# Reaction of Rosmarinic Acid with Nitrite Ions in Acidic Conditions: Discovery of Nitro- and Dinitrorosmarinic Acids as New Anti-HIV-1 Agents

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Rosmarinic acid was reacted with nitrite ions under acidic conditions to give 6′-nitro- and 6′,6″-dinitrorosmarinic acids according to the reaction time. Both compounds were active as HIV-1 integrase inhibitors at the submicromolar level. They also inhibited the viral replication in MT-4 cells with modest and similar selectivity indexes. The nitration of rosmarinic acid strongly improves the anti-integrase inhibition and the antiviral activity without increasing the cellular toxicity.

## Introduction

Rosmarinic acid is an active component of many culinary plants (sage, rosemary, mint, melissa, thyme, prunella, origan, sweet basil, etc.) mostly responsible for antiinfective, anti-inflammatory, and antioxidative activity of these herbs.[1] It has also been suggested that rosmarinic acid may have inhibitory effects in Alzheimer amyloid-$\beta$ peptide[2,3] and Parkinson $\alpha$-synuclein aggregation[4,5] and interesting properties in the field of cancer as Fyn kinase inhibitor[6] and as apoptosis of Jurkat and peripheral T-cells inducer.[7] It is also known as an aldose reductase inhibitor[8] (an enzyme implicated in type II diabetes) and possesses remarkable antioxidant properties as reactive oxygen species scavenger and lipid peroxidation inhibitor.[9]

Among 51 samples from 46 herb species, 45 showed significant inhibitory effects against HIV-1 induced cytopathogenicity in MT-4 cells. Particularly, *Melissa officinalis*, *Mentha*, and *Prunella vulgaris* extracts, where rosmarinic acid is the major phenolic component, showed potent anti-HIV-1 activity targeting reverse transcription step.[10,11] Earlier, rosmarinic acid was found to inhibit 3′-processing and strand transfer activities of HIV-1 integrase (IN[a]) in the presence of $Mn^{2+}$.[12] By use of a multiplate integration procedure, aqueous and ethanolic extracts of 50 Thai plants were screened for their inhibitory activity against HIV-1 integrase.[13] Rosmarinic acid, its methyl ester, and its calcium and magnesium salts were isolated from the ethanolic extract of *Coleus parvifolius* Benth. The calcium and magnesium rosmarinates were found to be 5 times more active than rosmarinic acid against IN activity. The same salts isolated from water extract of *Cordia spinescens* were found to be potent inhibitors of reverse transcriptase[14] but inactive against protease. Rosmarinic acid also directly inhibited reverse transcription ($IC_{50}$ between 100 and 200 $\mu$M) and affected distinct phases of early natural endogenous reverse transcription.[15]

One of the major exogenous sources of nitrosating species is represented by nitrite ions, which are present in high levels in human saliva (50–200 $\mu$M).[16] In the acidic environment of the stomach, nitrite ions are converted to nitrous acid that can react with catechols to give nitro derivatives.[17,18] The concentration of rosmarinic acid in plant infusion can reach millimolar levels,[19] and therefore, a reaction between rosmarinic acid and nitrite ions under acidic conditions in the stomach is not unrealistic. We therefore underwent to study such reaction and to isolate and purify the products.

Our convergent interests for antioxidant (reactivity toward reactive nitrogen species) and anti-integrase properties of polyphenols led us to evaluate their antiviral properties. Fortunately, we found a remarkable improvement of the anti-IN and antiviral activities of these products versus rosmarinic acid. In the present paper, we report the synthesis, the anti-integrase activities, and the antiviral properties of 6′-nitro and 6′,6″-dinitrorosmarinic acids. Finally magnesium-chelating properties of test compounds were investigated by UV–vis spectroscopy.

## Results and Discussion

**Chemistry.** The reaction of caffeic acid derivatives with nitrite ions under conditions simulating those within the stomach is well-documented, and nitration of the aromatic ring is known to occur only on caffeic esters.[20,21] Applying the same conditions to rosmarinic acid **1** afforded selectively mono or dinitro derivatives depending on the reaction time. When the reaction medium is saturated after 5 min by the addition of sodium chloride and immediately extracted by ethyl acetate, the 6′-nitrorosmarinic acid **2** can be easily isolated in 15% yield (Scheme 1). A reaction time of 10 min allowed us to isolate the 6′,6″-dinitrorosmarinic acid **3** in 25% yield. In both cases, mono- and dinitrorosmarinic acids are the main products but the yields dramatically decreased because of difficulty in purification (crystallization in water). The positions of the nitration on the aromatic ring can be easily determined from the aromatic portion of the $^1$H NMR spectra. The presence of two and four singlets for mono- and dinitrorosmarinic acid, respectively, attested that the remaining hydrogen atoms were

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[a] Abbreviations: BSA, bovine serum albumin; CPE, cytopathic effect; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; HIV, human immunodeficiency virus; EI, electronic impact; ESI, electrospray ionization; IN, integrase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PEG, polyethylene glycol.

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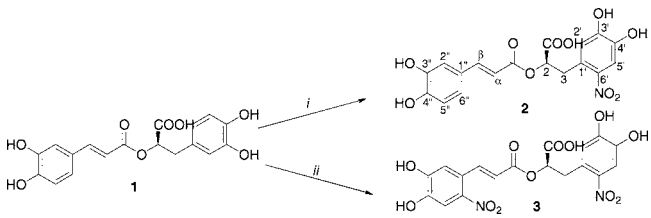
**Scheme 1.** Synthesis of Compounds **2** and **3**[a]



[a] Reagents and conditions: (i) $NaNO_2$, acetate buffer (0.2 M AcOH/0.2 M AcONa), room temperature, 5 min; (ii) $NaNO_2$, acetate buffer (0.2 M AcOH/0.2 M AcONa), room temperature, 10 min.

in the para position. The catechol where the nitration took place in **2** was determined by comparison of the $^{13}C$ NMR spectra of mononitrorosmarinic acid and rosmarinic acid. The $^{13}C$ NMR signals of the caffeic acid moiety are not modified by the mononitration, whereas a high-field shift is observed for Cα and C$\beta$ signals after dinitration. Expectedly the catechol ring substituted by an alkyl group (3,4-dihydroxyphenyllactic acid part of rosmarinic acid) is the most reactive toward the aromatic electrophilic substitution, allowing us to control the mono- and the dinitration with the reaction time.

**Biology.** 6′-Nitro and 6′,6″-dinitrorosmarinic acids **2** and **3** were tested in IN inhibition assays, which have been recently reviewed.[22,23] The overall IN enzymatic activity has been evaluated using two different protocols in the presence (protocol 1, Table 1 column 2) and in the absence (protocols 1 and 2, Table 1, columns 3 and 4) of bovine serum albumin (BSA) with magnesium ions as cofactor. The 3′-processing and strand transfer reaction inhibition were evaluated using protocol 1 (Table 1, columns 5 and 6). Antiviral properties and cytotoxicity were evaluated in the viral replication in MT-4 cells (Table 1, columns 8 and 9, respectively). Comparison of the overall integrase inhibitions in the absence of BSA using two different protocols of purification of the enzyme and two reaction media shows that our compounds are very sensitive to the conditions of the assays. But the most dramatic differences are obtained in the presence of BSA. This is probably due to the formation of complexes between BSA and the polyphenolic drugs.[24] In all the pharmacological assays, 6′,6″-dinitrorosmarinic acid **3** is always more active than 6′-nitrorosmarinic acid **2** and rosmarinic acid **1**, indicating that the nitration of the caffeic acid moiety allows for better anti-IN and anti-HIV activities. **3** presents a pronounced selectivity toward the strand transfer reaction (see column 7 of Table 1) and therefore was found to have the most powerful antiviral activity. Whereas **1** had weak antiviral properties limited by an important cytotoxicity, **2** and **3** were found to present noticeable antiviral activities in the MT-4 cells assays with modest therapeutic indexes of about ∼4. Nevertheless one must recognize that these antiviral activities remain modest in comparison to those of the naphtyridine reference compound, L-870,810. The IN assays on the overall reaction in the presence of BSA seem to be the most relevant ones compared to MT-4 cell assays.

**Physicochemical Studies.** In the overall integrase inhibition experiments, $IC_{50}$ values obtained in presence of BSA were considerably higher than those in absence of BSA, suggesting the possible formation of complexes between BSA and the polyphenolic drugs,[24] which could drastically decrease the catechol availability and therefore the IN inhibitory activities of the compounds. This was confirmed by a study of the interaction of rosmarinic derivatives with BSA by a fluorescence-based method.[25] All the compounds quenched significantly BSA tryptophan fluorescence, and quenching constants ($K_{SV}$) were determined using the Stern−Volmer equation to provide a measure of the binding affinity between the compounds and BSA (Figure 1). The binding affinities ranked in the following order: 6′-nitrorosmarinic acid **2** ($K_{SV} = 17460 \times 10^3\ M^{-1}$) > 6′,6″-dinitrorosmarinic acid **3** ($K_{SV} = 3300 \times 10^3\ M^{-1}$) > rosmarinic acid **1** ($K_{SV} = 20 \times 10^3\ M^{-1}$). The best binding affinities were obtained for the nitrated species, when compared to rosmarinic acid.

The biological studies also seemed to show an influence of the nitro groups on the anti-HIV and particularly on the anti-IN properties of the rosmarinic derivatives. This led us to investigate their magnesium-chelating capacities. UV–vis spectra of compounds were not affected by the addition of $MgCl_2$. This was not the case for $Mg(OAc)_2$. Figure 2 shows a slight modification of the UV–vis spectrum of rosmarinic acid in the presence of magnesium acetate. In contrast, in the same conditions, UV–vis spectra of the nitrated species were sharply modified with large hypochromic effects at 248 and 334 nm for **2** (Figure 3), a large hypochromic effect at 280 nm, and the simultaneous apparition of a new peak at 322 nm for **3** (Figure 4). Since the solutions took a slight orange color upon addition of $Mg(OAc)_2$, the formation of phenolates was very probable. Spectra of the fully deprotonated compounds in the presence of a large excess of NaOH were investigated. They were completely different (Figure 2 and Supporting Information) and exhibited a large peak around 385 nm (**1**, **2**) and 430 nm (**3**). In the presence of NaOAc, the spectra of all compounds were slightly modified (Supporting Information). All these results undoubtedly attest that the weak base acetate partially deprotonates the nitrated compounds, which leads to the formation of a complex with the magnesium ion. This situation was not encountered for rosmarinic acid. Thus, the nitration favorably affects the chelating properties of the rosmarinic derivatives, and this supports the best anti-IN activity obtained for the dinitrated species **3**.

## Conclusion

In the present paper, we have found that rosmarinic acid may readily react with nitrite ions under acidic conditions mimicking acidity in gastric juice. Two new nitrorosmarinic acids were isolated and revealed remarkable activities against HIV-1 integrase and antiviral properties. The anti-IN activities of **2** and **3** are expected to be due to their $Mg^{2+}$-chelating properties. The antiviral potency of infusion of rosmarinic acid-rich plants (such as sage) has to be evaluated in order to propose an alternative (or a complementation) to the classical antiretroviral therapy.

Since the concentrations of nitrite ions in stomach may reach millimolar levels, nitrorosmarinic acids may be considered as possible metabolites of rosmarinic acid. The great number of biological properties of rosmarinic acid indicates that the properties of its nitro derivatives have to be checked, particularly in the field of diabetes, cancer, and neurodegenerative diseases. The nitration of rosmarinic acid strongly affects the binding properties to BSA. Compounds **2** and **3** will be therefore tested in the inhibition of A$\beta$, α-synuclein, and $\tau$ filament formation.

Since the discovery of diketoacids,[26] nitrorosmarinic acids constitute the first new family of selective strand transfer inhibitors with antiviral properties. Two issues have to be addressed: (i) the caffeic and lactic parts of **3** must be synthesized separately and tested in order to define the contribution of these potential pharmacophores to the anti-IN and antiviral activities; (ii) the therapeutic index and therefore the antiviral activities must be increased.

**Table 1.** Inhibition of HIV-1 IN Catalytic Activities, Antiviral Activity, and Cytotoxicity of Rosmarinic Acid **1**, 6′-Nitrorosmarinic Acid **2**, 6′,6″-Dinitrorosmarinic Acid **3**, and Reference Compound L-870,810

| | $IC_{50}$ ($\mu$M) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| compd | overall[a] + BSA[f] | overall[a] | overall[b] | 3′-P[a] | ST[a] | 3′P/ST[g] | $EC_{50}$[c] ($\mu$M) | $CC_{50}$[d] ($\mu$M) | SI[e] |
| **1** | 63.5 ± 15 | NT[h] | NT[h] | NT[h] | NT[h] | | >55 | 55 | |
| **2** | 54.7 ± 17.1 | 1.4 ± 1.5 | 0.026 ± 0.005 | >247 | 3.7 ± 1.9 | >67 | 39 ± 9 | 160 ± 18.5 | 4.0 |
| **3** | 20.8 ± 14.9 | 0.15 ± 0.09 | 0.030 ± 0.005 | 52.2 | 0.07 ± 0.02 | 746 | 12 ± 3 | 45.5 ± 4.5 | 3.8 |
| L-870,810 | NT[h] | 0.0005 ± 0.0003 | NT[h] | 0.12 ± 0.03 | 0.0025 ± 0.0007 | 48 | 0.0047 ± 0.0007 | 2.2 ± 0.2 | 457 |

[a] Concentration required to inhibit by 50% the in vitro integrase activity assays using protocol 1. [b] Concentration required to inhibit by 50% the in vitro integrase activity assays using protocol 2. [c] Effective concentration required to reduce HIV-1-induced cytopathic effect by 50% in MT-4 cells. [d] Cytotoxic concentration to reduce MT-4 cell viability by 50%. [e] Selectivity index: $CC_{50}/EC_{50}$ ratio. [f] Bovine serum albumin was added in the medium (0.1 mg/mL). [g] $IC_{50}$(3′-P)/$IC_{50}$(ST) ratio. [h] NT: not tested.

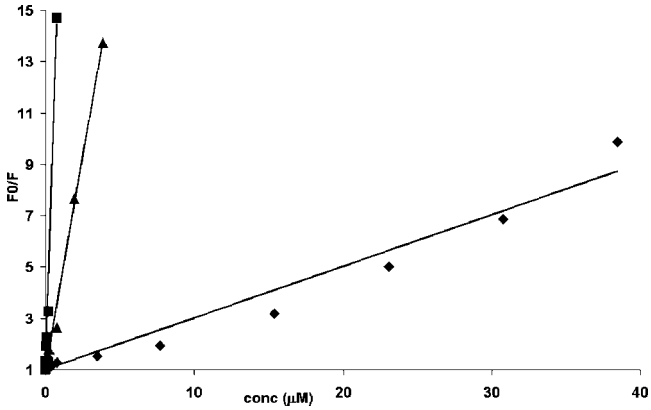



**Figure 1.** Stern−Volmer plots describing BSA tryptophan quenching at pH 7.4 caused by rosmarinic acid derivatives association: (■) 6′-nitrorosmarinic acid **2**, $y = 17.4x + 1$, $R^2 = 0.99$; (▲) 6′,6″-dinitrorosmarinic acid **3**, $y = 3.3x + 1$, $R^2 = 0.96$; (◆) rosmarinic acid **1**, $y = 0.02x + 1$, $R^2 = 0.96$.

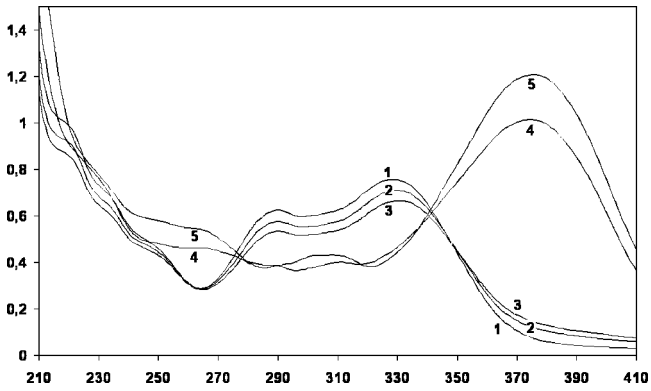


**Figure 2.** UV–visible spectra of rosmarinic acid **1** (50 $\mu$M in ethanol, **1**) in presence of 50 $\mu$M $Mg(OAc)_2$ (**2**), 100 $\mu$M $Mg(OAc)_2$ (**3**), 333 $\mu$M NaOH (**4**), and 666 $\mu$M NaOH (**5**).

Whereas 6-nitrocaffeic acid can be easily obtained by demethylation of the commercially available 6-nitro-3,4-dimethoxycinnamic acid using boron tribromide according to a previously reported method,[27] attempts to obtain and isolate the 6-nitro-3,4-dihydroxyphenyllactic acid were unsuccessful because of an instability of the formed nitrated species occurring during column chromatography.

## Experimental Section

**Chemistry. General Details.** All reagents and solvents were purchased from Aldrich-Chimie (Saint-Quentin-Fallavier, France) of ACS reagent grade and were used as provided. TLC analyses were performed on plastic sheets precoated with silica gel 60F254 (Merck). $SiO_2$, 200–400 mesh (Merck), was used for column chromatography. NMR spectra were obtained on an AC 200 Bruker

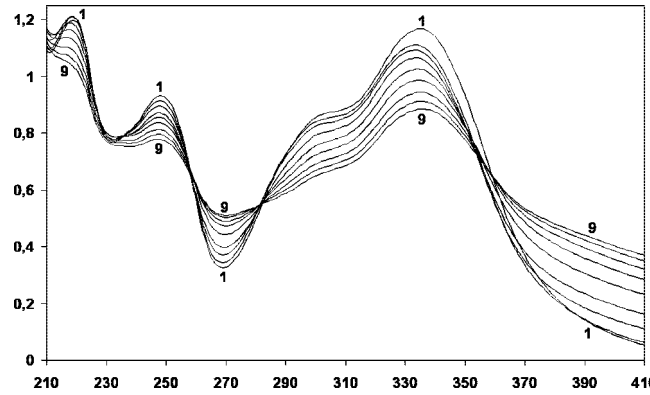


**Figure 3.** UV–visible spectra of 6′-nitrorosmarinic acid **2** (50 $\mu$M in ethanol, **1**) in presence of $Mg(OAc)_2$ (16.67 $\mu$M **2**, 33.33 $\mu$M **3**, 50.00 $\mu$M **4**, 66.66 $\mu$M **5**, 83.33 $\mu$M **6**, 100.00 $\mu$M **7**, 116.67 $\mu$M **8**, 133.33 $\mu$M **9**).

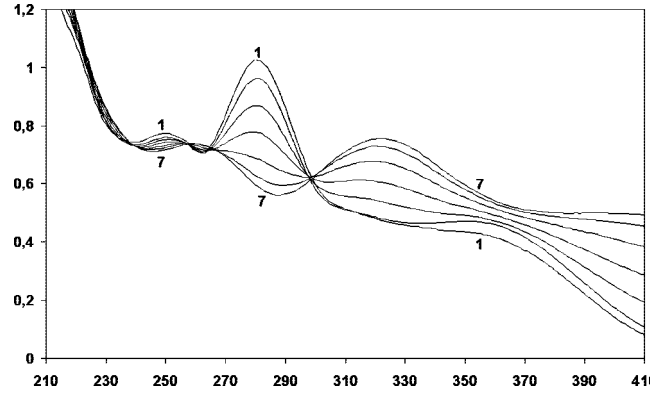


**Figure 4.** UV–visible spectra of 6′,6″-dinitrorosmarinic acid **3** (50 $\mu$M in ethanol, **1**) in presence of $Mg(OAc)_2$ (16.67 $\mu$M **2**, 33.33 $\mu$M **3**, 50.00 $\mu$M **4**, 66.66 $\mu$M **5**, 83.33 $\mu$M **6**, 100.00 $\mu$M **7**).

spectrometer in the appropriate solvent with TMS as internal reference. Melting points were obtained on a Reichert Thermopan melting point apparatus equipped with a microscope and are uncorrected. Mass spectra were recorded on a Thermo-Finnigan PolarisQ mass spectrometer (70 eV, electron impact). HRMS were obtained on an Apex Qe 9.4 T Bruker Daltonics spectrometer. Elemental analyses were performed by CNRS Laboratories (Vernaison).

**Synthesis of 2 and 3.** To a solution of 544 mg (7.9 mmol) of sodium nitrite in 50 mL of acetate buffer (0.2 M AcOH/0.2 M AcONa) was added 360 mg (1.0 mmol) of rosmarinic acid. After 5 min (**2**) or 10 min (**3**) the solution was saturated with sodium chloride and extracted with AcOEt (3 × 50 mL). The combined organic layers were dried over $Na_2SO_4$ and concentrated in vacuo. The crude product was crystallized in water (two times for **2**) to give **3** as an orange powder and **2** as a yellow powder.

**2:** $\alpha_D$ (MeOH) +61.9 ° (*c* 0.17 g/L); mp 168 °C; $^1$H NMR (acetone-$d_6$) 3.40 (dd, 1H, $^2J = 14.1$ Hz, $^3J = 9.4$ Hz, H3), 3.71

(dd, 1H, $^2J$ = 14.1 Hz, $^3J$ = 4.2 Hz, H3), 5.38 (dd, 1H, $^3J$ = 9.4 Hz, $^3J$ = 4.2 Hz, H2), 6.26 (d, 1H, $^3J$ = 15.8 Hz, Hα), 6.89 (d, 1H, $^3J$ = 8.2 Hz, H5″), 7.01 (s, 1H, H2′), 7.06 (dd, 1H, $^3J$ = 8.2 Hz, $^4J$ = 2.0 Hz, H6″), 7.18 (d, 1H, $^4J$ = 2.0 Hz, H2″), 7.55 (d, 1H, $^3J$ = 15.8 Hz, Hβ), 7.61 (s, 1H, H5′); $^{13}$C NMR 171.2 (COOH), 166.7 (C═O), 151.2 (C3′), 149.1 (C4″), 146.8 (C3″), 146.4 (Cβ), 145.2 (C4′), 142.3 (C6′), 127.5 (C1″), 126.0 (C1′), 122.8 (C6″), 120.0 (C2′), 116.5 (C5″), 115.3 (Cα), 114.7 (C2″), 113.3 (C5′), 72.7 (C2), 35.1 (C3); MS (EI) 404 ($M^{+0}$ − 1, 57%), 242 (lactic fragment, 100%), 180 (caffeic fragment, 50%); HRMS (nano-ESI-FT-ICR)(negative mode) calcd for $C_{18}H_{15}NO_{10}$ 404.062 32, found 404.062 33. Anal. ($C_{18}H_{15}NO_{10}$) C, H, N.

**3:** $\alpha_D$ (MeOH) +25.3 ° (*c* 0.21 g/L); mp 45–46 °C; $^1$H NMR (acetone-$d_6$) 3.36 (dd, 1H, $^2J$ = 13.9 Hz, $^3J$ = 9.0 Hz, H3), 3.69 (dd, 1H, $^2J$ = 13.9 Hz, $^3J$ = 4.3 Hz, H3), 5.40 (dd, 1H, $^3J$ = 9.0 Hz, $^3J$ = 4.3 Hz, H2), 6.33 (d, 1H, $^3J$ = 15.9 Hz, Hα), 6.98 (s, 1H, H2′), 7.23 (s, 1H, H2″), 7.58 (s, 1H, H5′), 7.61 (s, 1H, H5″), 8.12 (d, 1H, $^3J$ = 15.9 Hz, Hβ); $^{13}$C NMR 170.9 (COOH), 166.0 (C═O), 151.5 (C3″), 151.1 (C3′), 147.8 (C4″), 145.1 (C4′), 142.8 (Cβ), 142.6 (C6″) 142.2 (C6′), 125.8 (C1′), 124.4 (C1″), 120.3 (Cα), 120.0 (C2′), 115.4 (C2″), 113.3 (C5′), 113.1 (C5″), 73.0 (C2), 35.1 (C3); MS (EI) 449 ($M^{+0}$ − 1, 45%), 242 (lactic fragment, 100%), 226 (caffeic fragment, 52%); HRMS (nano-ESI-FT-ICR) (negative mode) calcd for $C_{18}H_{14}N_2O_{12}$ 449.047 40, found 449.043 22. Anal. ($C_{18}H_{14}N_2O_{12}$) C, H, N.

**Integrase Inhibition (Protocol 1).** To determine the susceptibility of the HIV-1 integrase enzyme toward different compounds, we used an enzyme-linked immunosorbent assay. This assay uses an oligonucleotide substrate of which one oligo (5′-ACTGCTAGAGATTTTCCACACTGACTAAAAGGGTC-3′) is labeled with biotin on the 3′ end and the other oligo is labeled with digoxigenin at the 5′ end. For the overall integration assay the second 5′-digoxigenin labeled oligo is 5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA**GT**-3′. The integrase was diluted in 750 mM NaCl, 10 mM Tris, pH 7.6, 10% glycerol, and 1 mM β-mercaptoethanol. To perform the reaction, 4 μL of diluted integrase (corresponds to 1.6 μM WT integrase[22]) and 4 μL of annealed oligos (7 nM) were added to a final reaction volume of 40 μL containing 10 mM $MgCl_2$, 5 mM DTT, 20 mM HEPES, pH 7.5, 5% PEG, and 15% DMSO. The reaction was carried out for 1 h at 37 °C. These reactions were followed by an immunosorbent assay on avidin coated plates.[28]

**Integrase Inhibition (Protocol 2).** Oligonucleotides were purchased from Eurogentec and further purified on 18% acrylamide/urea denaturing gel: U5B, GTGTGGAAAATCTCTAGCA; U5A, 5′-ACTGCTAGAGATTTTCCACAC. Wild-type HIV-1 integrase was purified as described previously.[29] The assay was performed in a reaction volume of 20 μL containing 0.025 pmol of labeled U5A/U5B double-stranded DNA substrate and 1 pmol of integrase in buffer [20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 25 mM NaCl, and 1 mM DTT]. Products were separated on an 18% acrylamide/urea denaturing gel and quantified on a phosphoimager using ImageQuant software (Amersham Pharmacia Biotech).

**In Vitro Anti-HIV and Drug Susceptibility Assays.** The inhibitory effect of antiviral drugs on the HIV-1-induced cytopathic effect (CPE) in human lymphocyte MT-4 cell culture was determined by the MT-4/MTT-assay.[30] This assay is based on the reduction of the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan derivative, which can be measured spectrophotometrically. The 50% cell culture infective dose ($CCID_{50}$) of the HIV-1 ($III_B$) strain was determined by titration of the virus stock using MT-4 cells. For the drug susceptibility assays MT-4 cells were infected with 100–300 $CCID_{50}$ of the virus stock in the presence of 5-fold serial dilutions of the antiviral drugs. The concentrations of various compounds achieving 50% protection against the CPE of the different HIV strains, which are defined as $EC_{50}$, were determined. In parallel the 50% cytotoxic concentration ($CC_{50}$) was determined.

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**Supporting Information Available:** Additional chemical and biological information. This material is available free of charge via the Internet at http://pubs.acs.org.

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