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Discovery of TSAO derivatives with an unusual HIV-1 activity/resistance profile

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Abstract

The very first TSAO derivative that lacks the 4″-amino group at the 3′-spiro moiety (compound **3**) has been prepared and the effect of this modification on the activity/resistance profile has been evaluated. This molecule proved HIV-1 specific (NNRTI-characteristic). A mixture of wild-type and V106V/A or L234L/I mutations were found in the RT of some, but not all compound **3**-resistant virus strains. Compound **3** does not select for the TSAO-specific E138K mutation in the RT. However, the compound markedly lost its antiviral potential against a variety of virus strains that contain NNRTI-characteristic mutations in RT including E138K. The deaminated TSAO compound must fit differently in the HIV-1 RT enzyme than its prototype TSAO-m^{3}T.

Keywords: Deaminated TSAO; TSAO compounds; Non-nucleoside HIV-1 RT inhibitors; Spironucleosides

1. Introduction

Reverse transcriptase (RT) is an attractive target for the development of new drugs useful in AIDS therapy (Jonckheere et al., 2000). This key enzyme plays an essential and multifunctional role in the replication of the human immunodeficiency virus (HIV). An important class of inhibitors targeted at the viral RT, are the so-called non-nucleoside RT inhibitors (NNRTIs) (Balzarini, 1999; Tavel et al., 1999; Campiani et al., 2002; De Clercq, 1998; Drake, 2000), Currently, only three NNRTIs, namely nevirapine, delarvirdine and efavirenz, are available in clinical practice. Although NNRTIs generally exhibit low toxicity and favorable pharmacokinetic properties, one of the major problems associated with them is the rapid emergence of drug-resistant virus strains (Balzarini, 1999, 2004; De Clercq, 1999; Vandamme et al., 1998). In fact, HIV-1 resistance to NNRTIs is primarily associated with mutations of amino acids that line the lipophilic NNRTI-specific binding pocket (Balzarini, 1999).

Among the NNRTIs, [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″, 2″-dioxide) nucleosides (TSAOs), developed in the last decade in our research group, represent a rather unique class of compounds (Balzarini et al., 1992; Camarasa et al., 1992). The prototype compound of this family is the thymine derivative designated as TSAO-T (**1**) and one of the most selective compound is its 3-*N*-methyl substituted derivative TSAO-m^{3}T (**2**) (Fig. 1). TSAO-T and TSAO-m^{3}T select in cell culture for the Glu138Lys mutation in the HIV-1 RT (Balzarini et al., 1993b,c; Jonckheere et al., 1994; Pelemans et al., 2001). The mutant Glu138Lys RT HIV-1 strains are resistant to TSAO derivatives but retain marked sensitivity to many other NNRTIs as well to nucleoside RT inhibitors (Camarasa et al., 2004). The Glu138 amino acid is part of the β7–β8 loop of the p51 subunit of RT, which forms the "floor" of the NNRT-binding pocket (Esnouf et al., 1997). The interaction of this loop with the p66 subunit of HIV-1 RT is essential to form a stable heterodimeric enzyme and thus for the catalytic function of the p66 subunit (Pandey et al., 2001, 2002). The catalytic activity

Abbreviations: TSAO, [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) nucleoside

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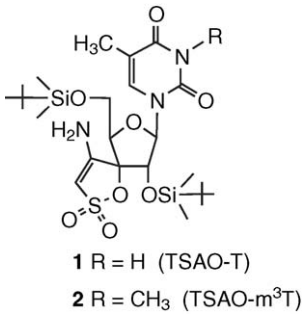


Fig. 1. Structures of TSAO-T and TSAO-m^{3}T.

of the RT is only observed with the heterodimeric form of the enzyme (Restle et al., 1990, 1992). Thus, compounds that interfere with the p66/p51 interface may be potential selective dimerization inhibitors of RT (Rodríguez-Barrios et al., 2001). Both modeling studies and experimental work provide enough evidence to hypothesize that there is a H-bond between the carboxylic acid group of Glu138 and the 4″-amino group of the spiro moiety of the TSAO molecule (Rodríguez-Barrios et al., 2001) (Fig. 2). Recent reports on the mechanism of TSAO-activity/resistance and structural modelling suggested that TSAO interferes with the dimerization process of the enzyme by destabilizing the RT heterodimer due to structural and conformational perturbations at the RT subunit interface (Rodríguez-Barrios et al., 2001; Sluis-Cremer et al., 2000). Therefore, TSAO compounds are a unique class among the NNRTIs, and represent the first example of small non-peptidic molecules that can interfere with the dimerization process of HIV-1 RT (Camarasa et al., 2004).

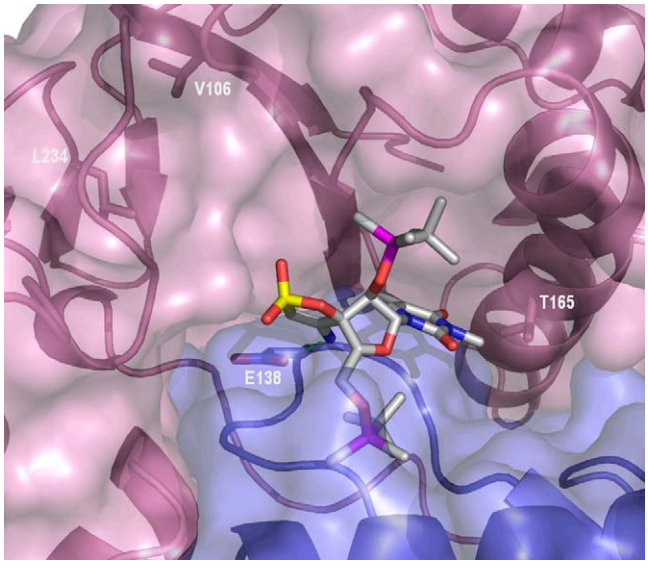


Fig. 2. Proposed molecular model (Rodríguez-Barrios et al., 2001) depicting the interaction of TSAO-m^{3}T with HIV-1 reverse transcriptase, whose p66 and p51 subunits are shown as ribbons enveloped by a semitransparent surface colored in pink and blue, respectively. The putative hydrogen bond between the carboxylic acid group of Glu138 and the 4″-NH_2 of the TSAO molecule is displayed as a green dashed line. The side chains of Val106, Thr165, Glu138, and Leu234 are shown as sticks.

Since we first synthesized TSAO-T (**1**) in 1992 (Balzarini et al., 1992; Camarasa et al., 1992), we have reported on extensive SAR studies on this lead that helped to reveal the structural requirements of this family of compounds for their optimal interaction with the HIV-1 RT (Camarasa et al., 2004). A crucial interaction with the RT, as supported by experimental data and molecular modeling studies, is a hydrogen bond between the amino group of the spiro moiety of the TSAO molecule and the carboxylic acid residue of 138Glu in the β7/β8 loop of the p51 subunit (Balzarini et al., 1993b; Jonckheere et al., 1994; Camarasa et al., 2004). As part of our medicinal chemistry program, we considered of interest to remove this amino group and evaluate the effect of this modification on the activity/resistance profile of the corresponding deaminated TSAO derivative. In this manuscript we report on the synthesis and biological studies of the very first TSAO derivatives that lack the amino group at the 3′-spiro moiety.

2. Materials and methods

2.1. Synthesis

2.1.1. General methods

Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. Electrospray mass spectra were measured on a quadropole mass spectrometer equipped with an electrospray source (Hewlett Packard, LC/MS HP 1100). ^{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 and 50 MHz with Me_4Si as internal 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), flow rate (5 ml/min).

2.1.2. *[1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-methylthymine]-3′-spiro-5″-(1″,2″-oxathiole-2″,2″-dioxide) and [1-[2′,5′-bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-methylthymine]-3″-spiro-5″-(4″-amino-3″-nitro-1″,2″-oxathiole-2″,2″-dioxide) (**3** and **4**)*

A mixture of TSAO-m^{3}T (**2**) (Pérez-Pérez et al., 1992) (0.25 g, 0.41 mmol) and freshly distilled *n*-pentyl nitrite (1.15 ml, 8.3 mmol) in dry THF (8 ml) was heated at 80 °C while being maintained under an argon atmosphere. Stirring and heating continued under direct incadescent illumination (160 W bulb) for 3 h. The reaction mixture was then cooled, concentrated under reduced pressure, and purified twice by CCTLC on the Chromatotron (first using hexane:ethyl acetate, 2:1 and then dichloromethane:methanol, 200:1). From the faster moving fractions compound **3** (0.036 g, 15%) was obtained as an amorphous solid. ^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ: 0.81, 0.99 (2s, 18H, 2*t*-Bu), 1.92 (d, 3H, $J = 1.2$ Hz, CH_3-5), 3.25 (s,

3H, CH_3-3), 3.91 (dd, 1H, $J_{4',5'a}$ = 2.7, $J_{5'a,5'b}$ = 12.1 Hz, H-5′a), 4.07 (dd, 1H, $J_{4',5'b}$ = 4.3 Hz, H-5′b), 4.36 (dd, 1H, H-4′), 4.66 (d, 1H, $J_{1',2'}$ = 8.2 Hz, H-2′), 6.19 (d, 1H, H-1′), 7.23 (d, 1H, $J_{3'',4''}$ = 6.5 Hz, H-4″), 7.44 (d, 1H, H-3″), 7.48 (d, 1H, H-6). ^{13}C NMR [75 MHz, $(CD_3)_2CO$] δ: 13.15 (CH_3-5), 18.40, 18.88 [$(CH_3)_3$–C–Si], 25.79, 26.48 [$(CH_3)_3$–C–Si], 28.04 (CH_3-3), 63.24 (C-5′), 76.31 (C-2′), 85.43 (C-4′), 88.14 (C-1′) 96.41 (C-3′), 113.39 (C-5), 129.72 (C-6), 133.78, 137.57 (C-3″, C-4″), 152.11 (C-2), 163.44 (C-4). MS (ES+) *m/z* 611.3 $(M + Na)^+$. Anal. calcd. for $C_{25}H_{44}N_2O_8SSi_2$: C, 50.99; H, 7.53; N, 4.76; S, 5.45. Found: C, 51.03; H, 7.51; N, 4.91; S, 5.55.

The slowest moving fractions gave 0.078 g (29%) of **4** as an amorphous solid. ^{1}H NMR [300 MHz $(CD_3)_2CO$] δ: 0.79, 0.90 (2s, 18H, 2*t*-Bu), 1.92 (s, 3H, CH_3-5), 3.26 (d, 3H, *J* = 1.2 Hz, CH_3-3), 4.15 (m, 2H, 2H-5′), 4.51 (m, 1H, H-4′), 4.98 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-2′), 6.11 (d, 1H, H-1′), 7.50 (s, 1H, H-6), 8.99 (bs, 1H, NH), 9.33 (bs, 1H, NH). MS (ES+) *m/z* 649.3 $(M + 1)^+$. Anal. calcd. for $C_{25}H_{44}N_4O_{10}SSi_2$: C, 46.28; H, 6.83; N, 8.63; S, 4.94. Found: C, 46.26; H, 6.79; N, 8.65; S, 5.00.

*2.1.3. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-methylthymine]-3′-spiro-5″-(3″-iodo-1″,2″-oxathiole-2″,2″-dioxide) (**6**)*

Following the diazotation/deamination procedure described for compound **3**, iodo nucleoside **5** (0.15 g, 0.20 mmol) was treated with isoamyl nitrite (1.07 ml, 8 mmol) in dry THF (15 ml) at reflux under direct incandescent illumination (160 W bulb) for 12 h. The final residue was purified by CCTLC on the Chromatotron (hexane:ethyl acetate, 10:1). The fastest moving fractions gave 0.028 g (23%) of the deaminated product **6**. ^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ: 0.80, 0.97 (2s, 18H, 2*t*-Bu), 1.91 (s, 3H, CH_3-5), 3.24 (s, 3H, CH_3-3), 3.94 (dd, 1H, $J_{4',5'a}$ = 3.2, $J_{5'a,5'b}$ = 12.8 Hz, H-5′a), 4.10 (dd, 1H, $J_{4',5'b}$ = 5.9 Hz, H-5′b), 4.35 (dd, 1H, H-4′), 4.74 (d, 1H, $J_{1',2'}$ = 8.2 Hz, H-2′), 6.12 (d, 1H, H-1′), 7.38 (s, 1H, H-4″), 7.47 (s, 1H, H-6). MS (ES+) *m/z* 737.6 $(M + Na)^+$. Anal. calcd. for $C_{25}H_{43}IN_2O_8SSi_2$: C, 42.01; H, 6.06; N, 3.92; S, 4.49. Found: C, 42.10; H, 6.12; N, 4.03; S, 4.53.

The slowest moving band gave unreacted starting material **5** (0.095 g, 75%).

*2.1.4. (5-O-Benzoyl-1,2-O-isopropylidene-α-D-ribofuranose)-3-spiro-5′-(1′,2′-oxathiole-2′,2′-dioxide) and (5-O-benzoyl-1,2-O-isopropylidene-α-D-ribofuranose)-3-spiro-5′-(4′-amino-3′-nitro-1′,2′-oxathiole-2′,2′-dioxide) (**8** and **9**)*

Compound **7** (Camarasa et al., 1999) (0.80 g, 2.0 mmol) was treated with isoamyl nitrite (10.75 ml, 80 mmol) in dry THF (80 ml) at reflux under direct incandescent illumination (160 W bulb) for 12 h following a similar diazotation/deamination procedure described for compound **3**. The final residue was purified by CCTLC on the Chromatotron (hexane:ethyl acetate, 15:1). The fastest moving fractions gave 0.274 g (36%) of **8** as a white foam. ^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ: 1.35, 1.62 (2s, 6H, $(CH_3)_2C$), 4.65 (2H, d, H-5), 4.85 (m, 2H, H-4, H-2), 6.18 (d, 1H, H-1′), 7.40 (d, 1H, $J_{3',4'}$ = 6.5 Hz, H-4′), 7.65 (d, 1H, H-3′), 7.58–8.04 (m, 5H, OBz). MS (ES+) *m/z* 405.4 $(M + Na)^+$. Anal. calcd. for $C_{17}H_{18}O_8S$: C, 53.40; H, 4.74; S, 8.39. Found: C, 53.51; H, 4.65; S, 8.45.

The slowest moving band gave the 3′-nitro derivative **9** (0.193 g, 22%) as a pale yellow foam. ^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ: 1.33, 1.61 (2s, 6H, $(CH_3)_2C$), 4.75 (2H, d, H-5), 4.91 (t, 1H, $J_{4,5}$ = 5.3 Hz, H-4), 4.80 (d, 1H, $J_{1,2}$ = 3.8 Hz, H-2), 6.08 (d, 1H, H-1), 7.60–8.05 (m, 5H, OBz), 9.28 (bs, 1H, NH), 9.67 (bs, 1H, NH). MS (ES+) *m/z* 465.4 $(M + Na)^+$. Anal. calcd. for $C_{17}H_{18}N_2O_{10}S$: C, 46.15; H, 4.10; N, 6.33; S, 7.25. Found: C, 46.21; H, 4.12; N, 6.43 S, 7.29.

*2.1.5. (1,2-Bis-O-acetyl-5-O-benzoyl-D-ribofuranose)-3-spiro-5′-(1′,2′-oxathiole-2′,2′-dioxide) (**10**)*

A solution of **8** (0.25 g, 0.65 mmol) in 1.4 ml of a (9:1) mixture of trifluoroacetic acid:water was stirred at room temperature for 4 h. The solvent was evaporated to dryness, and the residue was treated with acetic anhydride (1 ml) and pyridine (3 ml) and stirred at room temperature overnight. The solvents were evaporated under reduced pressure, and the residue was purified by CCTLC on the Chromatotron (hexane:ethyl acetate, 20:1) to afford **10** (0.26 g, 96%) as a syrup. The ^{1}H NMR spectrum showed that it was a mixture (1.5:1) of the α and β anomers. ^{1}H NMR [300 MHz, $CDCl_3$] δ: 2.05, 2.21 (2s, 6H, 2OAc), 4.86 (2H, m, H-5), 5.02 (m, 1H, H-4), 5.70 (d, 1H, $J_{1,2}$ = 5.0 Hz, H-2α), 5.87 (d, 1H, $J_{1,2}$ = 1.1 Hz, H-2β), 6.30 (d, 1H, H-1β), 6.71 (d, 1H, H-1α), 7.49 (d, 1H, $J_{3',4'}$ = 6.4 Hz, H-4′), 7.74 (d, 1H, H-3′), 7.60–8.05 (m, 5H, OBz). MS (ES+) *m/z* 449.1 $(M + Na)^+$. Anal. calcd. for $C_{18}H_{18}O_{10}S$: C, 50.70; H, 4.25; S, 7.52. Found: C, 50.71; H, 4.27; S, 7.55.

2.2. Biological methods

2.2.1. Cells and viruses

Human immunodeficiency virus type 1 [HIV-1 (III_B)] 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 isolation and characterization of the mutant (i.e. Glu138Lys RT) HIV-1 strains were described before (Balzarini et al., 1993a).

2.2.2. Activity assay of test compounds against HIV-1 and HIV-2 in cell culture

A total number of 4×10^5 CEM or 3×10^5 MT-4 cells per milliliter were infected with HIV-1 (III_B) or HIV-2(ROD) or mutant HIV-1 (III_B) strains at ~100 CCID50 (50% cell culture infective dose) per milliliter of cell suspension. Then, 100 μl of the infected cell suspensions were transferred to 96-well microtiter plates 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

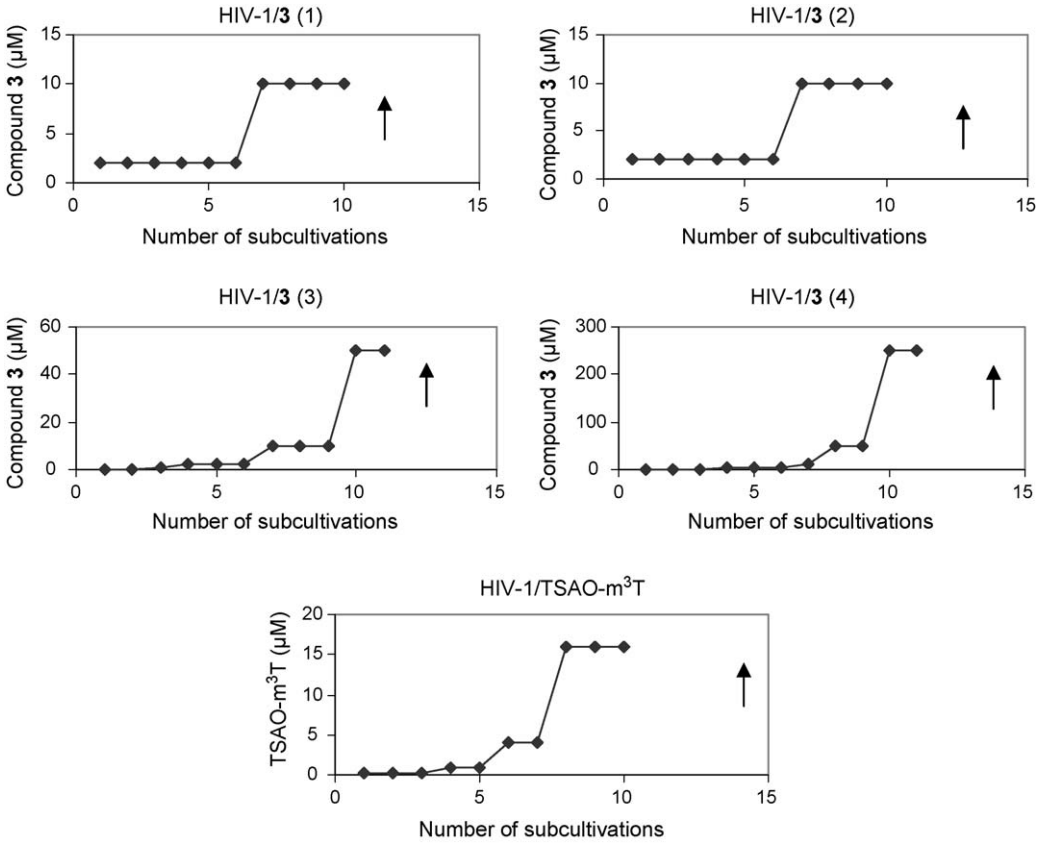


Fig. 3. Resistance selection for compound **3** in HIV-1-infected CEM cell cultures. Arrows indicate the isolation of the mutant virus strain, as referred to in Table 2.

(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%.

2.2.3. Selection of drug-resistant virus strains and identification of mutations in HIV-1 reverse transcriptase

Drug resistance was selected in four independent experiments for compound **3** against HIV-1 (IIIB) in 1 ml cell cultures (48-well plates) under escalating drug regimens. TSAO-m^3T was included for comparative reasons (Fig. 3). The compound concentrations that were added at initiation of the drug selection were ~2 to 3 times their EC_{50} value. Then, as soon as full cytopathicity appeared, the concentrations were gradually increased by two- or three-fold (Fig. 3). The cultures were passaged every 3 to 4 days by adding ~900 μl fresh cell culture medium to 50–100 μl from the virus-infected cell cultures. Four virus strains derived from independent selection experiments were isolated and designated HIV-1/**3**-(1) to HIV-1/**3**-(4). The mutations that appeared in the RT genes of the drug-exposed virus strains were determined according to previously published procedures (Auwerx et al., 2004; Balzarini et al., 1993a).

2.2.4. Anti-reverse transcriptase assays

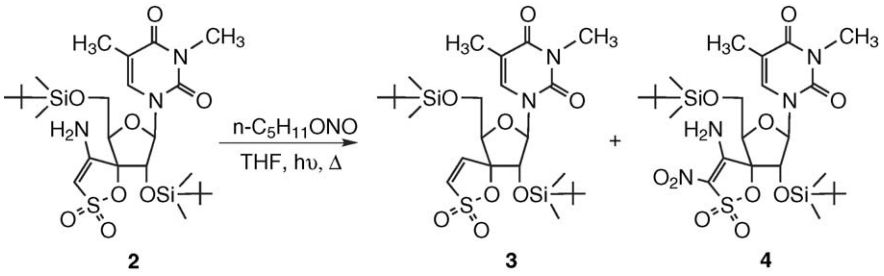
The source of the reverse transcriptases used were either recombinant HIV-1 RT (derived from HIV-1 III_B) and mutated recombinant HIV-1 RT, constructed and prepared as described before (Balzarini et al., 1994). The HIV-2 RT was a kind gift of Prof. D. Stammers (Oxford, UK).

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%.

3. Results and discussion

3.1. Chemical results

One of the most characteristic features of TSAO derivatives is the presence of the 4-amino-1,2-oxathiole-2,2-dioxide spiro ring. From a chemical point of view, the reactivity of this heterocyclic system has been scarcely studied (Lobatón et al., 2002; de Castro et al., 2005). Moreover, the presence of the *tert*-



Scheme 1. Synthesis of deaminated TSAO derivative **3**.

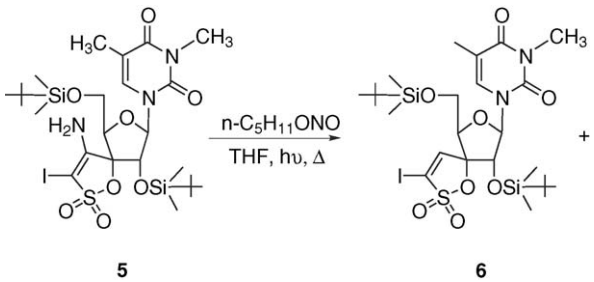
butyldimethylsilyl (TBDMS) groups at positions 2′ and 5′ of the sugar moiety, being crucial for antiviral activity and highly sensitive to both basic and acidic media, requires the selection of smooth reaction conditions compatible with such groups. The amino group attached to the double bond in the spiro moiety is a peculiar type of amino group. Experimental and theoretical studies indicated that the enamine form is the preferred tautomer both in polar and apolar solvents (Camarasa et al., 1999). Thus, hydrogenolysis of enamines (derived from pyrrolidine) with alanes, was initially attempted (Coulter et al., 1968). However, when TSAO-m^{3}T **2** was reacted with AlH_3 in refluxing ether, only unreacted starting material was recovered. This result may not be surprising due to the special primary enamine character of the molecule.

Although the 4″-amino group of the sultone moiety of TSAO-m^{3}T **2** is not aromatic, ab initio calculations suggested that this nitrogen has mainly an sp^2 character but with slight non-planarity (certain sp^3 tetrahedral geometry) (Camarasa et al., 1999). Thus, hydrogenolysis methods of aromatic amines were next explored. A general method for the replacement of a primary aromatic amino group by hydrogen is reduction of aromatic diazonium salts, prepared by diazotation (typically by treatment of the primary amine with sodium or potassium nitrite in strongly acidic aqueous media) (Engel, 1990). Conversion to the diazonium salts may also be accomplished in nonaqueous media using either acetic acid or alkyl nitrites (Cadogan and Molina, 1973). It is described in the literature that reduction of diazonium salts has been carried out using classical reagents such as alcohols, hypophosphrous acid, sodium stannite or other new reagents such as sodium borohydride, triethylsilane, tributylstannane among others (Engel, 1990). The synthesis of deaminated TSAO derivative was carried out by an anhydrous diazotation/radical deamination procedure developed by Nair and Richardson (1980) for aromatic amines using *n*-pentyl nitrite as the nitrosating agent and dry THF as the hydrogen atom-donating solvent (Nair and Richardson, 1980).

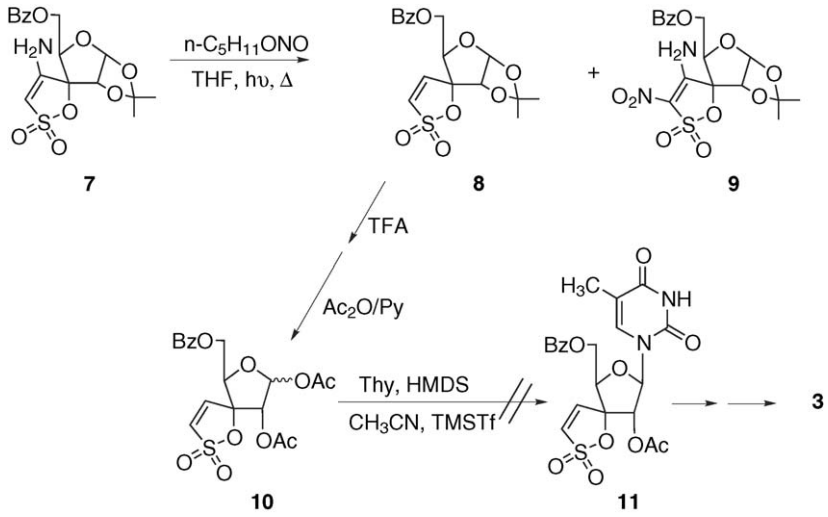
These non-aqueous non-acidic reductive deamination conditions were selected to be compatible with the crucial TBDMS groups. Thus, when TSAO-m^{3}T **2** was reacted with 40 equivalents of *n*-pentyl nitrite in dry THF (Scheme 1), under an argon atmosphere, and the resulting mixture was warmed at 80 °C and photolyzed with visible light (160 W bulb) for 10 h, the corresponding deaminated TSAO derivative **3** was isolated in low yield (15%) together with the 3″-nitro-4″-amino spiro derivative **4** (38%). Nitration, as side-reaction, in this procedure has been previously described (Driscoll et al., 1996). Attempts to improve the yield of **3** using other alkyl nitrites (isoamyl nitrite or *tert*-butyl nitrite), other hydrogen atom donating solvents (dioxan or ethanol) or other reducing agents such us tributylstannane, triethylsilane, or sodium borohydride were not successful. In order to avoid the undesired formation of the 3″-nitro derivative **4**, the corresponding 3″-iodo TSAO derivative **5** (which showed potent anti-HIV-1 activity comparable to that of the prototype TSAO-m^{3}T **2)** was subjected to the diazotation/deamination procedure (Scheme 2). However, when **5** was heated in dry THF in the presence of isoamyl nitrite, under incandescent illumination for 10 h, the corresponding deaminated 3″-iodo derivative **6** was obtained, again in low yield (23%) together with unreacted starting material (75%).

Finally, the sugar spiro derivative **7** (Camarasa et al., 1999) was reacted with isoamyl nitrite in THF under similar conditions. In this reaction the deaminated derivative **8** was obtained in a 36% yield together with the 3′-nitroderivative **9** (22%) (Scheme 3). The higher yields obtained in the deamination of the sugar derivative prompted us to explore the sugar deamination/glycosidation approach as an alternative to the deaminated nucleoside **3**.

Thus, hydrolysis of the 1,2-*O*-isopropylidene group of **8**, with aqueous trifluoroacetic acid, followed by reaction with acetic anhydride/pyridine afforded a mixture of the two anomeric forms (α and β, 1:1.5) of the diacetate derivative **10** in a 90% yield (Scheme 3). However, all attempts of condensation of the corresponding 1,2-bis-*O*-acetyl deaminated derivative **10** with silylated thymine base using trimethylsilyl triflate as condensing reagent (Vorbrüggen et al., 1981), as described previously by us for the synthesis of TSAO-m^{3}T (Pérez-Pérez et al., 1992), to yield the 4″-deaminated nucleoside **11**, were unsuccessful.



Scheme 2. Synthesis of deaminated 3″-iodo TSAO derivative **6**.



Scheme 3. Synthesis of deaminated sugar derivatives **8** and **10**.

3.2. Biological results

The anti-HIV activity of the deaminated TSAO-m^{3}T (**3**), 3′′-nitro-TSAO-m^{3}T (**4**), the deaminated 3′′-iodo-TSAO-m^{3}T (**6**) and the deaminated sugar (**8**) derivatives were evaluated in MT-4 and CEM cell cultures and compared with the prototype compound TSAO-m^{3}T (Table 1). They were also evaluated for their inhibitory activity against recombinant HIV-1 RT. Surprisingly, the deaminated TSAO derivative **3** was not inactive (no amino group to interact with Glu-138, the interaction point of TSAO derivatives) but instead it showed significant activity. Compound **3** proved inhibitory to HIV-1 replication in CEM (EC_{50} = 0.15 μM) and MT-4 (EC_{50} = 0.53 μM) cell cultures at concentrations that were only four- to nine-fold higher than TSAO-m^{3}T (Table 1). The compound was also inhibitory against HIV-1 RT (IC_{50} = 3.3 μM). In contrast, **3** was not inhibitory against HIV-2 (ROD) in CEM and MT-4 cell cultures, nor was it active against HIV-2 RT in cell-free assays. In this respect, compound **3** behaves as TSAO-m^{3}T and other NNRTIs listed in Table 2 in that no activity either against HIV-2 RT or HIV-2 replication is observed. The deaminated 3′′-iodo-TSAO-m^{3}T derivative of **3** (compound **6**) showed comparable activity against wild-type HIV-1 to its parent compound **3** (EC_{50}: 0.17–0.31 μM) but proved markedly more cytotoxic than **3** (Table 1). Again, this compound was inactive against HIV-2. In contrast, the deaminated sugar derivative of **3** (compound **8**) showed no antiviral activity at all, and proved marginally cytotoxic (IC_{50}: 147 to ≥250 μM), suggesting that the presence of the pyrimidine base is a requisite for antiviral activity.

On the other hand, the unwanted 3′′-nitro-4′′-amino spiro derivative **4** showed activity against HIV-1 replication in cell culture (EC_{50} = 7.0 μM in CEM and 6.0 μM in MT-4) at the lower micromolar range although the observed activity was found to be very close to its cytostatic activity. The same holds also for compound **6**, questioning the antiviral selectivity of these compounds. Interestingly, compound **4** showed evidence of marginal activity against HIV-2 replication at subtoxic concentrations (EC_{50} = ≥10 μM in MT4 and CEM cell cultures), an observation that is rather unusual for NNRTIs, and that has only previously been observed with certain PETT derivatives (Ren et al., 2000; Auwerx et al., 2004) and with some 3′′-substituted TSAO derivatives for which the structure–activity relationship for having both anti-HIV-1 and anti-HIV-2 activity is currently unclear (Lobatón et al., 2002). To exclude HIV-2 RT as a potential target for **4**, the compound was evaluated for its inhibitory activity against recombinant HIV-2 RT and found to be inactive at 100 μM. This compound was also tested against a mutant HIV-1 RT that contains the TSAO-characteristic Glu138Lys resistance mutation and also against a mutant Glu138Lys RT HIV-1 strain. In both cases, a much decreased drug susceptibility was observed.

Table 1
Inhibitory activity of test compounds against HIV-1 (III_B) and HIV-2 in MT-4 and CEM cell cultures

Compound	EC_{50}[a] (μM)				CC_{50} (μM)	
	MT-4		CEM		MT-4	CEM
	HIV-1	HIV-2	HIV-1	HIV-2		
3	0.53 ± 0.40	>50	0.15 ± 0.09	>50	135 ± 42	≥150
4	6.0 ± 1.2	9.82 ± 6.76	7.0 ± 4.2	≥10	14 ± 5	15.0 ± 3.7
6	0.17 ± 0.02	>0.4	0.31 ± 0.16	>0.4	0.81 ± 0.05	1.0 ± 0.05
8	>50	>50	>50	>50	147 ± 14	≥250
TSAO-m^{3}T	0.06 ± 0.09	>250	0.04 ± 0.01	>250	230 ± 7.3	≥100

[a] 50% Effective concentration or compound concentration required to inhibit HIV-induced cytopathicity in CEM cell cultures by 50%.

Table 2
Inhibitory activity of test compounds against wild-type HIV-1 and a 4″-H-TSAO-T-resistant HIV-1 strains

Compound	EC_{50}[a] (μM)				
	HIV-1/WT	HIV-1/**3** (isolate 1)[b]	HIV-1/**3** (isolate 2)[b]	HIV-1/**3** (isolate 3)[b]	HIV-1/**3** (isolate 4)[b]
3	0.15 ± 0.09	≥10–50[c]	≥1–10[c]	≥5–50[c]	≥5–50[c]
TSAO-m^3T (**2**)	0.058 ± 0.042	0.22 ± 0.10	0.088 ± 0.110	0.10 ± 0.04	0.11 ± 0.03
TSAO-T (**1**)	0.034 ± 0.017	0.31 ± 0.22	0.12 ± 0.06		
Nevirapine	0.11 ± 0.10	0.044 ± 0.014	0.034 ± 0.0	0.29 ± 0.07	0.11 ± 0.0
Delavirdine	0.016 ± 0.014	0.049 ± 0.025	0.012 ± 0.0	–	
Efavirenz	0.0095 ± 0.0063	0.0025 ± 0.0002	0.0025	–	
Emivirine	0.070 ± 0.005	0.033 ± 0.0	0.017 ± 0.007	–	
Quinoxaline GW420867	0.0036 ± 0.0014	0.011 ± 0.003	0.0018	–	
AZT	0.003 ± 0.001	0.008 ± 0.003	0.008 ± 0.0025	–	
3TC	0.044 ± 0.0	0.10 ± 0.05	0.061 ± 0.026	–	

[a] 50% Effective concentration or compound concentration required to inhibit HIV-induced cytopathicity in CEM cell cultures by 50%.
[b] The four virus isolates (isolated as indicated in Fig. 2) and designated as HIV-1/**3** (1), HIV-1/**3** (2), HIV-1/**3** (3) and HIV-1/**3** (4).
[c] No dose-dependent further increase of antiviral activity was observed at the indicated concentration ranges, since the indicated concentration range (i.e. between 10 and 50 μM, 1 and 10 μM or, 5 and 50 μM) invariably resulted in inhibition of virus-induced cytopathicity that varied around the EC_{50} value.

At least four different drug-resistant HIV-1 strains were independently selected under pressure of escalating concentrations of **3** (Fig. 3) and used for sensitivity/resistance testing (Table 2) against a variety of NNRTIs including nevirapine, delavirdine, efavirenz, emivirine, quinoxaline, several TSAO derivatives and the NRTIs AZT and 3TC. In a parallel experiment, TSAO-m^3T resistance selection was included. In all cases, phenotypic resistance development occurred within 7–10 subcultivations. As also observed earlier (Balzarini et al., 1993a,b,c), the TSAO-m^3T-exposed virus strains contained the E138K mutation in its RT and showed resistance against other virus strains that contain NNRTI-characteristic mutations in their RT. Interestingly, the drug (**3**)-resistant virus strains kept pronounced sensitivity to all NRTIs and NNRTIs, including TSAO-m^3T, TSAO-T (Table 2) and a TSAO-triazole derivative (not shown). In contrast, substantial resistance was exclusively observed for the deaminated TSAO derivative **3**, in the presence of which the virus strains were selected. Although a certain degree of antiviral activity was observed at concentrations around 5–10 μM of **3**, no complete inhibition of virus-induced cytopathicity could be observed at the higher compound concentrations tested. Instead, the highest concentrations (i.e. 50 μM) sometimes even resulted in a weaker inhibitory effect than that achieved by the lower drug concentrations, as inferred from the increased number of syncytia in the cell cultures, which is a relevant parameter related to the amount of virus-infected cells. Such a phenomenon was not seen for the other (aminated) TSAO derivatives. Our data clearly indicate that the deaminated TSAO derivative **3** must have selected for mutations in the HIV-1 genome that are different from the NNRTI-characteristic mutations.

Cross-resistance studies were also performed with compound **3** and a variety of mutant HIV-1 strains resistant to NNRTIs (Table 3). Intriguingly, all HIV-1 mutant strains that contain NNRTI-specific mutations in the reverse transcriptase (including the TSAO-m^3T-characteristic E138K substitution) proved completely cross-resistant to compound **3** while some antiviral activity was retained against the mutant L100I RT HIV-1 strain. This points to an interaction of compound **3** with the NNRTI binding pocket of RT. Interestingly, when the RT gene of the deaminated-TSAO (**3**)-resistant virus strains was analyzed, the TSAO-specific E138K mutation could not be detected (Table 4). This is somewhat intriguing, given the pronounced resistance of E138K RT-mutated HIV-1 against compound **3** (Table 3). However, it has also been observed in the past that TSAO-m^3T, although found to lose activity against several other NNRTI-specific mutant V106A, Y181C/I, Y188H/L RT HIV-1 strains, never selects for such mutant viruses in the presence of TSAO

Table 3
Inhibitory activity of test compounds against NNRTI-characteristic mutant virus strains

Compound	EC_{50}[a] (μM)					
	WT	L100I	K103N	V106A	E138K	Y181C
TSAO-m^3T	0.083	0.18	0.86	>15	>80	>15
3	0.15	0.80	>50	>50	>50	>50
Nevirapine	0.024	0.41	7.77	1.35	0.037	6.08
Delavirdine	0.014	2.88	2.51	0.10	0.021	0.93
Efavirenz	0.0016	0.032	0.13	0.0022	0.0095	0.0032
Emivirine	0.0066	0.083	1.99	0.046	0.063	2.98
Quinoxaline GW420867	0.0021	0.043	0.14	0.075	0.0057	0.086

[a] 50% Effective concentration or compound concentration required to inhibit HIV-1-induced cytopathicity in CEM cell cultures by 50%.

Table 4
Amino acid mutations present in the reverse transcriptase of HIV-1 strains selected in the presence of escalating concentrations of compound **3**

Amino acid location	Mutant HIV-1 strain			
	(1)	(2)	(3)	(4)
V106	–	–	V106V/A	V106V/A
I165	I165I/T	I165I/T	I165I/T	I165I/T
L234	L234L/I	–	–	–
G335	G335G/D	G335G/D	–	–
K/R461	K/R461K	–	K/R461R	K/R461R
T/P468	T/P468P	–	T/P468T	T/P468T
N/D471	N/D471N	–	–	–
N/S447	N/S447N	–	–	–

derivatives but exclusively selects for E138K RT-mutated HIV-1 strains. Profound studies revealed that the easiness of the appearance of a transition (G → A) mutation (E138 codon (GAG) to the K138 codon (AAG)), as well as a higher fitness of mutant E138K RT virus than other viral mutants, might contribute to this phenomenon (Pelemans et al., 2001). Although not proven, a similar situation can apply to compound **3**-resistant virus strains. Modeling studies as well as experimental data point to a direct interaction between the carboxylic group of E138 and the 4″-NH_2 of TSAO-m^3T (Fig. 2) (Rodríguez-Barrios et al., 2001). Such direct interaction cannot exist between compound **3** and E138 due to the lack of a 4″-NH_2 group in this novel TSAO derivative. Therefore, these results strongly suggest that the E138K mutation must induce a conformational change in HIV-1 RT that prevents optimal fitting of the TSAO derivative **3** into the enzyme's binding pocket. The long awaited crystal structure of a mutant enzyme–drug complex should help us understand these findings at the molecular level.

In many cases, mixtures of wild-type and mutated amino acids at certain HIV-1 RT locations were found. In several other cases, the wild-type HIV-1 virus contained a mixture of amino acids at certain RT positions at the start of the selection experiment, whereas the drug **3**-resistant virus pool at the end of the drug selection period contained one defined amino acid at this location. The only known NNRTI-characteristic mutation was found in strains (3) and (4) as a mixture with wild-type (V106V/A) (Vasudevachari et al., 1992) and in strain (1) as a mixture with wild-type (L234L/I) (Fujiwara et al., 1998). A number of other mutations have never been reported (i.e. I165T; G335D; K461R; T468P; D471N; S447N), several of them being located in the RNaseH domain of HIV-1 RT. However, there are clearly no consistent changes in the RT gene from the four independent virus isolates. Also, the often-occurring mixtures of wild-type and mutant amino acids makes the resistance spectrum hard to interpret. Clearly, the mutations found in each of the four drug-exposed virus strains are not present in one virus particle, and the virus isolates represent mixtures of several virus strains mutated at different amino acid positions in RT. Although a dual mechanism of RT and RNaseH inhibition cannot be ruled out, it may be unlikely to occur as no inhibition was found in HIV-2 strains. Thus, although it is clear that the deaminated TSAO derivative **3**, in contrast with aminated TSAOs, does not target E138 in HIV-1 RT, it is less clear how compound **3** fits into the HIV-1 RT enzyme, and what is the molecular basis for the development of resistance against **3**. Site-directed mutagenesis of the observed mutations, as such and in combination with each other, may possibly clarify the role of the observed amino acid mutations in the eventual (partial) resistance of the virus strains against compound **3**. However, all the results point to a different way of fitting **3** into the enzyme than that of TSAOs and NNRTIs. Further studies, including crystallographic investigations of RT/compound **3** complexes, are required to clarify this issue.

4. Conclusions

The amino group of the spiro moiety is the known interaction point of TSAO molecules with HIV-1 RT. When this amino group was removed (as in compound **3**), the molecule unexpectedly showed significant activity against HIV-1 replication. Compound **3** is unique among all the TSAO derivatives presently known as it shows an aberrant resistance spectrum. It still keeps HIV-1 specificity (characteristic for NNRTIs) but it does not consistently select for any of the classical NNRTI-specific mutations in the RT, such as E138K (characteristic for TSAO derivatives). The resistant viruses that emerged under the pressure of the deaminated TSAO (**3**), showed only substantial resistance to this compound but not to other NNRTIs including any other TSAO compounds that contain the amino group on the spiro moiety. Thus, removal of the amino group on TSAO molecules resulted in a new type of molecule with HIV-1 specificity but most likely with an altered molecular mode of interaction with RT or associated proteins than the classical NNRTIs and TSAO family of compounds. The resistance profile of this molecule may serve as a parameter to suggest a different way of binding to HIV-1 RT and opens interesting perspectives for a new rationale to design new drugs that make use of a different set of interactions for binding to the enzyme.

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