# Synthesis, *in Vitro* Antiviral Evaluation, and Stability Studies of Bis(*S*-acyl-2-thioethyl) Ester Derivatives of 9-[2-(Phosphonomethoxy)ethyl]adenine (PMEA) as Potential PMEA Prodrugs with Improved Oral Bioavailability†

Samira Benzaria,‡,▽ Hélène Pélicano,‡ Richard Johnson,‡ Georges Maury,‡ Jean-Louis Imbach,‡ Anne-Marie Aubertin,§ Georges Obert,§,‖ and Gilles Gosselin*,‡

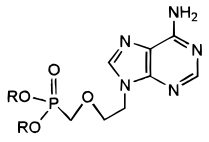
*Laboratoire de Chimie Bioorganique, case courrier 008, UMR CNRS-USTL 5625, Université Montpellier II, Sciences et Techniques du Languedoc, Place Eugène Bataillon, 34095 Montpellier Cédex 5, France, and Institut de Virologie de la Faculté de Médecine de Strasbourg, Unité INSERM 74, 3 Rue Koeberlé, 67000 Strasbourg, France*

*Received April 17, 1996*⊗

A new series of hitherto unknown 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) phosphonodiester derivatives incorporating carboxyesterase-labile *S*-acyl-2-thioethyl (SATE) moieties as transient phosphonate-protecting groups was prepared in an attempt to increase the oral bioavailability of the antiviral agent PMEA. We report here a direct comparison of the *in vitro* anti-HIV and anti-HSV activities as well as the *in vitro* stability between the bis(SATE) derivatives and the already known PMEA prodrugs, namely, bis[(pivaloyloxy)methyl (POM)]- and bis[dithiodiethyl (DTE)]PMEA. All of the compounds tested showed an enhanced *in vitro* antiviral activity compared to the parent PMEA. The bis(POM)- and bis(tBu-SATE)PMEA derivatives were the most effective. However, striking differences between these two compounds were found during the stability studies. In particular the bis(tBu-SATE)PMEA was found to be more stable than bis(POM)PMEA in human gastric juice and human serum, suggesting it could be considered as a promising candidate for further *in vivo* development.

## Introduction

9-[2-(Phosphonomethoxy)ethyl]adenine [PMEA (**1**); Figure 1] has demonstrated broad spectrum antiviral activity against human immunodeficiency virus (HIV) and other retroviruses.[1,2] PMEA is also active against various DNA viruses, including hepatitis B virus, herpes simplex virus (HSV), cytomegalovirus, and Epstein-Barr virus.[1,2] In addition to *in vitro* activity, PMEA has demonstrated *in vivo* efficacy when administrated intravenously, intraperitoneally, or intramuscularly.[1,2] Thus, PMEA is of interest both as a potential antiretroviral drug for HIV infections and for the treatment of some of the opportunistic infections associated with AIDS. It has undergone phase I/II clinical trials where it exhibited activity against HIV *in vivo*.[3] However, the potential therapeutic use of PMEA could be limited by its poor oral bioavailability, which has been reported to be <1% in monkeys[4] and 7.8−11% in rats.[5,6] The poor oral bioavailability is due to the phosphonate negative charges that are present in PMEA at physiological pH. Therefore, the concept of temporarily masking these charges with neutral substituents to form more lipophilic derivatives capable of crossing the gastrointestinal wall and reverting back to the parent PMEA in plasma was attempted.



| | | R = |
|---|---|---|
| **1** | PMEA | H |
| **2** | bis(DTE)PMEA | $HO(CH_2)_2S\text{-}S(CH_2)_2$ |
| **3** | bis(POM)PMEA | $(CH_3)_3C\text{-}C(O)\text{-}O\text{-}CH_2$ |
| **4a** | bis(Me-SATE)PMEA | $CH_3\text{-}C(O)\text{-}S(CH_2)_2$ |
| **4b** | bis(iPr-SATE)PMEA | $(CH_3)_2CH\text{-}C(O)\text{-}S(CH_2)_2$ |
| **4c** | bis(tBu-SATE)PMEA | $(CH_3)_3C\text{-}C(O)\text{-}S(CH_2)_2$ |
| **4d** | bis(Ph-SATE)PMEA | $C_6H_5\text{-}C(O)\text{-}S(CH_2)_2$ |

**Figure 1.** Structure of PMEA and its phosphonodiester derivatives studied.

Compared to prodrugs of nucleoside monophosphates,[7−9] relatively few examples of PMEA derivatives have so far been reported in the literature. One PMEA prodrug has been synthesized by linking a synthetic polymer bearing mannosylated residues to PMEA.[10] More recently, several derivatives, including hydrogenophosphinate,[11] mono- or bis(phosphonoamidate),[6] and mono- or bis(phosphonoester)[6,12−16] functionalities, have been prepared as potential prodrugs of PMEA. The best results were obtained when PMEA was esterified with two transient, enzyme-labile phosphonate-protecting groups, which were later removed by a specific enzymatic system. For instance, we have previously reported[16] that the bis[dithiodiethyl (DTE)]PMEA (**2**;

† This work is taken, in part, from the Ph.D. Dissertation of S. Benzaria, Université de Montpellier II, June 23, 1995. It has been presented in preliminary form at the Eleventh International Round Table on Nucleosides, Nucleotides, and their Biological Applications, Sept. 7−11, 1994, Leuven, Belgium. For the proceedings, see: Benzaria, S.; Gosselin, G.; Pélicano, H.; Maury, G.; Aubertin, A.-M.; Obert, G.; Kirn, A.; Imbach, J.-L. New Prodrugs of 9-(2-phosphonomethoxyethyl)adenine [PMEA]: synthesis and stability studies. *Nucleosides Nucleotides* **1995**, *14*, 563−565.

* Author for correspondence. Tel: (33) 4-67-14-38-55. Fax: (33) 4-67-04-20-29. E-mail: gosselin@univ-montp2.fr.

‡ Université Montpellier II.

§ Unite INSERM 74.

▽ Present address: Laboratory of Medicinal Chemistry, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

‖ Deceased on June 20, 1995.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

S0022-2623(96)00289-0 CCC: $12.00 © 1996 American Chemical Society

**Scheme 1.** Synthesis of Bis(POM)PMEA (**3**)

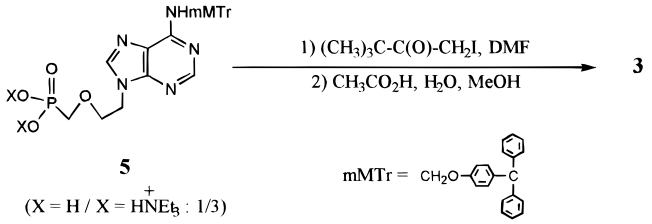



Figure 1), whose activation is mediated by reductases, showed an increase in the *in vitro* anti-HIV activity of PMEA. More importantly, (acyloxy)alkyl groups have also been studied as carboxyesterase-mediated bioreversible groups,[6,17] and the bis[(pivaloyloxy)methyl (POM)]PMEA (**3**; Figure 1) was examined as an efficient PMEA prodrug.[6,15] Antiretroviral activity and pharmacokinetics of orally administrated bis(POM)PMEA in mice have been reported,[18−21] and recent studies have demonstrated that this compound improved PMEA oral bioavailability 2-fold in rats[6] and 5-fold in monkeys,[22] despite its low aqueous solubility and stability.[22,23] Phase I/II clinical trials are currently ongoing to evaluate its efficacy following oral administration in AIDS patients.[24,25]

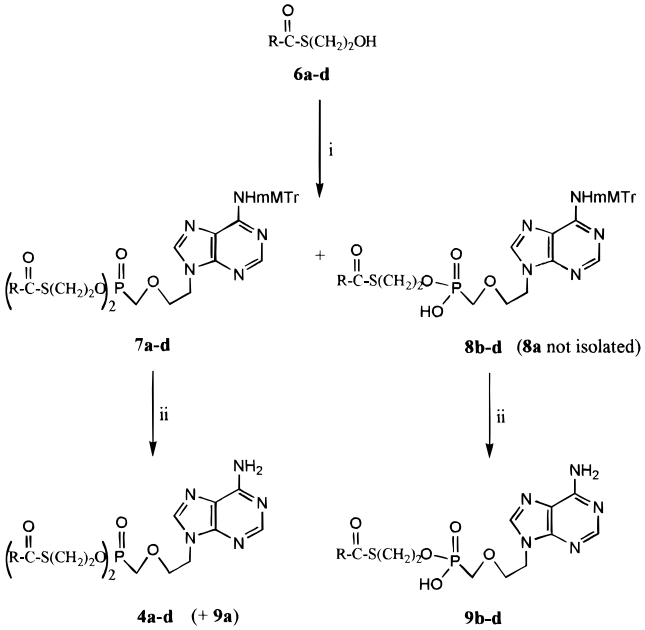
In our laboratory, we have independently developed the carboxyesterase-labile *S*-acylthioethyl (SATE) group for the transient protection of nucleoside monophosphates.[26−30] Based on the favorable results obtained with the SATE protection of various drugs, in terms of *in vitro* anti-HIV activity and preliminary stability, we decided to prepare and study a series of PMEA bis(SATE) derivatives (**4a**−**d**; Figure 1). We report herein the synthesis of these new PMEA prodrugs, as well as their *in vitro* anti-HIV and anti-HSV activities, and their chemical and enzymatic stabilities compared to the two known PMEA prodrugs, bis(DTE)- and bis(POM)-PMEA.

## Results and Discussion

**Chemistry.** PMEA (**1**) was prepared according to Holy's procedure[31] with some modifications[32−35] that improved the overall yield and facilitated the execution of some steps, which were performed more rapidly, or sometimes without any preliminary purification. The synthesis of bis(DTE)PMEA (**2**) was effected as previously reported[16] and involved the condensation of a hydroxylated derivative with a base-protected PMEA. Concerning the synthesis of the bis(POM)PMEA (**3**), we did not use the published procedure,[6,15] which consisted of reacting PMEA with chloromethyl pivaloate in the presence of the hindered base *N*,*N*′-dicyclohexylmorpholinecarboxamidine, to allow solubilization in DMF. We opted to overcome the solubility problem by using the $N^6$-monomethoxytritylated derivative (**5**)[16] of PMEA (Scheme 1), which is soluble in organic solvents. Thus, reaction of **5**[16] with iodomethyl pivaloate,[36] followed by treatment with acid, gave a 18% yield of the bis(POM)-PMEA (**3**).

For the synthesis of the title compounds **4a**−**d**, we chose an approach similar to that developed in the case of bis(DTE)PMEA. The hydroxythioester precursors **6a**−**d**, which were prepared by reacting 2-iodoethanol with the corresponding thio acid,[26] were condensed with **5** in pyridine in the presence of 1-mesitylene-2-sulfonyl-3-nitro-1,2,4-triazole (MSNT) to afford the corresponding protected phosphonodiesters **7a**−**d** in yields of 58−86% (Scheme 2). The monoesters **8b**−**d** were also isolated as byproducts. Treatment of **7a**−**d** with acetic acid provided the target PMEA prodrugs **4a**−**d** as oils in yields of 75−82% after purification by silica gel column chromatography. It is noteworthy that derivatives **4a**−**c** could be crystallized, unlike **4d**, bis(DTE)-PMEA (**2**), and bis(POM) PMEA (**3**). Furthermore, authentic samples of the corresponding mono(SATE) derivatives (**9a**−**d**) of PMEA were required as standards to identify the decomposition products from the stability studies in different media. The monoesters **9b**−**d** were prepared by acid treatment of **8b**−**d** and purified on Dowex resin (acetate form).[31] The mono(Me-SATE)-PMEA (**9a**) was obtained as a byproduct (3%) during the detritylation of **7a**.

**Scheme 2.** Synthesis of Bis(SATE)PMEA Prodrugs **4a**−**d** and Their Corresponding Monoesters **9a**−**d**[a]




[a](i) **5**, MSNT, pyridine; (ii) $CH_3CO_2H/H_2O$/MeOH. **a**, R = Me; **b**, R = iPr; **c**, R = tBu; **d**, R = Ph.

**Table 1.** Antiviral Activity of the Phosphonodiester Derivatives **2**−**4** Compared to That of PMEA (**1**) in Two Cell Lines Infected with HIV-1[a]

| | MT-4 | | CEM-SS | |
|---|---|---|---|---|
| compd | $EC_{50}$[b] | $CC_{50}$[c] | $EC_{50}$[b] | $CC_{50}$[c] |
| **1** | >10 | >10 | 0.42 | >10 |
| **2** | 6.9 | 50 | 2 | 67 |
| **3** | 0.08 | 0.74 | 0.04 | 4.4 |
| **4a** | 1.1 | 7.6 | 0.1 | 23 |
| **4b** | 0.51 | 3.8 | 0.09 | 13 |
| **4c** | 0.65 | 1.5 | 0.03 | 5.8 |
| **4d** | 0.41 | 2.4 | 0.05 | 3.9 |

[a] All data represent average values for at least three separate experiments. The variation of these results under standard operating procedures is below ±10%. [b] $EC_{50}$, 50% effective concentration (in $\mu$M) or concentration required to inhibit the replication of HIV-1 by 50%. [c] $CC_{50}$, 50% cytotoxic concentration (in $\mu$M) or concentration required to reduce the viability of uninfected cells by 50%.

**Antiviral Activity.** PMEA (**1**) and the phosphonodiester derivatives **2**, **3**, and **4a**−**d** were evaluated for their inhibitory effects on the replication of HIV-1 in two cell culture systems (Table 1). Under the assay conditions, all the tested prodrugs enhanced the *in vitro*

**Table 2.** Measured Partition Factors (between Octanol and Water) of the Phosphonodiesters **2**−**4** Compared to That of PMEA (**1**)

| | 1 | 2 | 3 | 4a | 4b | 4c | 4d |
|---|---|---|---|---|---|---|---|
| log $P^a$ | −4.11 (±0.03) | 0.21 (±0.01) | 2.48 (±0.01) | 0.32 (±0.05) | 2.31 (±0.04) | 3.41 (±0.01) | 3.93 (±0.01) |

[a]The determination of partition coefficients was carried out by HPLC by using a microscale method adapted from that described previously for various nucleoside analogues (Ford, H., Jr.; Merski, C. L.; Kelley, J. A. A rapid microscale method for the determination of partition coefficients by HPLC. *J. Liquid Chromatogr.* **1991**, *14*, 3365−3386).

anti-HIV activity of PMEA. The lower $EC_{50}$ values for the neutral phosphonodiester derivatives **2**−**4**, compared to that of the parent molecule **1**, can be attributed to an increase in cellular uptake followed by intracellular release of PMEA. The prodrugs appear to be lipophilic enough to cross the cellular membrane through simple diffusion, unlike the PMEA, which seems to require an endocytosis-like process,[37] or the ATP membrane receptor[1] for intracellular transport. The bis(POM)PMEA (**3**) and the bis(tBu-SATE)PMEA (**4c**) were found to be the most potent antiviral agents. The DTE derivative **2** showed limited efficacy, presumably due to its lower lipophilicity compared to the other diesters, as reflected by their measured log *P* values (Table 2).

We can tentatively conclude from the results presented in Tables 1 and 2 that lipophilicity is not the only factor that influences the antiviral activity of these enzyme-labile prodrugs. For instance, compound **4d** is more lipophilic than **3**, but it is not more potent. The enzymatic system involved in prodrug activation and the prodrugs' relative stabilities are also likely to be crucial factors.

PMEA (**1**) and its phosphonodiester derivatives **2**−**4** were also evaluated against HSV-1 and -2 in two cell lines (Table 3). Again, compounds **3** and **4c** proved to be the most potent, with $EC_{50}$ values of 0.91 and 0.97 $\mu$M, respectively, in HSV-2-infected MRC-5 cells, compared to 33.5 $\mu$M for PMEA. The inhibiting capacity of the derivatives followed the order **3** > **4c** ∼ **4b** > **4d** > **4a** > **2** ∼ **1**.

It should be noted that every prodrug-related gain in antiviral potency was in all cases accompanied by a proportional enhancement of cytotoxicity, as reflected by the $CC_{50}$ values (Tables 1 and 3). Although it is possible that these toxic effects are due to the release of the phosphonate-protecting groups, we do not believe this to be the case. Indeed, we have recently shown that use of SATE groups as transient phosphate protections of 5'-mononucleotides does not induce any additive toxicity compared to the parent nucleoside (manuscript in preparation). A more likely explanation would be that the derivatization of PMEA increases not only its intracellular concentration but the consequent intracellular concentration of the phosphorylated anabolites, which are the active and cytotoxic forms of the drug.[1] Therefore, in the case of PMEA, it appears that the prodrug approach is not well-suited to improve the antiviral selectivity index (ratio $CC_{50}/EC_{50}$). Nevertheless, this strategy may prove useful to deliver the PMEA into plasma after oral administration, as already demonstrated for the bis(POM)PMEA.[6,22]

**Stability Studies.** For a designed oral prodrug to be effective, it must be resistant enough to any hydrolysis that might occur before it reaches the bloodstream, and it should be lipophilic enough to cross the gastrointestinal wall. Therefore, to test the usefulness of **2** and the newly synthesized bis(SATE) derivatives **4a**−**d**, it was necessary to study their chemical and enzymatic stabilities. The bis(POM) ester **3** was chosen as a reference compound for this evaluation because it is the most effective PMEA oral prodrug reported to date.[6,22]

In order to measure the relative chemical and enzymatic stabilities of the PMEA prodrugs, the decomposition pathways and kinetic data for compounds **2**, **3**, and **4a**−**d** (initial concentration $5 \times 10^{-5}$ M) were studied at 37 °C (i) in water, (ii) in pH 7.2 buffer, (iii) in RPMI 1640 containing 10% heat-inactivated fetal calf serum (culture medium), (iv) in RPMI 1640 alone, (v) in pH 2 buffer, (vi) in human gastric juice, and (vii) in human serum. These various media were thought to be valid *in vitro* models for the different types of degradation that may affect the prodrugs during oral administration. Crude aliquots of incubates were directly analyzed by using the recently described,[26] on-line HPLC cleaning method. The use of a reverse-phase (RP) precolumn (Guard-Pak, $\delta$-pak C18, Waters) in this technique allowed the elimination of proteins before the sample reached the RP analytical column. During this cleaning step, we used an ion-pairing reagent (tetrabutylammonium sulfate) in order to avoid the coelimination of the polar PMEA and monoester derivatives produced after degradation of the prodrugs in the various media.

We observed that all the prodrugs tested in water and in pH 7.2 buffer had the same decomposition pathway, giving rise to the corresponding monoesters. For **4a**−**d** the decomposition products were unambiguously identified by HPLC coinjection of the corresponding mono(SATE) derivatives **9a**−**d**. Due to the lack of authentic samples in the case of **2** and **3**, we made an extrapolation based on the retention times and UV spectra of the compounds formed. Table 4 shows that the bis(SATE) prodrugs **4a**−**d** were the most stable toward chemical hydrolysis at neutral (pH 7.2) or slightly acidic (milliQ water, pH 5.5) pH.

The comparable decomposition rates obtained for **4a**−**d** in water and at pH 7.2 suggest that the variation of the SATE chain does not influence the reactivity of this protecting group under the conditions of the assay. This result is in accordance with a hydrolysis mechanism involving nucleophilic attack at the α carbon of the phosphorous atom[38] furthest from the acyl thioester moiety (Scheme 3).

For the study in RPMI 1640, which represents a free-enzyme system but a nucleophile-enriched medium, only **2**, **3**, and **4a** were selected for testing. The bis(POM)-PMEA (**3**) was the least stable under these conditions (Table 5). It proved to be more sensitive to chemical hydrolysis than the bis(DTE) derivative **2** (half-life of 5 h for **3**, compared to >24 h for **2**), which was not apparent in simple media, such as water and pH 7.2 buffer. The corresponding monoesters were detected as single decomposition products for the three compounds tested.

In culture medium, we additionally observed PMEA formation (as ascertained by coinjection with authentic sample), presumably following the decomposition pathway: phosphonodiester derivative → phosphonomonoester → PMEA. We have recently published[26] the

**Table 3.** Anti-HSV-1 and -2 Activity of the Phosphonodiester Derivatives **2**−**4** Compared to That of PMEA (**1**) in Two Infected Cell Lines[a]

| | Vero | | | MRC-5 | | |
|---|---|---|---|---|---|---|
| | $EC_{50}$[b] | | | $EC_{50}$[b] | | |
| compd | HSV-1 | HSV-2 | $CC_{50}$[c] | HSV-1 | HSV-2 | $CC_{50}$[c] |
| **1** | 38.5 ± 6.9 | 32.7 ± 10.5 | >100 | 29.7 ± 7.6 | 33.5 ± 9.6 | >100 |
| **2** | 22.3 ± 7.4 | 27.4 ± 8.5 | >100 | 32.3 ± 8.3 | 26.5 ± 6.8 | >100 |
| **3** | 0.87 ± 0.62 | 1.15 ± 0.39 | 22.56 ± 8.4 | 1.23 ± 0.73 | 0.91 ± 0.32 | 15.7 ± 5.1 |
| **4a** | 14.2 ± 2.5 | 10.2 ± 3.1 | 126 ± 38 | 12.6 ± 4.6 | 8.9 ± 2.1 | 148 ± 27 |
| **4b** | 1.35 ± 0.45 | 1.14 ± 0.39 | 23 ± 11.4 | 1.69 ± 0.37 | 1.20 ± 0.54 | 30.2 ± 9.6 |
| **4c** | 1.07 ± 0.24 | 1.12 ± 0.39 | 36.8 ± 6.3 | 1.43 ± 0.28 | 0.97 ± 0.38 | 48.5 ± 8.7 |
| **4d** | 2.56 ± 0.89 | 2.89 ± 0.97 | 12.7 ± 1.8 | 3.48 ± 1.23 | 4.75 ± 2.1 | 18.7 ± 3.4 |

[a] All data represent average values for at least three different experiments. [b,c] See the corresponding footnotes in Table 1.

**Table 4.** Calculated Rate of Decomposition in Water (Milli-Q, pH 5.5) and at pH 7.2 (Ammonium Acetate Buffer, 0.02 M) for the Phosphonodiester Derivatives **2**−**4**

| compd | water | pH 7.2 |
|---|---|---|
| **2** | $t_{1/2}$ 3.9 days | 13.9%[b] |
| **3** | $t_{1/2}$ 3.7 days | 32.7%[c] |
| **4a** | 13.2%[a] | 4%[c] |
| **4b** | 8.9%[a] | 1.6%[c] |
| **4c** | 14.2%[a] | 3%[c] |
| **4d** | 10.2%[a] | 1.7%[c] |

[a] Percent of decomposition after 9 days of incubation. [b] Percent of decomposition after 26 h of incubation. [c] Percent of decomposition after 24 h of incubation.

**Scheme 3.** Proposed Hydrolysis Mechanism of PMEA Prodrugs **4a**−**d** in Water (Milli-Q, pH 5.5) and at pH 7.2 (Ammonium Buffer, 0.02 M)

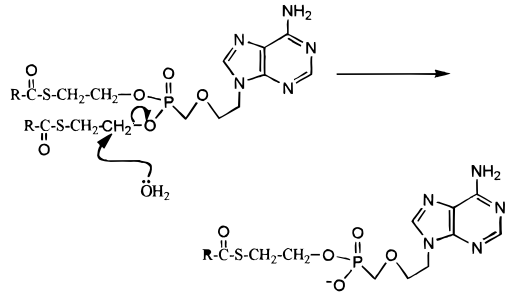



**Table 5.** Calculated Half-Lives of the Phosphonodiester Derivatives **2**−**4** and the Phosphonomonoesters **9a**−**d** in RPMI 1640 and in Culture Medium

| | $t_{1/2}$ | |
|---|---|---|
| compd | RPMI 1640 | culture medium |
| **2** | >24 h | 1.6 days |
| **3** | 5 h | 5 h |
| **4a** | >24 h | 3.7 h |
| **4b** | ND[a] | 8.3 h |
| **4c** | ND | 3.4 days |
| **4d** | ND | 25.7 h |
| **9a** | ND | 19.2 h |
| **9b** | ND | 1.2 days |
| **9c** | ND | 9.6 days |
| **9d** | ND | 2.7 days |

[a] ND, not determined.

proposed mechanisms for the decomposition of SATE prodrugs and the release of the parent mononucleotide in such culture media. It should be noted that we confirmed hereby the formation of the hypothesized intermediates corresponding to the mono(SATE) derivatives. The half-life of **4a** in culture medium was shorter than in RPMI alone (Table 5). This suggests that in the heat-inactivated serum added to RPMI there remains (i) carboxyesterase activity, which leads to a faster elimination of the first enzyme-labile SATE group, and (ii) phosphodiesterase activity, which leads to the elimination of the second SATE protecting group (we also report in Table 5 the half-life of the PMEA monoester derivatives **9a**−**d**). It is noteworthy that except for **4a**, all the SATE prodrugs proved to be more stable than bis(POM)PMEA (**3**), for which the measured half-life of 5 h compares favorably with the previously reported value.[22] This result suggests that the SATE thioester might be more stable toward carboxyesterase activity than a simple ester. The relative stability of **2** is quite surprising (half-life 1.6 days) and might be attributed to the lack of reductases in the medium or to the poor affinity of **2** toward the reductases. As Table 5 clearly points out, the bis(tBu-SATE)PMEA (**4c**) emerged as the most stable prodrug in media such as culture medium, which represents an appropriate model for neutral conditions where chemical and enzymatic hydrolysis can occur.

**Table 6.** Calculated Rate of Hydrolysis at pH 2 (Glycine/HCl Buffer) in Human Gastric Juice and Human Serum for the Phosphonodiester Derivatives **2**−**4**

| compd | pH 2 | human gastric juice[a] | human serum[b] |
|---|---|---|---|
| **2** | $t_{1/2}$ 4.4 days | $t_{1/2}$ 4.8 days | $t_{1/2}$ <5 min |
| **3** | $t_{1/2}$ 3.2 days | $t_{1/2}$ 2.4 days | $t_{1/2}$ <5 min |
| **4a** | 20.8%[c] | $t_{1/2}$ 5.3 days | $t_{1/2}$ <5 min |
| **4b** | 12.8%[c] | 25.7%[d] | $t_{1/2}$ 11 min |
| **4c** | 7.8%[c] | 11.1%[d] | $t_{1/2}$ 4 h |
| **4d** | 9%[c] | 5%[d] | $t_{1/2}$ 1.5 h |

[a] The human gastric juice (pH 1.3) [obtained from Dr J.-C. Cuber (Institut National de la Santé et de la Recherche Médicale, U-45, Lyon, France)] was centrifuged for 15 min at 3000 rpm at 4 °C. The corresponding compounds were dissolved in the filtered gastric juice up to a 50 $\mu$M concentration. After incubation at 37 °C, aliquots were periodically removed and directly analyzed by HPLC. [b] Human serum from healthy volunteers was a gift from the Centre Regional de Transfusion Sanguine (Montpellier, France). [c] Percent of decomposition after 7 days of incubation. [d] Percent of decomposition after 5 days of incubation.

For their acid stability, the PMEA prodrugs were evaluated not only in the usual pH 2 test buffer but also in human gastric juice (pH 1.3). The values reported in Table 6 show a good correlation for these two media, for which we observed the same decomposition pathway, consisting of the formation of the corresponding monoester derivative as the sole product for all the prodrugs tested. In each case, the SATE prodrugs **4a**−**d** proved to be more acid resistant than the already known PMEA prodrugs **2** and **3**. This last result leads us to suppose that **4a**−**d** could be more stable than **2** and **3** in the gastric environment following oral administration, which would increase their absorption. However, as reflected by their half-lives in human serum (Table 6), the two SATE prodrugs **4a**,**b**, as well as **2** and **3**, are likely to be hydrolyzed immediately to PMEA once in

circulation. On the other hand, the two other SATE prodrugs (**4c**,**d**) showed greater stability in human serum (half-life, respectively, of 4 and 1.5 h), which indicated that they could show *in vivo* bioavailability and biodistribution different from (and probably better than) the parent PMEA.

## Conclusion

The present results demonstrate that the bis(SATE)-PMEA prodrugs **4a**−**d** are chemically and enzymatically more stable than the already known bis(DTE)- and bis(POM)PMEA, **2** and **3**, respectively. This greater stability in neutral and acidic conditions, as well as in serum, indicates that the bis(SATE) derivatives might enhance PMEA oral bioavailability. Moreover, the crystalline form of **4a**−**c** might facilitate the formulation of oral forms of administration. Among the SATE series, the tBu-SATE derivative **4c** emerged as the most promising compound, combining an antiviral potency similar to that of the currently developed bis(POM)PMEA (**3**) with a markedly greater chemical and enzymatic stability. *In vivo* studies with **4c** are presently ongoing to determine its antiretroviral efficacy after oral administration vis-à-vis the bis(POM) derivative **3** and the parent compound PMEA (**1**).

## Experimental Section

**Chemical Synthesis.** Evaporation of solvents was carried out on a rotary evaporator under reduced pressure. Melting points were determined with a Gallenkamp MFB-595-010-M apparatus and are uncorrected. $^1$H NMR spectra were run at ambient temperature in $(CD_3)_2SO$ (DMSO-$d_6$) or DMSO-$d_6$ + $D_2O$ with a Bruker AC 250 spectrometer. Chemical shifts are given in $\delta$ values, $(CD_3)(CD_2H)SO$ being set at $\delta_H$ 2.49 as a reference. Deuterium exchange and decoupling experiments were performed in order to confirm proton assignments. $^{31}$P NMR spectra were recorded at ambient temperature on a Bruker AC 250 spectrometer with proton decoupling. Chemical shifts are reported relative to external $H_3PO_4$. FAB mass spectra were recorded in the positive-ion or negative-ion mode on a JEOL DX 300 mass spectrometer operating with a JMA-DA 5000 mass data system. Xe atoms were used for the gun at 3 kV with a total discharge current of 20 mA. The matrix used was 3-nitrobenzyl alcohol (NBA) or a mixture (50:50, v/v) of glycerol and thioglycerol (G/T). High-resolution mass spectra (HRMS) were obtained by using FAB$^+$. UV spectra were recorded on an Uvikon 810 (Kontron) spectrometer. Elemental analyses were performed by the Service de Microanalyses du CNRS, Division de Vernaison (France), and the results were within ±0.4% of the theoretical values. TLC was performed on precoated aluminum sheets of silica gel 60 $F_{254}$ (Merck, article 5554), visualization of products being accomplished by UV absorbance followed by charring with 10% ethanolic sulfuric acid with heating; phosphorous-containing compounds were detected by spraying with Hanes molybdate reagent.[39] Column chromatography was carried out on silica gel 60 (Merck, article 9385) at atmospheric pressure. High-performance liquid chromatography (HPLC) studies were carried out on a Waters Assoc. unit equipped with a model 600E multisolvent delivery system, a model 600E system controller, a model U6K sample injector, a 486 tunable absorbance detector, and a base line 810 data workstation. The column was a reverse-phase analytical column (Hypersil, C18, 100 × 4.6 mm, 3 $\mu$m) protected by a prefilter and a precolumn (Guard Pak, C18). The compound to be analyzed was eluted using the same system as indicated below in the Stability and Decomposition Studies section. The different retention times are also indicated in this section.

The test compounds were found to be pure by rigorous HPLC analysis, high-field multinuclear NMR spectroscopy, and high-resolution mass spectroscopy.

**9-{2-[*O,O′*-Bis[(pivaloyloxy)methyl]phosphonomethoxy]-ethyl}adenine (3).** Iodomethyl pivaloate[36] (966.75 mg, 3.99 mmol) was added to a solution of **5**[16] (544 mg, 0.78 mmol) in anhydrous pyridine (10 mL) and stirred at room temperature for 36 h. The reaction mixture was neutralized with an aqueous solution of 1 M triethylammonium bicarbonate buffer (pH 7.5, 8 mL), evaporated under reduced pressure, and coevaporated with toluene and methanol. The crude residue was treated with a (8:1:1, v/v/v) mixture of acetic acid, water, and methanol (25 mL) and stirred at room temperature for 12 h. The residue obtained after evaporation was dissolved in chloroform (50 mL), and the organic layer was washed with water, dried over sodium sulfate, filtered, and evaporated under reduced pressure. Column chromatography of the residue on silica gel with a stepwise gradient of methanol (0−3%) in dichloromethane afforded the title compound **3** as an oil (67.5 mg, 18%): $^1$H NMR (DMSO-$d_6$) $\delta$ 8.14 and 8.09 (2s, 1H and 1H, 2-H and 8-H), 7.33 (s, 2H, $NH_2$), 5.54 (d, 4H, $J$ = 12.7 Hz, 2 × -O-$CH_2$-), 4.32 (t, 2H, $J$ = 5.1 Hz, -$CH_2$-N), 3.95 (d, 2H, $J$ = 7.8 Hz, -$CH_2$-P-), 3.88 (t, 2H, $CH_2$-$CH_2$-N), 1.12 (s, 18H, 2 × $(CH_3)_3C$-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.2; FAB MS (>0, NBA) $m/e$ 502 (M + H)$^+$; FAB MS (<0, G/T) $m/e$ 386 (M − Piv-O-$CH_2$)$^-$, 272 (M − 2Piv-O-$CH_2$ + H)$^-$; HRMS 502.2000 (M + H), calcd for $C_{20}H_{33}N_5O_8P$ 502.2067; UV (ethanol) $\lambda_{max}$ 260 nm ($\epsilon$ 12 000), $\lambda_{min}$ 228 nm ($\epsilon$ 3700).

**General Procedure for the Preparation of the Phosphonodiester Derivatives 7a−d and the Phosphonomonoesters 8b−d.** 1-Mesitylene-2-sulfonyl-3-nitro-1,2,4-triazole (MSNT; 3 equiv) was added to a solution of **5**[16] and the appropriate hydroxythioester **8**[26] in anhydrous pyridine (24 mL/mmol of **5**). After stirring at room temperature for 12 h, the reaction mixture was neutralized with an aqueous solution of 1 M triethylammonium hydrogenocarbonate buffer (pH 7.5, 2 times the number of moles of MSNT) and extracted with chloroform and water. The organic layer was dried over sodium sulfate, filtered, and evaporated to dryness under reduced pressure.

***N*$^6$-(4-Monomethoxytrityl)-9-{2-[*O,O′*-bis[(*S*-acetylthio)-ethyl]phosphonomethoxy]ethyl}adenine (7a).** Silica gel chromatography [eluent, stepwise gradient of methanol (0−2%) in dichloromethane] gave **7a** as a solid (59%) after lyophilization in dioxane: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.15 and 7.90 (2s, 1H and 1H, 2-H and 8-H), 7.3−6.8 (m, 15H, NH, 14H aromatic), 4.32 (2H, t, $J$ = 4.7 Hz, $CH_2N$), 4.0−3.8 (m, 8H, 2 × S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 3.70 (s, 3H, -$OCH_3$), 3.01 (t, 4H, $J$ = 6.4 Hz, 2 × S-$CH_2$-$CH_2$-O), 2.30 (s, 6H, 2 × $CH_3$-C(O)-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.5; FAB MS (>0, G/T) $m/e$ 750 (M + H)$^+$.

***N*$^6$-(4-Monomethoxytrityl)-9-{2-[*O,O′*-bis[(*S*-isobutyrylthio)ethyl]phosphonomethoxy]ethyl}adenine (7b) and *N*$^6$-(4-Monomethoxytrityl)-9-{2-[*O*-mono[(*S*-isobutyrylthio)ethyl]phosphonomethoxy]ethyl}adenine (8b).** Silica gel chromatography [eluent, stepwise gradient of methanol (0−100%) in dichloromethane] gave **7b** as a solid (86%) after lyophilization in dioxane and the more polar **8b** as a powder (8%) after lyophilization in a solution of dioxane and water. **7b**: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.15 and 7.90 (2s, 1H and 1H, 2-H and 8-H), 7.3−6.8 (m, 15H, NH, 14H aromatic), 4.32 (t, 2H, $J$ = 4.8 Hz, $CH_2N$), 4.0−3.9 (m, 8H, 2 × S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 3.70 (s, 3H, -$OCH_3$), 3.01 (t, 4H, $J$ = 6.4 Hz, 2 × S-$CH_2$-$CH_2$-O), 2.8−2.7 (m, 2H, 2 × $(CH_3)_2CH$-), 1.08 (d, 12H, $J$ = 6.9 Hz, 2 × $(CH_3)_2CH$); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.45; FAB MS (>0, G/T) $m/e$ 806 (M + H)$^+$; FAB MS(<0, G/T) $m/e$ 804 (M − H)$^-$, 674 (M − iPr-C(O)-S-$CH_2$-$CH_2$)$^-$, 604 (M − iPr-C(O)-S-$CH_2$-$CH_2$ − iPr-C(O) + H)$^-$.

**8b**: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.28 and 7.88 (2s, 1H and 1H, 2-H and 8-H), 7.3−6.8 (m, 15H, NH, 14H aromatic), 4.27 (t, 2H, $J$ = 4.7 Hz, $CH_2N$), 3.8−3.6 (m, 9H, S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N, -$OCH_3$), 2.86 (t, 2H, $J$ = 6.7 Hz, S-$CH_2$-$CH_2$-O), 2.8−2.7 (m, 1H, $(CH_3)_2CH$-), 1.04 (d, 6H, $J$ = 6.8 Hz, $(CH_3)_2CH$); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 12.5; FAB MS (<0, G/T) $m/e$ 674 (M − H)$^-$, 544 (M − iPr-C(O)-S-$CH_2$-$CH_2$)$^-$.

***N*$^6$-(4-Monomethoxytrityl)-9-{2-[*O,O′*-bis[(*S*-pivaloylthio)ethyl]phosphonomethoxy]ethyl}adenine (7c) and *N*$^6$-(4-Monomethoxytrityl)-9-{2-[*O*-mono[(*S*-pivaloylthio)-ethyl]phosphonomethoxy]ethyl}adenine (8c).** Silica gel

chromatography [eluent, stepwise gradient of methanol (0−100%) in dichloromethane] gave **7c** as a solid (58%) after lyophilization in dioxane and **8c** as a 3:97 mixture of the acidic and triethylammonium forms (10%) after lyophilization in a solution of dioxane and water. **7c**: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.15 and 7.89 (2s, 1H and 1H, 2-H and 8-H), 7.3−6.8 (m, 15H, NH, 14H aromatic), 4.32 (t, 2H, $J$ = 4.8 Hz, $CH_2$N), 4.0−3.8 (m, 8H, 2 × S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 3.70 (s, 3H, -$OCH_3$), 2.99 (t, 4H, $J$ = 6.4 Hz, 2 × S-$CH_2$-$CH_2$-O), 1.14 (s, 18H, 2 × $(CH_3)_3$C-C(O)-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.5; FAB MS (>0, G/T), $m/e$ 834 (M + H)$^+$; FAB MS (<0, G/T), $m/e$ 832 (M − H)$^-$, 688 (M − Piv-S-$CH_2$-$CH_2$)$^-$, 604 (M − Piv-S-$CH_2$-$CH_2$ − Piv + H)$^-$.

**8c**: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.28 and 7.89 (2s, 1H and 1H, 2-H and 8-H), 7.3−6.8 (m, 15H, NH, 14H aromatic), 4.28 (t, 2H, $J$ = 4.6 Hz, $CH_2$N), 3.8 (m, 6H, S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 3.70 (s, 3H, -$OCH_3$), 2.85 (t, 2H, $J$ = 6.7 Hz, S-$CH_2$-$CH_2$-O), 1.11 (s, 9H, $(CH_3)_3$C-C(O)-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 12.4; FAB MS (<0, G/T), $m/e$ 688 (M − H)$^-$, 544 (M − Piv-S-$CH_2$-$CH_2$)$^-$.

***N*$^6$-(4-Monomethoxytrityl)-9-{2-[*O,O′*-bis[(*S*-benzoylthio)ethyl]phosphonomethoxy]ethyl}adenine (7d) and *N*$^6$-(4-Monomethoxytrityl)-9-{2-[*O*-mono[(*S*-benzoylthio)-ethyl]phosphonomethoxy]ethyl}adenine (8d).** Silica gel chromatography [eluent, stepwise gradient of methanol (0−100%) in dichloromethane] gave **7d** as a solid (58%) after lyophilization in dioxane and **8d** as a 3:97 mixture of the acidic and triethylammonium forms (10%) after lyophilization in a solution of dioxane and water. **7d**: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.2−6.8 (m, 27H, 2-H, 8-H, NH, 24H aromatic), 4.31 (t, 2H, $J$ = 4.8 Hz, $CH_2$N), 4.08 (q, 4H, 2 × S-$CH_2$-$CH_2$-O), 3.94 (d, 2H, $J$ = 8.3 Hz, $CH_2$-P), 3.87 (t, 2H, $CH_2$-$CH_2$-N), 3.68 (s, 3H, -$OCH_3$), 3.23 (t, 4H, $J$ = 6.3 Hz, 2 × S-$CH_2$-$CH_2$-O); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.6; FAB MS (>0, G/T), $m/e$ 874 (M + H)$^+$; FAB MS (<0, G/T) $m/e$ 872 (M − H)$^-$, 708 (M − Bz-S-$CH_2$-$CH_2$)$^-$.

**8d**: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.3−6.8 (m, 22H, 2-H, 8-H, NH, 19H aromatic), 4.27 (t, 2H, $J$ = 4.4 Hz, $CH_2$N), 3.8−3.7 (m, 9H, S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N, -$OCH_3$), 3.08 (t, 2H, $J$ = 6.4 Hz, S-$CH_2$-$CH_2$-O); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 12.5; FAB MS (<0, G/T) $m/e$ 708 (M − H)$^-$, 544 (M − Bz-S-$CH_2$-$CH_2$)$^-$.

**General Procedure for the Preparation of the Phosphonodiester Derivatives 4a−d.** The corresponding **7** was treated with a (8:1:1, v/v/v) mixture of acetic acid, water, and methanol (75 mL/mol of **7**). After 12 h of stirring at room temperature, the reaction mixture was evaporated under reduced pressure.

**9-{2-[*O,O′*-Bis[(*S*-acetylthio)ethyl]phosphonomethoxy]-ethyl}adenine (4a) and 9-{2-[*O,O′*-Mono[(*S*-acetylthio)-ethyl]phosphonomethoxy]ethyl}adenine (9a).** Silica gel column chromatography [stepwise gradient of methanol (0−6%) in dichloromethane] gave **4a** (89%) which was crystallized from toluene (67%) and **9a** (3%) after evaporation of the appropriate fractions and purification on Dowex 1 × 2 resin (acetate form) [eluent: linear gradient of acetic acid (0.5−2 M)]. **4a**: mp 68−69 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 8.11 and 8.06 (2s, 1H and 1H, 2-H and 8-H), 7.19 (s, 2H, $NH_2$), 4.31 (t, 2H, $J$ = 5.0 Hz, $CH_2$N), 4.0−3.9 (m, 8H, 2 × S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 3.03 (t, 4H, $J$ = 6.4 Hz, 2 × S-$CH_2$-$CH_2$-O), 2.8−2.7 (m, 2H, 2 × $(CH_3)_2$$CH$-), 1.09 (d, 12H, $J$ = 6.9 Hz, 2 × $(CH_3)_2$CH-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.55; FAB MS (>0, G/T) $m/e$ 570 (M + G + H)$^+$, 478 (M + H)$^+$; FAB MS (<0, G/T) $m/e$ 374 (M − Ac-S-$CH_2$-$CH_2$)$^-$; HRMS 478.0852 (M + H), calcd for $C_{16}H_{25}N_5O_6PS_2$ 478.0984; UV (ethanol) $\lambda_{max}$ 260 ($\epsilon$ 14 100), 230 nm ($\epsilon$ 10 400), $\lambda_{min}$ 240 ($\epsilon$ 9200), 223 nm ($\epsilon$ 9800). Anal. ($C_{16}H_{24}N_5O_6PS_2$) C, H, N, P, S.

**9a**: $^1$H NMR ($D_2O$) $\delta$ 8.46 and 8.44 (2s, 1H and 1H, 2-H and 8-H), 4.57 (t, 2H, $J$ = 4.9 Hz, $CH_2$N), 4.00 (t, 2H, $CH_2$-$CH_2$-N), 3.8−3.7 (m, 4H, S-$CH_2$-$CH_2$-O, $CH_2$-P), 2.83 (t, 2H, $J$ = 6.5 Hz, S-$CH_2$-$CH_2$-O), 2.34 (s, 3H, $CH_3$-C(O)-); $^{31}$P NMR ($D_2O$) $\delta$ 17.8; FAB MS (<0, G/T) $m/e$ 749 (2M − H)$^-$, 374 (M − H)$^-$, 272 (M − $AcSCH_2CH_2$ + H)$^-$.

**9-{2-[*O,O′*-Bis[(*S*-isobutyrylthio)ethyl]phosphonomethoxy]ethyl}adenine (4b).** Silica gel column chromatography [stepwise gradient of methanol (0−6%) in dichloromethane] gave **4b** (81%) which was crystallized from $CCl_4$ (75%): mp 83−84 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 8.11 and 8.06 (2s, 1H and 1H, 2-H and 8-H), 7.19 (s, 2H, $NH_2$), 4.31 (t, 2H, $J$ = 5.0 Hz, $CH_2$N), 4.0−3.9 (m, 8H, 2 × S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 3.03 (t, 4H, $J$ = 6.4 Hz, 2 × S-$CH_2$-$CH_2$-O), 2.8−2.7 (m, 2H, 2 × $(CH_3)_2$$CH$-), 1.09 (d, 12H, 2 × $(CH_3)_2$CH-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.55; FAB MS (>0, G/T) $m/e$ 534 (M + H)$^+$, 136 $(BH_2)^+$; FAB MS (<0, NBA) $m/e$ 402 (M − iPr-C(O)-S-$CH_2$-$CH_2$)$^-$, 272 (M − 2iPr-C(O)-S-$CH_2$-$CH_2$ + H)$^-$; HRMS 534.1595 (M + H), calcd for $C_{20}H_{33}N_5O_6PS_2$ 534.1610; UV (ethanol) $\lambda_{max}$ 260 ($\epsilon$ 15 000), 231 nm ($\epsilon$ 11 000), $\lambda_{min}$ 240 ($\epsilon$ 10 400), 223 nm ($\epsilon$ 10 100). Anal. ($C_{20}H_{32}N_5O_6PS_2$) C, H, N, P, S.

**9-{2-[*O,O′*-Bis[(*S*-pivaloylthio)ethyl]phosphonomethoxy]ethyl}adenine (4c).** Silica gel column chromatography [stepwise gradient of methanol (0−6%) in dichloromethane] gave **4c** (76%) which was crystallized from toluene (60%): mp 66 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 8.11 and 8.06 (2s, 1H and 1H, 2-H and 8-H), 7.17 (s, 2H, $NH_2$), 4.31 (t, 2H, $J$ = 5.0 Hz, $CH_2$N), 4.0−3.9 (m, 8H, 2 × S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 3.01 (t, 4H, $J$ = 6.4 Hz, 2 × S-$CH_2$-$CH_2$-O), 1.16 (s, 18H, 2 × $(CH_3)_3$C-C(O)-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.6; FAB MS (>0, NBA) $m/e$ 562 (M + H)$^+$; FAB MS (<0, NBA) $m/e$ 416 (M − Piv-S-$CH_2$-$CH_2$)$^-$, 272 (M − 2Piv-S-$CH_2$-$CH_2$ + H)$^-$; HRMS 562.1876 (M + H), calcd for $C_{22}H_{37}N_5O_6PS_2$ 562.1923; UV (ethanol) $\lambda_{max}$ 260 ($\epsilon$ 16 100), 231 nm ($\epsilon$ 11 700), $\lambda_{min}$ 242 ($\epsilon$ 11 100), 223 nm ($\epsilon$ 10 400). Anal. ($C_{22}H_{36}N_5O_6PS_2$) C, H, N, P, S.

**9-{2-[*O,O′*-Bis[(*S*-benzoylthio)ethyl]phosphonomethoxy]-ethyl}adenine (4d).** Silica gel column chromatography [stepwise gradient of methanol (0−6%) in dichloromethane] gave **4d** (75%) as an oil: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.11 and 8.07 (2s, 1H and 1H, 2-H and 8-H), 7.9−7.5 (m, 10H, 2 Ph), 7.18 (s, 2H, $NH_2$), 4.30 (t, 2H, $J$ = 5.0 Hz, $CH_2$N), 4.10 (q, 4H, 2 × S-$CH_2$-$CH_2$-O), 3.94 (d, 2H, $J$ = 8.2 Hz, $CH_2$-P), 3.88 (t, 2H, $J$ = 5.0 Hz, $CH_2$-$CH_2$-N), 3.26 (t, 4H, $J$ = 6.3 Hz, 2 × S-$CH_2$-$CH_2$-O); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 22.7; FAB MS (>0, G/T) $m/e$ 602 (M + H)$^+$; FAB MS (<0, G/T) $m$/e 436 (M − Bz-S-$CH_2$-$CH_2$)$^-$, 272 (M − 2Bz-S-$CH_2$-$CH_2$ + H)$^-$; HRMS 602.1316 (M + H), calcd for $C_{26}H_{29}N_5O_6PS_2$ 602.1297; UV (ethanol) $\lambda_{max}$ 260 ($\epsilon$ 30 900), 244 nm ($\epsilon$ 27 800), $\lambda_{min}$ 250 ($\epsilon$ 26 500), 223 nm ($\epsilon$ 12 600).

**General Procedure for the Preparation of the Phosphonomonoesters 9b−d**. The corresponding **8** was treated with a (8:1:1, v/v/v) mixture of acetic acid, water, and methanol (70 mL/mol of **8**). After 12 h of stirring at room temperature, the reaction mixture was evaporated under reduced pressure and extracted with water and chloroform. The aqueous layer was evaporated under reduced pressure.

**9-{2-[*O,O′*-Mono[(*S*-isobutyrylthio)ethyl]phosphonomethoxy]ethyl}adenine (9b).** Dowex 1 × 2 resin (acetate form) chromatography [eluent: linear gradient of acetic acid (0−2 M)] gave **9d** (46%) as a solid after lyophilization in water: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.20 and 8.18 (2s, 1H and 1H, 2-H and 8-H), 7.70 (s, 2H, $NH_2$), 4.33 (t, 2H, $J$ = 5.0 Hz, $CH_2$N), 3.9−3.7 (m, 6H, S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 2.98 (t, 2H, $J$ = 6.5 Hz, S-$CH_2$-$CH_2$-O), 2.8−2.7 (m, 1H, $(CH_3)_2$$CH$-), 1.09 (d, 6H, $J$ = 6.9 Hz, $(CH_3)_2$CH-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 19.1; FAB (<0, NBA) $m/e$ 805 (2M − H)$^-$, 402 (M − H)$^-$, 272 (M − iPr-C(O)-S-$CH_2$-$CH_2$)$^-$.

**9-{2-[*O,O′*-Mono[(*S*-pivaloylthio)ethyl]phosphonomethoxy]ethyl}adenine (9c).** Dowex 1 × 2 resin (acetate form) chromatography [eluent: linear gradient of acetic acid (0−2 M)] gave **9c** (54%) as a solid after lyophilization in water: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.20 and 8.18 (2s, 1H and 1H, 2-H and 8-H), 7.72 (s, 2H, $NH_2$), 4.34 (t, 2H, $J$ = 5.1 Hz, $CH_2$N), 3.9−3.7 (m, 6H, S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 2.97 (t, 2H, $J$ = 6.6 Hz, S-$CH_2$-$CH_2$-O), 1.15 (s, 9H, $(CH_3)_3$C-C(O)-); $^{31}$P NMR (DMSO-$d_6$) $\delta$ 19.1; FAB MS (<0, G/T) $m/e$ 833 (2M − H)$^-$, 416 (M − H)$^-$, 272 (M − Piv-S-$CH_2$-$CH_2$)$^-$.

**9-{2-[*O,O′*-Mono[(*S*-benzoylthio)ethyl]phosphonomethoxy]ethyl}adenine (9d).** Dowex 1 × 2 resin (acetate form) chromatography [eluent: linear gradient of acetic acid (0−2 M)] gave **9d** (49%) as a solid after lyophilization in a mixture of water and dioxane (9:1, v/v): $^1$H NMR ($D_2O$ + DMSO-$d_6$) $\delta$ 8.57 and 8.38 (2s, 1H and 1H, 2-H and 8-H), 8.0−7.7 (m, 5H, Ph), 4.62 (t, 2H, $J$ = 5.0 Hz, $CH_2$N), 4.1−3.8 (m, 6H, S-$CH_2$-$CH_2$-O, $CH_2$-P, $CH_2$-$CH_2$-N), 3.22 (t, 2H, $J$ = 6.3

Hz, S-$CH_2$-$CH_2$-O); $^{31}$P NMR ($D_2O$ + DMSO-$d_6$) $\delta$ 16.8; FAB MS (<0, G/T) $m/e$ 873 $(2M - H)^-$, 436 $(M - H)^-$.

**Biological Methods. Anti-HIV Assays on Cell Culture.** The origin of the viruses and the techniques used for measuring inhibition of virus multiplication have previously been described.[16] Briefly, in MT-4 cells, the determination of the antiviral activity of the pronucleotides was based on a reduction of HIV-1-IIIB-induced cytopathogenicity, the metabolic activity of the cells being measured by the property of mitochondrial dehydrogenases to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan.[16,40,41] For CEM cells, the production of virus HIV-LAI was measured by quantification of the reverse transcriptase activity associated with the virus particle released in the culture supernatant.[16,41] Cells, MT-4 and CEM-SS, were respectively incubated with 50 or 100 $TCID_{50}$ of viruses during 30 min; after virus adsorption, unbound particles were eliminated by two washes, and cells were cultured in the presence of different concentrations of test compounds for 5 days before virus production determination. The 50% effective concentration ($EC_{50}$) was derived from the computer-generated median effect plot of the dose−effect data.[42] In parallel experiments, cytoxicity of the test compounds was measured after an incubation of 5 days in their presence using the colorimetric MTT test.[43] The 50% cytotoxic concentration ($CC_{50}$) is the concentration at which $OD_{540}$ was reduced by one-half and was calculated using the program mentioned above.

**Anti-HSV Assays on Cell Culture.** The anti-HSV assays were performed by a method adapted from that described by De Clercq.[44,45] The herpes simplex virus type 1 [HSV-1, strain F (ATCC VR-733)] and the herpes simplex virus type 2 [HSV-2, strain G (ATCC VR-734)] were propagated on Vero cells and human embryonic fibroblasts (cell line MRC-5).

All assays were carried out on confluent cells in 96-well microtiter plates. The cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). They were infected with 100 $CCID_{50}$ of virus for 1 h at 37 °C and immediately thereafter exposed to various concentrations of the test compounds (from 1 mM to 1 $\mu$M). The viral cytopathic effect (CPE) was recorded daily. Antiviral activity was expressed as $EC_{50}$ (50% effective concentration), that is, the concentration of compound required to reduce the viral CPE by 50% when it had reached completion in the control virus-infected cell cultures.

Drug cytotoxicity was determined using the quantitative colorimetric MTT assay.[43] In this assay, different dilutions of the test compounds were added to 96-well plates containing about $3 \times 10^3$ cells (approximately 30% confluent). When cells in the control wells (without drug) reached confluence, 0.05 mL of MTT (5 mg/mL) was added to each well. After a 3 h incubation at 37 °C, acidified 2-propanol was added to each well. The optical density in each well of the plate was determined using a 96-well plate photometer at 540 nm. Toxicity was considered to occur if the monolayer remained less than 50% confluent.

**Stability and Decomposition Studies.** We used the previously described HPLC procedure.[26] Briefly, the cleaning precolumn was a Guard-Pak insert (Delta-Pak C18 100 Å) in a Guard-Pak holder (Waters, Saint Quentin, France). The analytical column used was a Hypersil C18, 3 $\mu$m, 120 Å, 4.6 × 100 mm (Shandon, Eragny, France). The elution system was prepared as follows: stock solution S, 1.0 M, pH 6.0, from ammonium acetate and acetic acid *pro analysis* (Merck, Darmstadt, Germany); eluent A 2.5 mM buffered tetrabutylammonium sulfate [one bottle of PIC A reagent (Waters) in 2.0 L of water]; eluent B (v/v), S 10, water 90; eluent C (v/v/v), S 10, acetonitrile 50, water 40. The crude sample (80 $\mu$L, initial concentration of **2**, **3**, or **4a**−**c** of $5 \times 10^{-5}$ M) was injected into the precolumn and eluted with eluent A during 5 min. Then, the switching valve for connecting the precolumn to the column was activated, and a linear gradient from eluent B to eluent C programmed over 30 min was used. The retention times (min) were **1**, 13.2; **2**, 22.1; **3**, 32.1; **4a**, 23.9; **4b**, 33.3; **4c**, 37; **4d**, 36.3; **9a**, 16.8; **9b**, 20.7; **9c**, 22.8; **9d**, 23.1; mono(DTE)PMEA, 16.6; mono(POM)PMEA, 20.5.

**Acknowledgment.** These investigations were supported by grants from the CNRS, Agence Nationale de Recherches sur le SIDA (ANRS, France), and Association pour la Recherche sur le Cancer (ARC, France). Two of us are particularly grateful to ANRS (S.B.) and ARC (H.P.) for a fellowship. We warmly thank S. Schmidt and G. Albrecht for excellent assistance in performing the anti-HIV-1 assays. We are indebted to Dr. Victor Marquez (NIH, Bethesda, MD) for critical reading of the manuscript. The assistance of G. S. Hanrahan in typing this manuscript is also greatly appreciated.

## References

(1) For a review, see: Naesens, L.; Balzarini, J.; De Clercq, E. Therapeutic potential of PMEA as an antiviral drug. *Med. Virol.* **1994**, *4*, 147−159.
(2) De Clercq, E. Broad-spectrum anti-DNA virus and anti-retrovirus activity of phosphonylmethoxyalkylpurines and -pyrimidines. *Biochem. Pharmacol.* **1991**, *42*, 963−972.
(3) Walker, R. E.; Vogel, S. E.; Jaffe, H. S.; Polis, M. A.; Kovacs, J. A.; Faloon, J.; Davey, R. T.; Ebeling, D.; Cundy, K.; Paar, D.; Markowitz, N.; Masur, H.; Lane, H. C. A phase I/II study of PMEA in HIV infected patients. *Abstracts of Papers*; 1st National Conference on Human Retroviruses and Related Infections, Washington, DC, 1993; Abstract 522.
(4) Balzarini, J.; Naesens, L.; Slachmuylders, J.; Niphuis, H.; Rosenberg, I.; Holy, A.; Schellekens, H.; De Clercq, E. 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) effectively inhibits retrovirus replication *in vitro* and simian immunodeficiency virus infection in rhesus monkeys. *AIDS* **1991**, *5*, 21−28.
(5) Bronson, J. J.; Ghazzouli, I.; Hitchcock, M. J. M.; Russel, J. W.; Klunk, L. J.; Kern, E. R.; Martin, J. C. In vivo anti-retrovirus and anti-cytomegalovirus activity of 9-((2-phosphonylmethoxy)-ethyl)adenine (PMEA). *Abstracts of Papers*; 5th International Conference on AIDS, Montreal, Canada, 1989; Abstract M.C.P. 74.
(6) Starrett, J. E., Jr.; Tortolani, D. R.; Russel, J.; Hitchock, M. J. M.; Whiterock, V.; Martin, J. C.; Mansuri, M. M. Synthesis, oral bioavailability determination, and *in vitro* evaluation of prodrugs of the antiviral agent 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA). *J. Med. Chem.* **1994**, *37*, 1857−1864.
(7) Alexander, P.; Holy, A. Prodrugs of analogs of nucleic acid components. *Collect. Czech. Chem. Commun.* **1994**, *59*, 2127−2165.
(8) Périgaud, C.; Girardet, J.-L.; Gosselin, G.; Imbach, J.-L. Comments on nucleotide delivery forms. In *Advances in antiviral drug design;* De Clercq, E., Ed.; JAI Press Inc.: Greenwich, CT, 1996; Vol. *2*, pp 147−172.
(9) Jones, R. J.; Bischofberger, N. Minireview: nucleotides prodrugs. *Antiviral Res.* **1995**, *27*, 1−17.
(10) Midoux, P.; Negre, E.; Roche, A. C.; Mayer, R.; Monsigny, M.; Balzarini, J.; De Clercq, E.; Mayer, E.; Ghaffar, A.; Gangemi, J. D. Drug targeting: anti-HSV-1 activity of mannosylated polymer-bond 9-(2-phosphonylmethoxyethyl)adenine. *Biochem. Biophys. Res. Commun.* **1990**, *167*, 1044−1049.
(11) Alexander, P.; Holy, A.; Masojidkova, M. Synthesis of 9-(2-phosphinomethoxyethyl)adenine and related compounds. *Collect. Czech. Chem. Commun.* **1994**, *59*, 1870−1878.
(12) Alexander, P.; Holy, A.; Masojidkova, M. Preparation of 9-(2-phosphonomethoxyethyl)adenine esters as potential prodrugs. *Collect. Czech. Chem. Commun.* **1994**, *59*, 1853−1869.
(13) Glazier, A. Potent topical anti-herpes activity of a lipophilic phosphorus prodrug for the antiviral agent PMEA. *Antiviral Res.* **1995**, *26*, A306.
(14) Glazier, A.; Buckheit, R.; Yanachkova, M.; Yanachkov, I.; Wright, G. E. Lipophilic phosphorus prodrugs for the antiviral agent PMEA. *Antiviral Res.* **1994**, *23* (Suppl. 1), 65.
(15) Starrett, J. E., Jr.; Tortolani, D. R.; Hitchock, M. J. M.; Martin, J. C.; Mansuri, M. M. Synthesis and *in vitro* evaluation of a phosphonate prodrug: bis(pivaloyloxymethyl)9-(2-phosphonylmethoxyethyl)adenine. *Antiviral Res.* **1992**, *19*, 267−273.
(16) Puech, F.; Gosselin, G.; Lefebvre, I.; Pompon, A.; Aubertin, A. M.; Kirn, A.; Imbach, J.-L. Intracellular delivery of nucleoside monophosphates through a reductase-mediated activation process. *Antiviral Res.* **1993**, *22*, 155−174.
(17) Srinivas, R. V.; Robbins, B. L.; Connely, M. C.; Gong, Y. F.; Bischofberger, N.; Fridland, A. Pivaloyloxymethyl esters of acyclic nucleoside phosphonates. *Int. Antiviral News* **1994**, *2*, 53−55.
(18) Naesens, L.; Neyts, J.; Balzarini, J.; Bischofberger, N.; De Clercq, E. Antiviral efficacy in mice of oral bis(POM)-PMEA, the bis-(pivaloyloxymethyl) ester prodrug of 9-(2-phosphonylmethoxyethyl)adenine. *Antiviral Res.* **1995**, *26*, A276.

(19) Naesens, L.; Neyts, J.; Balzarini, J.; Bischofberger, N.; De Clercq, E. Pharmacokinetics in mice of bis(POM)-PMEA, the bis-(pivaloyloxymethyl) ester prodrug of 9-(2-phosphonylmethoxyethyl)adenine. *Antiviral Res.* **1995**, *26*, A277.
(20) Naesens, L.; Neyts, J.; Balzarini, J.; Bischofberger, N.; De Clercq, E. *In vivo* antiretroviral efficacy of oral bis(POM)-PMEA, the bis(pivaloyloxymethyl)prodrug of 9-(2-phosphonylmethoxyethyl)-adenine (PMEA). *Nucleosides Nucleotides* **1995**, *14*, 767−770.
(21) Naesens, L.; Balzarini, J.; Bischofberger, N.; De Clercq, E. Antiretroviral activity and pharmacokinetics in mice of oral bis-(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine, the bis(pivaloyloxymethyl) ester prodrug of 9-(2-phosphonylmethoxyethyl)adenine. *Antimicrob. Agents Chemother.* **1996**, *40*, 22−34.
(22) Srinivas, R. V.; Robbins, B. L.; Connely, M. C.; Gong, Y. F.; Bischofberger, N.; Fridland, A. Metabolism and *in vitro* antiretroviral activities of bis(pivaloyloxymethyl)prodrugs of acyclic nucleoside phosphonates. *Antimicrob. Agents Chemother.* **1993**, *37*, 2247−2250.
(23) Cundy, K. C.; Fishback, J. A.; Shaw, J.-P.; Lee, M. L.; Socke, K. F.; Yisor, G.C.; Lee, W. A. Oral bioavailability of the antiretroviral agent 9-(2-phosphonylmethoxyethyl)adenine (PMEA) from three formulations of the prodrug bis(pivaloyloxymethyl)-PMEA in fasted male cynomolgus monkeys. *Pharm. Res.* **1994**, *11*, 839−843.
(24) GS840, a PMEA prodrug, enters trials for HIV-infection. *Antiviral Agents Bull.* **1994**, *7*, 232−233.
(25) Barditch-Crovo, P. A.; Toole, J.; Burgee, H.; Wachsman, M.; Ebeling, D.; Cundy, K. C.; Jaffe, H. S.; Lietman, P. S. A randomized, double-blind, placebo-controlled phase I/II evaluation of 9-[-2-(bispivaloyloxy-methyl) phosphonylmethoxyethyl] adenine (bis-POM PMEA), an orally bioavailable prodrug of the anti-HIV nucleotide, PMEA. *Antiviral Res.* **1995**, *26*, A229.
(26) Lefebvre, I.; Périgaud, C.; Pompon, A.; Aubertin, A.-M.; Girardet, J.-L.; Kirn, A.; Gosselin, G.; Imbach, J.-L. Mononucleoside phosphotriester derivatives with S-acyl-2-thioethyl bioreversible phosphate-protecting groups: intracellular delivery of 3′-azido-2′,3′-dideoxythymidine 5′-monophosphate. *J. Med. Chem.* **1995**, *38*, 3941−3950.
(27) Périgaud, C.; Gosselin, G.; Lefebvre, I.; Girardet, J. L.; Benzaria, S.; Barber, I.; Imbach, J. L. Rational design for cytosolic delivery of nucleoside monophosphates: "SATE" and "DTE" as enzyme-labile transient phosphate protecting groups. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2521−2526.
(28) Périgaud, C.; Aubertin, A. M.; Benzaria, S.; Pélicano, H.; Girardet, J.-L.; Maury, G.; Gosselin, G.; Kirn, A.; Imbach, J.-L. Equal inhibition of the replication of human immunodeficiency virus in human T-cell culture by ddA bis(SATE)phosphotriester and 3′-azido-2′,3′-dideoxythymidine. *Biochem. Pharmacol.* **1994**, *48*, 11−14.
(29) Benzaria, S.; Girardet, J.-L.; Périgaud, C.; Aubertin, A. M.; Pélicano, H.; Maury, G.; Gosselin, G.; Kirn, A.; Imbach, J.-L. The SATE pronucleotide derivative of ddA : a more potent HIV inhibitor than AZT. *Nucleic Acids Symp. Ser.* **1994**, *31*, 129−130.
(30) Girardet, J.-L.; Périgaud, C.; Aubertin, A.-M.; Gosselin, G.; Kirn, A.; Imbach, J.-L. Increase of the anti-HIV activity of D4T in human T-cell culture by the use of the SATE pronucleotide approach. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2981−2984.
(31) Holy, A.; Rosenberg, I. Synthesis of 9-[2-(phosphonylmethoxy)-ethyl]adenine and related compounds. *Collect. Czech. Chem. Commun.* **1987**, *52*, 2801−2809.
(32) Bronson, J. J.; Ghazzouli, I.; Hitchcock, M. J. M.; Webb, R. R., II; Martin, J. C. Synthesis and antiviral activity of the nucleotide analogue (S)-1-[3-hydroxy-2(phosphonylmethoxy)propyl]cytosine. *J. Med. Chem.* **1989**, *32*, 1457−1463.
(33) Bronson, J. J.; Kim, C. U.; Ghazzouli, I.; Hitchcock, M. J. M.; Kern, E. R.; Martin, J. C. Synthesis and antiviral activity of phosphonylmethoxyethyl derivatives of purine and pyrimidine bases. In *Nucleotides analogues as antiviral agents*; Martin, J. C., Ed.; American Chemical Society: Washington, DC; 1989; Vol. 401, pp 72−87.
(34) Bailey, W. F.; Rivera, A. D. Acetolysis of cyclic acetals : regioselective acylative cleavage of cyclic formals. *J. Org. Chem.* **1984**, *49*, 4958−4964.
(35) Foye, W. O.; Kauffman, J. M.; Kim, Y. H. Synthesis of 4-alkyl-4H-benzo[*e*]- and 4H-pyrido[2,3-*e*]-1,2,4-triazin-3-one 1-oxides for potential anticancer activity. *J. Heterocycl. Chem.* **1982**, *19*, 497−501.
(36) Miyake, A.; Yamaoka, M. Method for producing 1-iodoalkyl acylates. Eur. Patent Appl. E.P. 143 601, June 5, 1985; *Chem. Abstr.* **1986**, *104*, 5589z.
(37) Palu, G.; Stefanelli, S.; Rassu, M.; Parolin, C.; Balzarini, J.; De Clercq, E. Cellular uptake of phosphonylmethoxyalkylpurine derivatives. *Antiviral Res.* **1991**, *16*, 115−119.
(38) Blackburn, G. M.; Gait, M. J. *Nucleic acids in chemistry and biology;* IRL Press: Oxford, New York, Tokyo, 1990; pp 71−135.
(39) Hanes, C. S.; Isherwood, F. A. Separation of the phosphoric esters on the filter paper chromatogram. *Nature* **1949**, *154*, 1107−1112.
(40) Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn, P.; Desmyter, G.; De Clercq, R. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods* **1988**, *20*, 309−321.
(41) Moog, C.; Wick, A.; Le Bur, P.; Kirn, A.; Aubertin, A.-M. Bicyclic imidazo derivatives, a new class of highly selective inhibitors for the human immunodeficiency virus type 1. *Antiviral Res.* **1994**, *24*, 274−288.
(42) Chou, J.; Chou, T.-C. Dose-effect analysis with microcomputers: quantitation of $ED_{50}$, $LD_{50}$, synergism, antagonism, low-dose risk, receptor binding and enzyme kinetics. *Computer software for Apple II series and IBM-PC and instruction manual*; Elsevier-Biosoft, Elsevier Science Publishers: Cambridge, U.K., 1985; pp 19−28.
(43) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(44) De Clercq, R.; Holy, A.; Rosenberg, I.; Sakuma, T.; Balzarini, J.; Maudgal, P. L. A novel selective broad-spectrum anti-DNA virus agent. *Nature* **1986**, *323*, 464−467.
(45) De Clercq, E.; Descamps, J.; Verhelst, J.; Walker, R. T.; Jones, A. S.; Torrence, P. F.; Shugar, D. Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus. *J. Infect. Dis.* **1980**, *141*, 563−574.

JM960289O