refluxed for 2 h. Toluene and thionyl chloride were removed under reduced pressure, and the resulting mixture was purified by vacuum distillation (10 mmHg) to give 2.7 g of a colorless oil (**13**) in 84% yield (bp_{10} 84–86 °C, bp_{10} 64 °C[18a]).^{1}H NMR [200 MHz, $(CD_3)_2CO$] δ: 1.41 (t, 3H, J = 7.2 Hz, $CO_2CH_2CH_3$), 4.47 (q, 2H, J = 7.2 Hz, $CO_2CH_2CH_3$).

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[(3-[[[(ethyloxycarbonyl)hydroxyphosphonyl]-oxy]propyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (14). Ethyl (dichlorophosphonyl)formate (**13**) (0.047 g, 0.25 mmol) was dissolved in 2.5 mL of dry CH_2Cl_2, under an argon atmosphere. The mixture was cooled to −20 °C, and a solution of the alcohol **4** (0.1 g, 0.15 mmol) and NEt_3 (70 μL, 0.45 mmol) in dry CH_2Cl_2 (3.5 mL), also precooled to −20 °C, was added dropwise. The mixture was stirred for 5 min at −20 °C, and then the solvent was removed under reduced pressure. The remaining residue was dissolved in EtOAc (20 mL) and succesively washed with H_2O (4 × 10 mL) and brine (10 mL). The organic phase was dried (Na_2SO_4), filtered, and evaporated to dryness. The final residue was purified by CCTLC on the chromatotron (ethyl acetate: methanol, 5:1) to give compound **14** (0.036 g, 31%) as a white amorphous solid. IR (KBr) = 1710 cm-^{1}. ^{1}H NMR [400 MHz, CD_3OD] δ: 0.67, 0.83 (2s, 18H, 2*t*-Bu), 1.16 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.82 (m, 5H, CH_3-5, CH_2), 3.79–3.92 (m, 6H, $NCH_2CH_2CH_2O$, 2H-5′), 4.07 (q, 2H, CH_2CH_3), 4.19 (t, 1H, J = 3.6 Hz, H-4′), 4.41 (d, 1H, $J_{1',2'}$ = 8.2 Hz, H-2′), 5.48 (s, 1H, H-3″), 5.86 (d, 1H, H-1′), 7.36 (d, 1H, H-6). ^{13}C NMR [100 MHz, CD_3OD] δ: 13.11 (CH_3-5), 14.60 (CH_2CH_3), 18.79, 19.23 [$(CH_3)_3CSi$], 25.91, 26.49 [$(CH_3)_3CSi$], 30.07 (d, J_{P,CH_2} = 5.9 Hz, CH_2), 40.18 (NCH_2), 61.28 (d, J_{P,CH_2} = 4.2 Hz, $CO_2CH_2CH_3$) 63.21 (C-5′), 65.34 (d, J_{P,CH_2} = 6.2 Hz, CH_2OP), 75.98 (C-2′), 85.70 (C-4′), 88.75 (C-1′), 90.88 (C-3″), 93.16 (C-3′), 112.17 (C-5), 135.72 (C-6), 152.45 (C-2), 153.60 (C-4″), 164.51 (C-4), 172.34 (d, $J_{P,C}$ = 250 Hz, $CO_2CH_2CH_3$). ^{31}P NMR [161.88 MHz, CD_3OD] δ: −4.50. Anal. ($C_{30}H_{54}N_3O_{13}PSSi_2$) C, H, N, S.

[1-[2′-*O*-(*tert*-Butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[(3-[[(hydroxycarbonyl)hydroxyphosphonyl]oxy]-propyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) Sodium Salt (15). Method A. Compound **10**

(0.1 g, 0.13 mmol) was dissolved in dry THF (1.5 mL), and an aqueous solution of 0.1 N NaOH (0.75 mL, 0.2 mmol) was added. The mixture was stirred for 2 h at room temperature. The mixture was neutralized with DOWEX 50 Wx4 (H^+ form, previously washed with distilled water), filtered, and evaporated to dryness. The residue was purified by reverse phase chromatography using SPE cartridges (acetonitrile: water, 95:5). The fastest moving fractions afforded **15** (0.035 g, 53%) as a white amorphous solid. ^{1}H NMR [400 MHz, CD_3OD] δ: 1.01 (s, 9H, *t*-Bu), 2.12 (m, 5H, CH_2, CH_3-5), 3.97 (dd, 1H, $J_{5'a,5'b}$ = 12.3, $J_{4',5'a}$ = 2.5 Hz, H-5′a), 4.12 (dd, 1H, $J_{4',5'b}$ = 2.8 Hz, H-5′b), 4.26 (m, 2H, NCH_2CH_2CH_2O), 4.53 (t, 1H, H-4′), 4.90 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-2′), 5.83 (s, 1H, H-3″), 6.26 (d, 1H, H-1′), 8.14 (s, 1H, H-6). ^{13}C NMR [100 MHz, CD_3OD] δ: 13.34 (CH_3-5), 18.88 [(CH_3)$_3$*C*Si], 26.10 [(*C*H$_3$)$_3$CSi], 30.93 (d, J_{P,CH_2} = 4.0 Hz, CH_2), 40.42 (NCH_2), 61.67 (C-5′), 63.92 (d, J_{P,CH_2} = 6.1 Hz, *C*H$_2$O), 76.75 (C-2′), 86.29 (C-4′), 89.52 (C-1′), 91.28 (C-3″), 95.79 (C-3′), 112.13 (C-5), 137.11 (C-6), 153.05 (C-2), 153.17 (C-4″), 164.77 (C-4), 199.27 (d, $J_{P,C}$ = 230 Hz, CO_2Na). ^{31}P NMR [161.88 MHz, CD_3OD] δ: −12.19. Anal. ($C_{22}H_{34}N_3Na_2O_{13}PSSi$) C, H, N, S.

The slowest moving fractions gave [1-[2′-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-[[[(hydroxyphosphonoyl)oxy]propyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) sodium salt (**16**) (0.035 g, 43%) as a white amorphous solid. ^{1}H NMR [400 MHz, CD_3OD] δ: 0.67 (s, 9H, *t*-Bu), 1.79 (m, 5H, CH_2, CH_3-5), 3.59−4.01 (m, 6H, NCH_2-CH_2CH_2O, 2H-5′), 4.21 (m, 1H, H-4′), 4.57 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-2′), 5.51 (s, 1H, H-3″), 5.97 (d, 1H, H-1′), 6.59 (d, 1H, $J_{P,H}$ = 623 Hz, PH), 7.72 (s, 1H, H-6). ^{13}C NMR [100 MHz, CD_3OD] δ: 13.34 (CH_3-5), 18.75 [(CH_3)$_3$*C*Si], 26.09 [(*C*H$_3$)$_3$CSi], 30.74 (d, J_{P,CH_2} = 6.1 Hz, CH_2), 40.11 (NCH_2), 61.65 (C-5′), 62.92 (d, J_{P,CH_2} = 5.5 Hz, *C*H$_2$O), 76.75 (C-2′), 86.29 (C-4′), 89.52 (C-1′), 91.28 (C-3″), 95.66 (C-3′), 112.23 (C-5), 137.11 (C-6), 153.05 (C-2), 153.56 (C-4″), 163.47 (C-4).^{31}P NMR (161.88 MHz, CD_3OD) δ: 6.83. Anal. ($C_{21}H_{35}N_3NaO_{11}PSSi$) C, H, N, S.

Method B. Compound **10** (0.1 g, 0.13 mmol) was dissolved in dry THF (1.5 mL), and an aqueous solution of NaOH 0.45 N (0.75 mL, 0.2 mmol) was added. The mixture was stirred for 30 min at room temperature. The mixture was neutralized with DOWEX 50 Wx4 (H^+ form, previously washed with distilled water), filtered, and evaporated to dryness. The residue was purified by reverse phase chromatrography using SPE cartridges (acetonitrile: water, 95:5) to give **15** (0.063 g, 76%) as a white amorphous solid.

Benzyl [Bis(trimethylsilyl)phosphono]formate (18). Tris(trimethylsilyl) phosphite (12 mL, 36 mmol) was added dropwise to benzyl chloroformate **17** (5.4 mL, 36 mmol) in an ice bath under an argon atmosphere. The mixture was left stirring overnight at room temperature and distilled under 10 mmHg. Trimethylsilyl chloride distilled first, then bis(trimethylsilyl)phosphorous acid, and finally 11 g of a colorless oil benzyl [bis(trimethylsilyl)phosphono]formate **18** was obtained in 89% yield (bp_{10} 154−156 °C). ^{1}H NMR [200 MHz, $(CD_3)_2CO$] δ: 0.02 (m, 27H, 9CH_3), 5.21 (s, 2H, CH_2), 7.4 (m, 5H, Ph). ^{13}C NMR [100 MHz, $(CD_3)_2CO$] δ: 0.70, 1,96 (CH_3−Si), 67.40 (CH_2), 129,24, 129.34, 136.20, 167.45 (Ph), 170.20 (*C*O$_2$Bn). ^{31}P NMR [161.88 MHz, $(CD_3)_2CO$] δ: −21.81.

Benzyl (Dichlorophosphonyl)formate (19). Benzyl [bis-(trimethylsilyl)phosphono]formate **18** (2 g, 5.5 mmol) was dissolved in 10 mL of dry toluene, under an argon atmosphere. Thionyl chloride (1.1 mL, 16 mmol) was added, and the mixture was refluxed for 2 h. Toluene and thionyl chloride were removed under reduced pressure, and the resulting mixture was purified by vacuum distillation (10 mmHg) to give 0.4 g of a colorless oil (**19**) in 30% yield (bp_{10} 154−120−122 °C).^{1}H NMR [200 MHz, $(CD_3)_2CO$] δ: 5.25 (s, 2H, CH_2), 7.4 (m, 5H, Ph). ^{31}P NMR [161.88 MHz, $(CD_3)_2CO$] δ: 10.80.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[(3-[[[(benzyloxycarbonyl)hydroxyphosphonyl]-oxy]propyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (20). Benzyl (dichlorophosphonyl)formate (**19**) (0.06 g, 0.25 mmol) was dissolved in 2.5 mL of dry CH_2Cl_2, under an argon atmosphere. The mixture was cooled to −20 °C, and a solution of the alcohol **4** (0.1 g, 0.15 mmol) and NEt_3 (70 μL, 0.45 mmol) in dry CH_2Cl_2 (3.5 mL), also precooled to −20 °C, was added dropwise, according to the procedure described for the synthesis of **14**. The final residue after the workup was purified by CCTLC on the chromatotron (ethyl acetate:methanol, 5:1) to give compound **20** (0.07 g, 54%) as a white amorphous solid. IR (KBr) = 1710 cm^{-1}. ^{1}H NMR [400 MHz, CD_3OD] δ: 0.99, 1.15 (2s, 18H, 2*t*-Bu), 2.12 (m, 2H, CH_2), 2.15 (s, 3H, CH_3-5,), 3.91−4.20 (m, 6H, NCH_2CH_2CH_2O, 2H-5′), 4.51 (t, 1H, J = 3.6 Hz, H-4′), 4.77 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-2′), 5.39 (ABs, 2H, CH_2Ph), 5.81 (s, 1H, H-3″), 6.19 (d, 1H, H-1′), 7.53−7.46 (m, 3H, Ph), 7.60 (m, 2H, Ph), 7.70 (s, 1H, H-6). ^{13}C NMR [100 MHz, CD_3OD] δ: 9.30 (CH_2), 13.06 (CH_3-5), 18.76, 19.21 [(CH_3)$_3$*C*Si], 25.95, 26.48 [(*C*H$_3$)$_3$CSi], 29.94 (d, J_{P,CH_2} = 6.8 Hz, CH_2), 40.18 (NCH_2), 62.01 (C-5′), 65.31 (d, J_{P,CH_2} = 6.1 Hz, *C*H$_2$O), 66.71 (d, J_{P,CH_2} = 3.8 Hz, CH_2Ph,), 75.94 (C-2′), 85.73 (C-4′), 88.69 (C-1′), 90.91 (C-3″), 93.18 (C-3′), 112.15 (C-5), 129.14 (Ph), 129.48 (Ph), 135.70 (C-6), 137.41 (Ph), 152.43 (C-2), 153.62 (C-4″), 164.46 (C-4), 175.62 (d, $J_{P,C}$ = 230 Hz, *C*O$_2$CH$_2$Ph). ^{31}P NMR [161.88 MHz, CD_3OD] δ: −4.24. Anal. ($C_{35}H_{56}N_3O_{13}PSSi_2$) C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-[[[(hydroxycarbonyl)hydroxyphosphonyl]oxy]-propyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (6). Compound **20** (0.1 g, 0.12 mmol) was dissolved in methanol (10 mL) and hydrogenated in the presence of 10% Pd/C (0.01 g, 7.8 mmol) at 30 psi at 25 °C for 1 h. The mixture was filtered, washed with methanol (10 mL), and evaporated to give **6** (0.06 g, 60%) as an amorphous solid. ^{1}H NMR [400 MHz, CD_3OD] δ: 0.81, 0.97 (2s, 18H, 2*t*-Bu), 1,92 (m, 2H, CH_2), 1.96 (s, 3H, CH_3-5,), 3.74−4.08 (m, 6H, NCH_2CH_2CH_2O, 2H-5′), 4.33 (t, 1H, $J_{4',5'a}$ = 3.8, $J_{4',5'b}$ = 3.7 Hz, H-4′), 4.54 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-2′), 5.63 (s, 1H, H-3″), 6.03 (d, 1H, H-1′), 7.51 (s, 1H, H-6). ^{13}C NMR [100 MHz, CD_3OD] δ: −5.47, −4.94, −4.91, −4.24 (CH_3−Si), 9.30 (CH_2), 13.12 (CH_3-5), 18.78, 19.23 [(CH_3)$_3$*C*Si], 25.91, 26.48 [(*C*H$_3$)$_3$-CSi], 30.07 (d, J_{P,CH_2} = 6.8 Hz, CH_2), 40.16 (NCH_2), 63.22 (C-5′), 64.30 (d, J_{P,CH_2} = 6.1 Hz, *C*H$_2$O), 76.03 (C-2′), 85.71 (C-4′), 88.51 (C-1′), 90.96 (C-3″), 93.22 (C-3′), 112.18 (C-5), 135.62 (C-6), 152.48 (C-2),153.53 (C-4″), 164.55 (C-4), 182.01 (d, $J_{P,C}$ = 235 Hz, *C*O$_2$H). ^{31}P NMR [161.88 MHz, CD_3OD] δ: −12.20. Anal. ($C_{28}H_{50}N_3O_{13}PSSi_2$) C, H, N, S.

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). The P4/R5 cells were obtained from Ned Landau from the Salk Institute.

Activity Assay of Test Compounds against HIV-1 and HIV-2 in Cell Culture. 4×10^5 CEM or 3×10^5 MT-4 cells per milliliter were infected with HIV-1 or HIV-2 at ~100 $CCID_{50}$ (50% cell culture infective dose) per milliliter of cell suspension. Then, 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. Giant cell 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 (EC_{50}) 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 (CC_{50}) 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%.

Activity Assay of Test Compounds against TSAO-Resistant HIV-1 Strains in Cell Culture. CEM cells were suspended at 250 000 cells per milliliter of culture medium and infected with TSAO-resistant mutant HIV-1 strains at 100 50% cell culture infective doses per milliliter. Then 100 μL of the infected cell suspensions was added to 200-μL microtiter plate wells containing 100 μL of an appropriate dilution of the test compounds. After 4 days incubation at 37 °C, the cell

cultures were examined for syncytium formation. The EC_{50} was determined as the compound concentration required to inhibit syncytium formation by 50%.

Activity Assay of Test Compounds against HIV-1LAI (WT) and PFA-Resistant HIV-1_{W88G} Strain. Susceptibility assays were conducted in P4/R5 cells. The P4/R5 reporter cell line is a Hela cell line that had been stably transfected with a Tat-activated β-galactosidase gene under the control of an HIV-L TR promoter (provided by Ned Landau, Salk Institute). Drug dilutions were made 2- or 3-fold and added to the plate in triplicate. An amount of 5×10^4 cells/mL were infected with HIV_{LAI}(wild type) and HIV_{W88G} at an MOI of 0.05 $TCID_{50}$/mL. At 48 h post-infection, a lysis buffer and a luminescent substrate was added to each well. The amount of β-galactosidase produced by the P4/R5 cells was then determined using a luminometer.

Anti-Reverse Transcriptase Assays. The source of the reverse transcriptases used were either recombinant HIV-1 RT (derived from HIV-1 III_B) and Glu138Lys mutated recombinant HIV-1 RT, constructed and prepared as described before.[23] The RT assays contained in a total reaction mixture volume (50 μL) 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 300 mM glutathione, 500 μM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 μg of bovine serum albumin, labeled substrate [8-^{3}H]dGTP (specific radioactivity, 15.6 Ci/mmole) (2.5 μM) (Moravek Biochemicals, Brea, CA), a fixed concentration of the template/primer poly(C).oligo$(dG)_{12-18}$ (0.1 mM), 0.06% Triton X-100, 10 μL of inhibitor at various concentrations, and 1 μL of the RT preparation. The reaction mixtures were incubated at 37 °C for 30 min, at which time 100 μL of calf thymus DNA (150 μg/mL), 2 mL of $Na_4P_2O_7$ (0.1 M in 1 M HCl), and 2 mL of trichloroacetic acid (10% v/v) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analyzed for radioactivity. The IC_{50} for each test compound was determined as the compound concentration that inhibited HIV RT activity by 50%.

Stability Assay of Test Compounds in PBS and Cell Extracts. CEM cells were grown in RPMI-1640 medium containing 10% newborn calf serum, 2 mM L-glutamine, and 0.075% $NaHCO_3$. When reaching a cell density of $\sim 10^6$ to 2×10^6 cells/mL, the cell cultures were centrifuged at 1200 rpm in a Megafuge 3.0R (Vanderheyden, Brussels, Belgium) and washed twice with phosphate-buffered saline (PBS, pH 7.4). Then, a concentrated cell suspension of $\sim 50 \times 10^6$ cells/mL PBS was sonicated (3×10 s) on ice to lyse the cells. The crude cell extract was then centrifuged at 100 000*g* in an ultracentrifuge (PMSE–65) (Beun-de-Ronde), and the supernatant stored at −80 °C.

Test compound solutions were prepared in PBS and analyzed for chemical stability by HPLC/Reverse phase C-18 column (Waters 2690, Alliance, Brussels, Belgium). Also, compound solutions were exposed to the crude CEM cell extracts and analyzed for stability in the same assay system. The potential breakdown products that may arise in function of incubation time at 37 °C were separated by an acetonitrile/H_2O elution gradient.

Combination of TSAO-m^{3}T and PFA in HIV-1-Infected CEM Cell Cultures. A variety of TSAO-m^{3}T (i.e. 0.04, 0.02, 0.01, 0.005, 0.0025, 0.0012, 0.0006, 0.0003, and 0.0002 μg/mL) and PFA (i.e. 20, 10, 5, 2.5, and 1.25 μg/mL) concentrations were combined and added to virus-infected CEM cell cultures as described above. The inhibitory activity of all individual drug combinations was determined by microscopic estimation of syncytia formation in the cell cultures. The data were then plotted in an isobologram to visualize the nature of inhibition of the combined action of the drugs. From these plots, synergistic (points above the line that connect the fractional inhibitory concentration (FIC) values of 1.5 at the abscissa and ordinate), additive (points between the lines that connect the FIC values of 1.5 and 0.5 at the abscissa and ordinate), or antagonistic effects (points under the line that connect the FIC values of 0.5 at the abscissa and ordinate) can be revealed.

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