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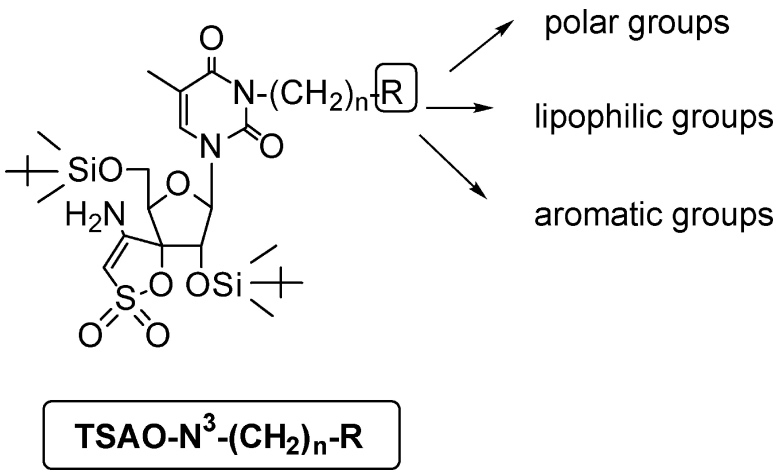

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Improving the Antiviral Efficacy and Selectivity of HIV-1 Reverse Transcriptase Inhibitor TSAO-T by the Introduction of Functional Groups at the N-3 Position

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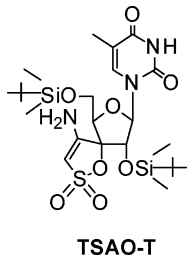
Novel derivatives of the anti-HIV-1 agent, TSAO-T, bearing at the N-3 position a variety of polar, lipophilic, or aromatic groups linked to that position through flexible polymethylene linkers of different length, were prepared and evaluated for their anti-HIV activity. Several compounds (within the series of polar bearing groups) exhibited a 2−6-fold improved antiviral potency with regard to the lead compound, TSAO-T. Moreover, some of the most active N-3 TSAO derivatives retain antiviral activity against the TSAO-T-resistant HIV-1 strain (Glu138 → Lys). Interestingly, the *N*-methylcarboxamide derivative **17** was 5- to 6-fold more active (IC_{50}: 0.56 μM) against recombinant HIV-1 reverse transcriptase than the lead compound, thus becoming the most active TSAO derivative synthesized so far. On the other hand, the N-3 methylcarboxamide TSAO derivative **12** turned out to have the highest selectivity index yet reported for this class of compounds (around ≥12 000).

Introduction

Reverse transcriptase (RT) plays a critical role in the life cycle of the human immunodeficiency virus (HIV), the primary causative agent of AIDS. Inhibition of this enzyme is a key target in the search for effective antiretroviral drugs.[1−4]

HIV-1 RT is an asymmetric heterodimer composed of two subunits of 66 and 51 kDa (designated as p66 and p51, respectively) that converts the genomic HIV RNA into proviral DNA by its RNA-dependent and DNA-dependent DNA polymerase and RNase H activities.[5−7] The larger subunit (p66) contains both DNA polymerase and RNase H activities, while the smaller subunit (p51) lacks these functions.[5−7] However, both the p66 and p51 monomers are functionally inactive when dissociated from each other.[8−10]

TSAO-T (Figure 1) is the prototype compound of a unique family of nonnucleoside RT inhibitors (NNRTIs) that have been synthesized and characterized in our laboratories.[11−14] Although TSAO derivatives are highly functionalized nucleosides, they behave mechanistically as all the other nonnucleoside RT inhibitors (NNRTIs). Our experimental data on TSAO derivatives strongly suggest a specific interaction of the amino group of the 3′-spiro moiety of TSAO molecules with the carboxylic group of a glutamic acid residue at position 138 of the p51 subunit of HIV-1 RT.[15−18] This residue is located in the β7−β8 loop of the p51 subunit of HIV-1 RT.[19,20] Moreover, other amino acids in the p66 RT subunit at the NNRTI binding pocket are also needed for an optimal interaction of TSAO derivatives with HIV-1 RT.[21] Therefore, TSAO compounds are 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

Figure 1. Structure of TSAO-T.

In addition, an interesting aspect observed with TSAO-T and also with its N-3 ethyl derivative (TSAO-e^{3}T) is that both compounds destabilize the p66/p51 dimeric forms of HIV RT.[22] To the best of our knowledge, these compounds are the first nonpeptidic small molecules to have such an effect.

We have reported a model of interaction of the N-3 methyl TSAO-T derivative (TSAO-m^{3}T) with the HIV-1 RT.[23] In this model, TSAO-m^{3}T straddles between both subunits at the p66/p51 interface but it does not make direct use of the NNRTI binding pocket. Because the N-3 substituent at the thymine base of TSAO-m^{3}T was found running parallel to the subunit interface, we reasoned that this N-3 position afforded a unique opportunity to explore the HIV-1 RT dimer interface. We then hypothesized that the presence of functional groups at this position might provide additional interactions with amino acids at or near the p51/p66 interface (e.g., Thr-A165, Lys-B49, Pro-B140, and Ile-B142) possibly endowing the N-3-substituted TSAO derivatives with an improved activity and/or resistance profile over

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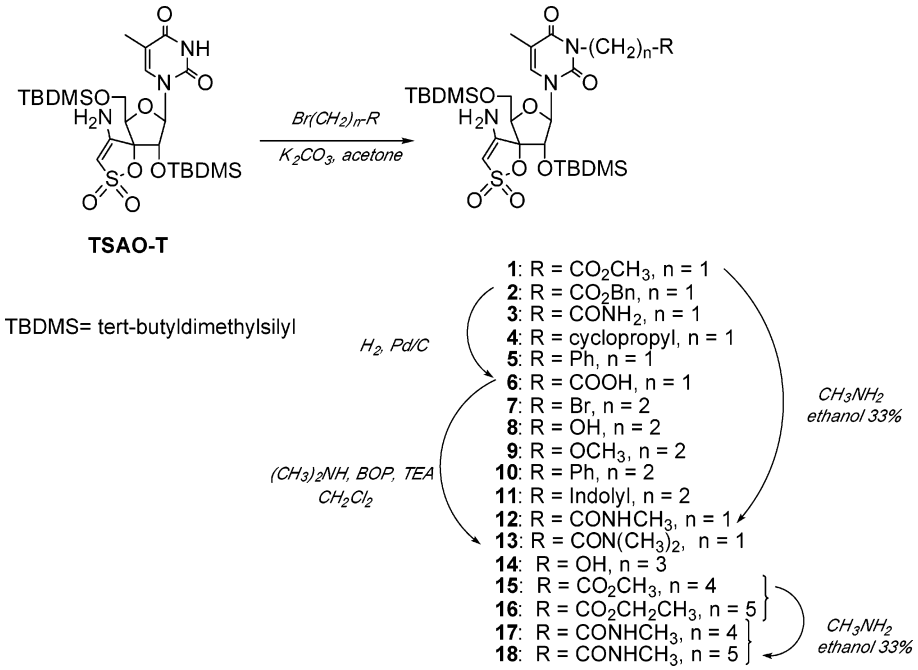
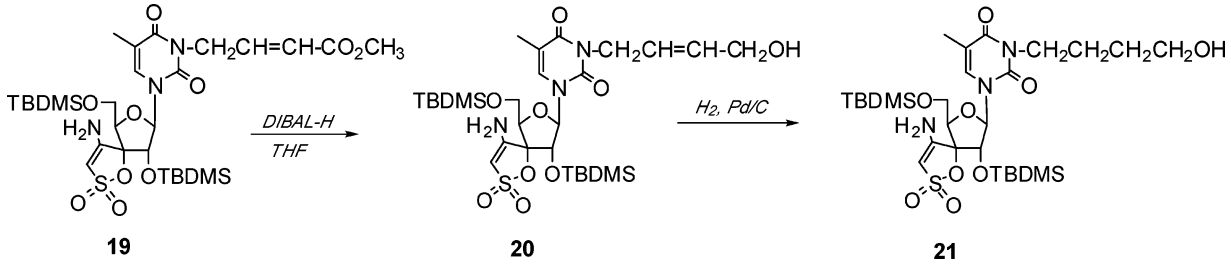
Scheme 1. Synthesis of N-3 TSAO Derivatives **1**−**18**



Scheme 2. Synthesis of N-3 TSAO Derivatives **19**−**21**



the prototype compound (TSAO-T). On the basis of this hypothesis, novel analogues of TSAO-T bearing at the N-3 position functional groups of diverse nature, that is, polar (alcohol, carboxylic acid, amide, amine, etc.), lipophilic, or aromatic, linked to that nitrogen through polymethylene linkers of different length, were prepared and evaluated for their anti-HIV activity. This broad chemical diversity strategy was complementary to the more focused targeted approach reported earlier using docked fragments from a commercial library.[23]

Results and Discussion

Synthesis. Most of these compounds bearing broad chemical diversity at the N-3 position of TSAO-T and only one or two methylene groups could be readily prepared following our previously described method for the selective N-3-alkylation of TSAO derivatives.[14] Thus, reaction of TSAO-T (Scheme 1) with the corresponding alkyl halides in the presence of anhydrous potassium carbonate afforded the N-3 derivatives: **1** (85%), **2**[24](85%), **3** (68%), **4** (83%), **5** (72%), **7** (41%), **8** (86%), **9** (30%), **10** (89%), and **11** (60%) (Scheme 1).

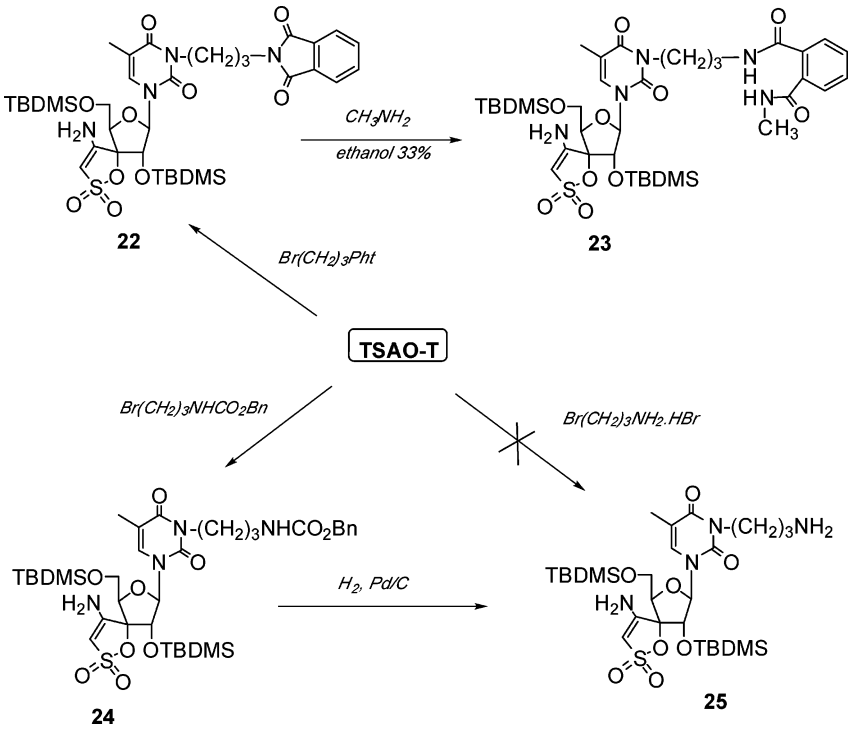
Catalytic hydrogenation of the N-3 benzyl ester derivative **2**[24] in the presence of 10% palladium on charcoal (Scheme 1) gave the corresponding free acid **6**[24] (97%).

Treatment of the ester derivative (**1**) with methylamine afforded the *N*-methylcarbamoyl derivative **12** in 70% yield (Scheme 1). However, reaction of **1** with dimethylamine did not afford the corresponding *N,N*-dimethylcarbamoyl derivative **13**. Instead, a mixture of 5′-deprotected derivatives was obtained. Then, we sought a different method, which consisted of coupling the N-3 carboxylic acid TSAO derivative **6**[24] with dimethylamine in the presence of BOP and triethylamine. Under these conditions, the desired *N, N*-dimethylcarbamoyl derivative **13** was obtained in 68% yield (Scheme 1).

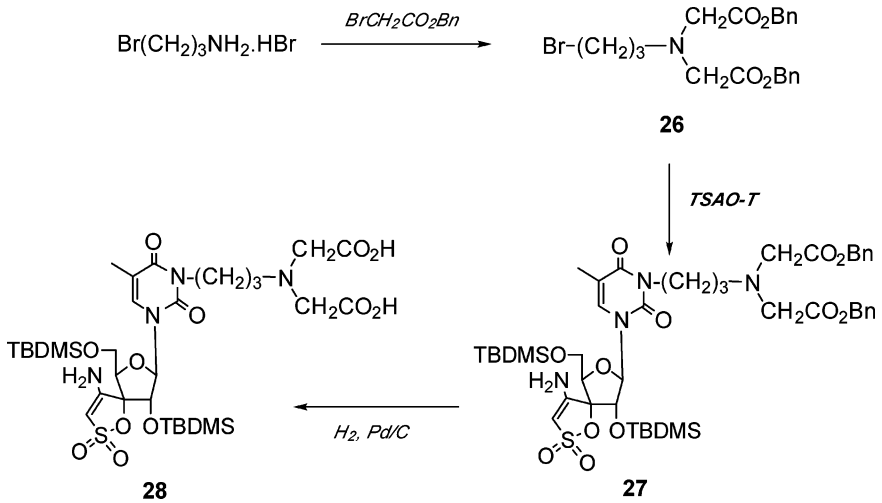
Compounds bearing polar groups linked at the N-3 position of the thymine through longer polymethylene linkers (n = 3−5) were also prepared. Following a similar procedure to that described for the synthesis of **1**−**11**, TSAO-T was treated with 3-bromopropanol, methyl-5-bromovalerate, or ethyl-6-bromohexanoate to give the N-3-alkyl derivatives **14**[23] (82%), **15** (71%), and **16** (80%) (Scheme 1). Treatment of **15** and **16** with methylamine afforded the *N*-methylcarbamoyl derivatives **17** and **18** in 50% and 40% yields, respectively (Scheme 1).

Attempts to prepare the N-3 hydroxybutyl derivative **21** (Scheme 2) by reaction of TSAO-T with 4-chlorobutanol using the previously mentioned alkylation conditions were unsuccessful. Thus, for the synthesis of this compound, we developed the three-step procedure outlined in Scheme 2. The first step involves the reaction of TSAO-T with 4-bromomethyl crotonate to give the N-3 methyl ester derivative **19** in 93% yield. Next,

Scheme 3. Synthesis of N-3 TSAO Derivatives **22**–**25**



Scheme 4. Synthesis of N-3 TSAO Derivatives **27** and **28**

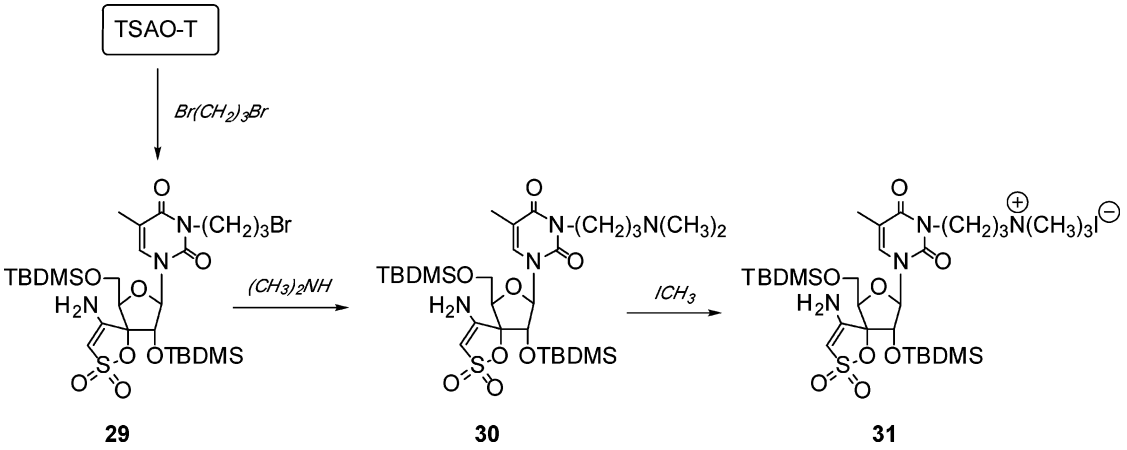


reduction of **19** with DIBAL-H followed by catalytic hydrogenation of the double bond, in the presence of 10% palladium on charcoal, afforded the hydroxybutyl derivative **21** in 75% yield.

Next, we focused on the synthesis of compound **25** (Scheme 3) in which a terminal amino group was separated from the N-3 position by an alkyl chain of three carbon atoms. Attempts to prepare the 3-*N*-propylamine TSAO derivative **25** by reaction of TSAO-T with 3-bromopropylamine hydrobromide were unsuccessful, and the starting material was recovered unaltered (Scheme 3). The synthetic approach that involved the synthesis of the N-3 phthalimidopropyl derivative **22** followed by deprotection of the phthalimide moiety with hydrazine hydrate at room temperature also failed to afford the expected amine derivative **25**. Instead, a mixture of 5′-deprotected derivatives was obtained. When methylamine was used in place of hydrazine hydrate to deprotect the phthalimide moiety, compound **23** was obtained exclusively in 79% yield (Scheme 3). Finally, the synthesis of the amine derivative **25** was achieved in a two-step procedure as follows. Reaction of TSAO-T with *N*-(benzyloxycarbonyl)-3-bromopropylamine,[25] prepared by reaction of 3-bromopropylamine hydrobromide with benzyl chloroformate in the presence of TEA, gave compound **24** in 87% yield. Catalytic hydrogenation of the (Cbz) amino-protected TSAO derivative **24** in the presence of 10% palladium on charcoal gave the target amino-deprotected TSAO derivative **25** in 75% yield.

Compound **28** bearing a terminal iminodiacetic acid moiety was also prepared. For the synthesis of this compound (Scheme 4), we designed a three-step procedure that consisted of treatment of 3-bromopropylamine hydrobromide with excess of benzyl bromoacetate in the presence of TEA to give the bromoalkyl derivative **26** that was further reacted with TSAO-T to give the protected TSAO-T derivative **27** (60%). Removal of the

Scheme 5. Synthesis of N-3 TSAO Derivatives **29**−**31**



benzyl groups by catalytic hydrogenation in the presence of 10% palladium on charcoal afforded the corresponding dicarboxyl TSAO-T derivative **28** in 97% yield (Scheme 4).

Finally, reaction of the bromopropyl derivative **29**[26] with dimethylamine (2 M in THF) afforded the *N*,*N*-dimethylamino derivative **30** (71%), and reaction of **30** with methyl iodide in acetone yielded its corresponding quaternary salt **31** (98%) (Scheme 5).

Biological Evaluation. Virtually all synthesized N-3 substituted TSAO derivatives were endowed with a marked antiviral potential, showing anti-HIV-1 activities that are in the nanomolar range. The only exceptions are compound **6**, bearing a terminal carboxylic acid, the iminodiacetic acid **28**, its benzyl-protected derivative **27**, and the N-3 quaternary amine derivative **31** that were active at EC_{50} values of 3.5−13 μM (Table 1). There is a good agreement for the anti-HIV activities obtained in both MT-4 and CEM cell cultures. As observed for the parent prototypes TSAO-T and TSAO-m^{3}T, none of the compounds were inhibitory against HIV-2 replication. Among the most active N-3 TSAO derivatives were the carboxamide derivative **3,** its *N*-methyl (**12**) and *N*,*N*-dimethyl (**13**) substituted analogues and the *N*-methylcarboxamide derivatives (**17** and **18**) (EC_{50}: 0.01−0.05 μM). Moreover, the 3-hydroxy-

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

	EC_{50} (μM)[a]					CC_{50} (μM)[b]	SI[c]	IC_{50}[d] (μM)
	MT-4		CEM			CEM	CEM	
compound	HIV-1	HIV-2	HIV-1	HIV-2	HIV-1/138K			HIV-1 RT
1	0.11 ± 0.03	>152	0.06 ± 0.04	>152	−	>152	>253	3.0
2	0.54 ± 0.08	>27	0.41 ± 0.19	>5	−	12.9 ± 1.3	31	>250
3	0.07 ± 0.04	>10	0.05 ± 0.02	>10	>50	13.3 ± 3.0	266	>250
4	0.20 ± 0.13	>50	0.18 ± 0.09	>10	>50	60.4 ± 14.8	335	>500
5	0.47 ± 0.37	>50	0.18 ± 0.04	>50	>50	53.0 ± 15.0	294	>500
6	6.22 ± 4.69	>155	3.48 ± 0.54	≥155	−	152 ± 4.2	44	15
7	0.17 ± 0.04	>50	0.03 ± 0.01	>50	>50	129 ± 19	4300	>500
8	0.06 ± 0.03	>10	0.04 ± 0.01	>10	8.0 ± 2.8	11.3 ± 3.9	282	3.1 ± 0.21
9	0.16 ± 0.02	>10	0.08 ± 0.0	>10	>10	38.8± 6.4	485	2.9 ± 0.6
10	0.84 ± 0.14	>2	0.62 ± 0.43	>10	>10	14.2 ± 9.2	23	>500
11	0.96 ± 0.18	>10	0.34 ± 0.10	>50	>50	27.6 ± 0.9	81	>500
12	0.03 ± 0.01	>250	0.02 ± 0.01	>50	3.5 ± 0.7	≥250	≥12500	3.1 ± 0.7
13	0.04 ± 0.01	>250	0.02 ± 0.01	>250	4.5 ± 0.7	245 ± 7.1	12250	3.5 ± 0.39
14	0.03 ± 0.00	>2	0.01 ± 0.01	>2	−	3.86 ± 0.29	386	3.0 ± 1.4
15	0.20 ± 0.20	>142	0.08 ± 0.02	≥142	−	125 ± 24	1563	327 ± 21
16	0.27 ± 0.17	>50	0.13 ± 0.08	>50	>10	40.6 ± 4.0	312	>500
17	0.05 ± 0.01	>10	0.01 ± 0.01	>10	≥2	10→50	1000−5000	0.56 ± 0.09
18	0.03 ± 0.00	>125	0.05 ± 0.01	>125	10	22.1 ± 1.3	442	2.4 ± 0.6
19	0.13 ± 0.07	>250	0.06 ± 0.04	>10	5.5 ± 0.7	142 ± 10.6	2367	30 ± 1.0
20	0.07 ± 0.05	>2	0.02 ± 0.00	>2	4.5 ± 0.7	4.41 ± 0. 1	220	4.5 ± 0.1
21	0.17 ± 0.02	>10	0.09 ± 0.01	>10	≥10	20.5 ± 1.0	227	13 ± 3.0
22	0.29± 0.13	>250	0.11 ± 0.01	>250	>250	>250	>2272	>500
24	0.17± 0.01	>50	0.15 ± 0.06	>50	>50	229.0± 30.4	1526	>500
25	0.52± 0.31	>10	0.14 ± 0.09	>10	>10	11.6 ± 8.2	83	8.1 ± 4.6
27	12.8± 11.2	>250	3.50 ± 0.70	>250	>250	>250	71	>500
28	8.32± 6.23	>50	4.00 ± 0.00	>10	>50	>250	>63	>500
30	0.16 ± 0.0	>10	0.17 ± 0.08	>10	>10	10.4 ± 4.8	61	6.6 ± 2.5
31	4.9 ± 0.1	>25	5.0 ± 0.0	>25	>25	110 ± 21.3	22	11 ± 3.5
TSAO-T[e]	0.06± 0.03	>20	0.06 ± 0.01	>20	>10	12 ± 3	200	−
TSAO-m^{3}T[e]	0.06± 0.01	>50	0.04 ± 0.01	>250	>10	115 ± 14	2875	3.1 ± 1.2

[a] 50% effective concentration required to inhibit HIV-induced cytopathicity by 50%. [b] 50% cytostatic concentration required to inhibit cell proliferation (CEM) by 50%. [c] Selectivity index = ratio of cytostatic concentration/effective concentration. [d] Inhibitory concentration = 50% of the compound concentration required to inhibit recombinant HIV-1 RT activity by 50%. [e] Data taken from ref 14.

propyl derivative **14**[23] proved very active, being 2- to 4-fold more active than the 2-hydroxyethyl derivative **8**. Also, the 4-hydroxybutenyl derivative **20** showed pronounced antiviral activity, whereas the closely related 4-hydroxybutyl analogue **21** was around 4-fold less active than **20**. It is noticeable that the N-3-substituted TSAO derivatives that contain an aromatic [i.e., benzyl (**2**, **27**), phenyl (**5**, **10**), indolyl (**11**)] or a cyclopropyl (**4**) entity attached to the N-3 position are, in the majority of cases, less potent antiviral agents. This may be due to steric hindrance, although some other compounds with bulky substituents such as **22** and **24** still show pronounced antiviral activity.

The cytostatic activity of the N-3-substituted TSAO compounds is found to vary considerably depending on the nature of the N-3 substituent. The most toxic compounds have CC_{50} values at the lower micromolar range ($CC_{50} \leq 25$ μM: compounds **10**, **14**, **18**, **20**, and **30**), whereas other compounds show poor if any cytostatic potential ($CC_{50} > 50$ μM: compounds **1**, **4**, **5**, **6**, **7**, **12**, **15**, **19**, **22**, **24**, **27**, **28**, and **31**) (Table 1). There is no correlation between the antiviral and the cytotoxic/static activity of the TSAO derivatives. For example, the most active compounds **12**, **13**, and **14** (EC_{50}: 0.01−0.04 μM) markedly differ in their cytotoxic/static potential (CC_{50}: ≥ 250, 245, and 3.86 μM, respectively). Therefore, toxicity seems not to be mediated by a better or worse cellular uptake of the compounds.

Several compounds (i.e., **7**, **17**, **19**, and **22**) showed selectivity indices (SI, ratios CC_{50}/EC_{50}) values comparable to or even 4-fold higher (i.e., **12** and **13**) than those found for TSAO-m^{3}T (up to now the most selective TSAO derivative) (Table 1). Interestingly, the latter increase is due to a more pronounced anti-HIV-1 activity, rather than to a decreased cytotoxic activity.

The majority of TSAO derivatives have also been evaluated for their inhibitory activity against a mutant HIV-1 strain that contains the E138K mutation in its RT, which is known to afford resistance to TSAO-T and TSAO-m^{3}T.[16] As a rule, all N-3-substituted TSAO derivatives lose marked inhibitory potential against the mutant virus strain. Interestingly, the most active anti-HIV N-3 TSAO derivatives (i.e., **8**, **12**, **13**, **19**, **20**) retain some antiviral activity (EC_{50}: 3.5−8.09 μM) against this mutant virus strain, although this activity is at least 100- to 500-fold lower than against the wild-type virus. Up to now, these compounds are the only TSAO derivatives that showed activity in the micromolar range against this mutant, resistant HIV-1 strain. Thus, the N-3-substituted TSAO derivatives reported herein seem to have overall a similar mechanistic basis of antiviral activity (and resistance) as the parent prototype compounds TSAO-T and TSAO-m^{3}T. However, it cannot be excluded that those TSAO derivatives that show residual activity against HIV-1/138K and that belong to the most active TSAO analogues ever reported, may show additional interactions with amino acids at the p66/p51 subunit interface of heterodimeric RT, thus accounting for the residual activity against HIV-1 viruses harboring the E138K mutation in their RT.

For a number of compounds, the inhibitory activity against recombinant HIV-1 RT has been determined (Table 1). The most active compounds **8**, **9**, **12**, **13**, **14**, **18**, and **20** showed IC_{50} values almost equal (IC_{50}: 2.4−4.5 μM) to those found for TSAO-m^{3}T (IC_{50}: 3.1 μM). Interestingly, the *N*-methylcarboxamide derivative **17** proved to be five to 6-fold more active (IC_{50}: 0.56 μM) than the lead compound, TSAO-m^{3}T, thus being the most active TSAO derivative against recombinant HIV-1 RT prepared so far. Loss of antiviral activity was usually accompanied by higher IC_{50} values against HIV-1 RT. In fact, those compounds having the lowest antiviral activity (containing aromatic/bulky substituents) were not found to be active at 500 μM. We hypothesize that these compounds might have difficulties reaching the pocket at the interface of the preformed RT heterodimer (explaining the inactivity against RT) in the cell-free RT assays, but are able—at least to some extent—to reach its RT binding site in virus-infected cells where p66 and p51 monomers need to dimerize to a heterodimer in the presence of the compounds.

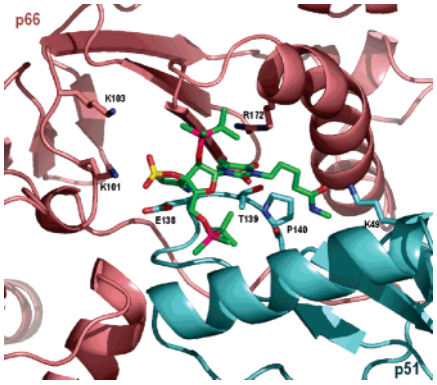


Figure 2. Schematic representation of the dimeric structure of HIV-1 RT in the complex with the *N*-methylcarboxamide derivative **17**. The protein C_α trace of each subunit is shown as a ribbon, colored pink for p66 and cyan for p51, whereas compound **17** is displayed as sticks with C atoms in green. Protein residues relevant to the discussion have been labeled, and their side chains are shown as sticks. The PyMOL molecular graphics program[27] was used for visualization and display.

Docking Studies. To explain the increased activity of **17** the *N*-3-methylcarboxamide moiety was built into the TSAO molecule that was docked into our proposed binding site at the p66(A)−p51(B) dimer interface of HIV-1 RT.[23] The resulting model showed the *N*-3-methylcarboxamide moiety of **17** running parallel to the subunit interface and establishing two putative hydrogen bonds with the enzyme (Figure 2), one between the amide nitrogen of **17** and the main-chain carbonyl oxygen of Pro-B140 and a second one between the amide carbonyl of **17** and the terminal amino group of the side chain of Lys-B49. The additional stabilization gained from these extra interactions might explain why **17** turns out to be a 6-fold better inhibitor than TSAO-m^{3}T. This outcome is fully consistent with the prediction[23] that additional interactions at or near the p51−p66 interface could result in compounds with improved binding affinity over the prototype TSAO-T, as shown earlier for the hydroxypropyl derivative (**14**), whose affinity was increased 2-fold relative to TSAO-T.[23]

Conclusions. In conclusion, the antiviral activity of the parent prototype TSAO-T has been improved by the introduction of hydroxy, carboxamide, *N*-methyl, or *N*,*N*-dimethylcarboxamide alkyl moieties at the N-3 position of the thymine ring. Our modeled TSAO-T/HIV-1 RT complex provided the foundation for these modifications

and now supports the experimental findings because the improved activity of these compounds could be explained by proposing that the functional moieties attached at the N-3 position of TSAO-T can establish additional interactions with one or more amino acids that make up the dimer interface near the structurally important $\beta7-\beta8$ loop.

Experimental Section

General Procedure for the N-3 Alkylation of TSAO-T Derivatives. To a solution of TSAO-T (1 mmol) in dry acetone (20 mL) was added dried and powdered K_2CO_3 (1.1 mmol) and the corresponding bromoalkyl reagent (2−8 mmol). The reaction mixture was refluxed for 5−10 h and then concentrated to dryness. The residue was dissolved in ethyl acetate (20 mL), washed with brine (2 × 20 mL), dried (Na_2SO_4), filtered, evaporated to dryness, and purified by CCTLC on the chromatotron.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[(methoxycarbonyl)methyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (1). Via the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with methyl-2-bromoacetate (0.05 mL, 0.34 mmol) for 5 h. The residue was chromatographed with hexane/ethyl acetate (4:1) to give **1** (0.09 g, 85%) as a white, amorphous solid. Anal. ($C_{27}H_{47}N_3O_{10}SSi_2$) C, H, N, S. MS (ES+) *m/z* 662.2 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(carbamoylmethyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (3). According to the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with 2-bromoacetamide (0.05 g, 0.34 mmol) for 9 h. The residue was chromatographed with dichlorometane/ethyl acetate (3:1) to give **3** (0.08 g, 68%) as a white, amorphous solid. Anal. ($C_{26}H_{46}N_4O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 647.2 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(cyclopropylmethyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (4). According to the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with (bromomethyl)cyclopropane (0.03 mL, 0.34 mmol) for 4 h. The residue was chromatographed with hexane/ethyl acetate (2:1) to give **4** (0.09 g, 83%) as a white, amorphous solid. Anal. ($C_{28}H_{49}N_3O_8SSi_2$) C, H, N, S. MS (ES+) *m/z* 644.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(benzyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (5). According to the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with benzyl bromide (0.03 mL, 0.34 mmol) for 3 h. The residue was chromatographed with hexane/ethyl acetate (2:1) to give **5** (0.05 g, 72%) as a white, amorphous solid. Anal. ($C_{31}H_{49}N_3O_8SSi_2$) C, H, N, S. MS (ES+) *m/z* 680.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(2-bromoethyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (7). According to the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with 1,2-dibromoethane (0.09 mL, 1.02 mmol) for 10 h. The residue was chromatographed with hexane/ethyl acetate (2:1) to give **7** (0.03 g, 41%) as a white, amorphous solid. Anal. ($C_{26}H_{46}BrN_3O_8SSi_2$) C, H, N, S. MS (ES+) *m/z* 696.1 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(2-hydroxyethyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (8). According to the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with 2-bromoethanol (0.12 mL, 1.70 mmol) for 8 h. The residue was chromatographed with hexane/ethyl acetate (1:1) to give **8** (0.09 g, 86%) as a white, amorphous solid. Anal. ($C_{26}H_{47}N_3O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 634.1 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(2-methoxyethyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (9). According to the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with 2-bromoethylmethyl ether (0.16 mL, 1.70 mmol) for 10 h. The residue was chromatographed with hexane/ethyl acetate (1:1) to give **9** (0.03 g, 30%) as a white foam. Anal. ($C_{27}H_{49}N_3O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 648.2 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(2-phenylethyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (10). According to the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with 2-phenylethyl bromide (0.05 mL, 0.34 mmol) for 6 h. The residue was chromatographed with hexane/ethyl acetate (2:1) to give **10** (0.10 g, 89%) as a white foam. Anal. ($C_{32}H_{51}N_3O_8SSi_2$) C, H, N, S. MS (ES+) *m/z* 694.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[2-(3-indolyl)ethyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (11). According to the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was reacted with 3-(2-bromoethyl) indole (0.114 g, 0.51 mmol) and tetrabutylammonium (0.31 g, 0.85 mmol) iodide for 6 h. The residue was chromatographed with dichloromethane/ethyl acetate (20:1) to give **11** (0.07 g, 60%) as a white foam. Anal. ($C_{34}H_{52}N_4O_8SSi_2$) C, H, N, S. MS (ES+) *m/z* 733.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[(*N*′-methylcarbamoyl)methyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (12). A solution of **1** (0.1 g, 0.15 mmol) in methylamine (33 wt% in ethanol, 3 mL) was stirred at room temperature for 2 h. Solvent was evaporated to dryness and the residue was purified by CCTLC on the chromatotron (dichloromethane/ethyl acetate, 4:1) to give **12** (0.07 g, 70%) as a white amorphous solid. Anal. ($C_{27}H_{48}N_4O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 661.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[(*N*′,*N*′-dimethylcarbamoyl)methyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (13). To a cooled (−20 °C) solution of compound **6**[24] (0.1 g, 0.16 mmol) and triethylamine (TEA) (0.02 mL, 0.16 mmol) in dry dichloromethane (4 mL) was added *N,N*-dimethylamine (2 M in THF) (0.02 mL, 0.40 mmol). After 15 min, benzotriazol-1-yloxy tris(dimethylamino)phosphoniumhexafluorophosphate (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% $NaHCO_3$ (5 mL), and brine (5 mL). The organic phase was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was purified by CCTLC on the chromatotron (hexane/ethyl acetate, 3:1) to give **13** (0.07 g, 68%) as a white foam. Anal. ($C_{28}H_{50}N_4O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 675.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[4-(methoxycarbonyl)butyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (15). Following the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was treated with methyl-5-bromovalerate (0.08 mL, 0.34 mmol) for 6 h. The residue was chromatographed with hexane/ethyl acetate (1:1) to yield **15** (0.09 g, 71%) as a white, amorphous solid. Anal. ($C_{30}H_{53}N_3O_{10}SSi_2$) C, H, N, S. MS (ES+) *m/z* 704.4 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[5-(ethoxycarbonyl)pentyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (16). Following the general alkylation procedure, TSAO-T (0.10 g, 0.17 mmol) was treated with ethyl-6-bromohexanoate (0.06 mL, 0.34 mmol) for 5 h. The residue was chromatographed with hexane/ethyl acetate (2:1) to yield **16** (0.10 g, 80%) as a white, amorphous solid. Anal. ($C_{32}H_{57}N_3O_{10}SSi_2$) C, H, N, S. MS (ES+) *m/z* 732.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[4-(*N*′-methylcarbamoyl)butyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (17). Compound **15** (0.1 g, 0.15 mmol) was treated with methylamine (33 wt% in ethanol, 5 mL) as previously described for **12**, affording a residue that was purified by CCTLC on the chromatotron (dichloromethane/methanol, 30:1) to give **17** (0.03 g, 50%) as a white foam. Anal. ($C_{30}H_{54}N_4O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 703.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[5-(*N*′-methylcarbamoyl)pentyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (18). Compound **16** (0.1 g, 0.15 mmol) was treated with methylamine (33 wt% in ethanol, 5 mL) as previously described for **12**, affording a residue that was purified by CCTLC on the chromatotron (dichloromethane/methanol, 30:1) to give **18** (0.02 g, 40%). Anal. ($C_{31}H_{56}N_4O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 717.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-(methoxycarbonyl)-2-propenyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (19). Via the general alkylation procedure, TSAO-T (0.20 g, 0.34 mmol) was reacted with methyl 4-bromocrotonate (0.08 mL, 0.68 mmol) for 4 h. The residue was chromatographed with hexane/ethyl acetate (1:1) to give **19** (0.22 g, 93%) as a white, amorphous solid. Anal. ($C_{29}H_{49}N_3O_{10}SSi_2$) C, H, N, S. MS (ES+) *m/z* 688.5 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(4-hydroxy-2-butenyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (20). DIBAL-H (1 M solution in THF, 0.58 mL, 0.58 mmol) was added dropwise into a solution of **19** (0.2 g, 0.29 mmol) in dry THF (0.5 mL) at 0 °C 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 NH_4Cl (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 **20** (0.11 g, 58%) as a white, amorphous solid. Anal. ($C_{28}H_{49}N_3O_9SSi_2$) C, H, N, S. Found: C, 50.40; H, 7.27; N, 6.58. MS (ES+) *m/z* 660.4 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(4-hydroxybutyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (21). A solution of spiro nucleoside **20** (0.1 g, 0.15 mmol) in methanol (15 mL) containing Pd/C (10%) (0.02 g, 20 wt%) was hydrogenated at 20 psi for 30 min. The reaction mixture was filtered. Evaporation of the filtrate gave a residue that was purified by CCTLC on the chromatotron using hexane/ethyl acetate (2:1) to give **21** (0.075 g, 75%) as a white, amorphous solid. Anal. ($C_{28}H_{51}N_3O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 662.4 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(3-phthalimidopropyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (22). According to the general alkylation procedure, TSAO-T (0.2 g, 0.34 mmol) and 3-bromopropylphthalimide (0.18 g, 0.68 mmol) were reacted for 6 h. The residue was chromatographed with hexane/ethyl acetate (2:1) to afford **22** (0.16 g, 60%)) as a white, amorphous foam. Anal. ($C_{35}H_{52}N_4O_{10}SSi_2$) C, H, N, S. MS (ES+) *m/z* 777.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[[3-*N*′-[benzoyl-2-(N″-methylcarbamoyl)]aminopropyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (23). Methylamine (33 wt% in ethanol, 1.8 mL) was added to a cooled (−20 °C) solution of **22** (0.1 g, 0.13 mmol) in ethanol (2 mL). Stirring was continued at −20 °C for 45 min. Solvent was evaporated to dryness, and the residue was purified by CCTLC on the chromatotron (dichloromethane/methanol, 30:1) to give **23** (0.08 g, 79%) as a white amorphous solid. Anal. ($C_{36}H_{57}N_5O_{10}SSi_2$) C, H, N, S. MS (ES+) *m/z* 808.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[(benzyloxycarbonyl)aminopropyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (24). According to the general alkylation procedure, TSAO-T (0.20 g, 0.34 mmol) and *N*-(benzyloxycarbonyl)-3-bromopropylamine[25] (0.28 mg, 1.02 mmol) were reacted for 6 h. The residue was chromatographed with dichloromethane/methanol (50:1) to afford **24** (0.23 g, 87%) as a white, amorphous foam. Anal. ($C_{35}H_{56}N_4O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 781.33 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-(3-aminopropyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (25). A solution of the protected nucleoside **24** (0.1 g, 0.16 mmol) in methanol (15 mL) containing Pd/C (10%) (0.02 g, 20 wt%) was hydrogenated at 20 psi for 30 min. The reaction mixture was filtered, and the filtrate was evaporated to dryness, under reduced pressure, to give **25** (0.07 g, 75%) as a white foam. Anal. ($C_{27}H_{50}N_4O_9SSi_2$) C, H, N, S. MS (ES+) *m/z* 647.3 $(M + 1)^+$.

3-[Bis(benzyloxycarbonylmethyl)amino]propyl Bromide (26). To a suspension of 3-Bromopropylamine hydrobromide (0.4 g, 2.0 mmol) in acetonitrile (14 mL) excess of distilled triethylamine (TEA) (0.92 mL, 13.2 mmol) was added. After 5 min at room temperature, benzyl-2-bromoacetate (0.4 mL, 6.6 mmol) was added and the reaction was stirred at room-temperature overnight and evaporated to dryness. The residue was chromatographed with dichloromethane/methanol (100:1) to afford **26** (0.40 g, 50%) as a light yellow oil. Anal. ($C_{21}H_{24}BrNO_4$) C, H, Br, N. MS (ES+) *m/z* 434.1 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-[*N*′,*N*′-bis(benzyloxycarbonyl)methyl)aminopropyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (27). According to the general alkylation procedure, TSAO-T (0.20 g, 0.34 mmol) was reacted with **26** (0.21 g, 0.68 mmol) for 14 h. The residue was chromatographed with dichloromethane/methanol (50:1) to give **27** (0.19 g, 60%) as a white, amorphous solid. Anal. ($C_{45}H_{66}N_4O_{12}SSi_2$) C, H, N, S. MS (ES+) *m/z* 943.4 $(M + 1)^+$.

[1-[2′,5′-Bis-O-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-[*N*′,*N*′-bis(carboxymethyl)aminopropyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (28). A solution of the protected nucleoside **27** (0.12 g, 0.12 mmol) in methanol (9 mL) containing Pd/C (10%) (0.02 g, 20 wt%) was hydrogenated at 30 psi at 40 °C for 3 h. The reaction mixture was filtered, and the filtrate was evaporated to dryness, under reduced pressure, to give **28** (0.09 g, 97%) as a white amorphous solid. Anal. ($C_{31}H_{54}N_4O_{12}SSi_2$) C, H, N, S. MS (ES+) *m/z* 763.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-[*N*′,*N*′-(dimethyl)aminopropyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (30). To a solution of **29**[26] (0.1 g, 0.14 mmol) in acetone (4 mL) was added dimethylamine (2 M in THF) (0.42 mL, 0.84 mmol). The solution was stirred under reflux for 12 h. The solvent was evaporated to dryness, and the residue was purified by CCTLC on the chromatotron (dichloromethane/methanol/ammonium hydroxide, 10:1:2%), to give **30** (0.07 g, 71%) as a white foam. Anal. ($C_{29}H_{54}N_4O_8SSi_2$) C, H, N, S. MS (ES+) *m/z* 675.3 $(M + 1)^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-[*N*′,*N*′,*N*′-(trimethyl)aminopropyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) iodide (31). A solution of **30** ((0.06 g, 0.09 mmol) and methyl iodide (0.006 mL, 0.09 mmol) in acetone (3 mL) was stirred at room temperature for 3 h. The solvent was removed in vacuo, and diethyl ether (5 mL) was added to the residue. The mixture was kept on ice (0 °C) overnight, and the solid formed was removed by filtration to give **31** (0.072 g, 98%) as a white amorphous solid. Anal. ($C_{30}H_{57}IN_4O_8SSi_2$) C, H, N, S. MS (ES+) *m/z* 690.3 $(M + 1)^+$.

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Supporting Information Available: Spectroscopic data (^1H and ^{13}C NMR chemical shift assignments), general methods (chemical procedures, biological evaluation), and elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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