Enantioselective Syntheses and Cytotoxicity of *N,O*-Nucleosides

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Enantiomers of 4′-aza-2′,3′-dideoxynucleosides have been prepared by two different synthetic approaches, on the basis of 1,3-dipolar cycloaddition of a chiral nitrone. Cytotoxicity and apoptotic activity have been investigated. (5′*S*)-5-Fluoro-1-isoxazolidin-5-yl-1*H*-pyrimidine-2,4-dione [(−)-AdFU], while showing low level of cytotoxicity, is a good inductor of apoptosis on lymphoid and monocytoid cells, acting as a strong potentiator of Fas-induced cell death.

Introduction

The development of novel drugs in the therapy of infections caused by viruses and also in the treatment of certain neoplastic diseases has been remarkably exploited in these last 10 years.[1] In this context, unnatural nucleoside analogues have emerged as major therapeutic agents: since the discovery that nucleoside analogues can effectively protect cells from the lethal action of some viruses, including the human immunodeficiency virus (HIV), herpes simplex virus, hepatitis C virus, and Cytomegalovirus, several reports have appeared concerning their synthesis, therapeutic applications, and mechanism of action.[2]

In the search for effective, selective, and nontoxic agents, a variety of strategies has been devised to design nucleoside analogues. These strategies have involved several structural modifications of the naturally occurring nucleosides at the level of the sugar moiety and/or the heterocyclic base. A series of new compounds, endowed with a relevant biological activity, can be originated from alteration of the carbohydrate fragment: in this context, the design of novel "ribose" rings has resulted in the discovery of effective biological agents with lower toxicity and higher biological effects. Promising results have been in fact obtained from a new generation of nucleoside analogues in which the furanose ring has been replaced by an alternative carbo- or heterocyclic rings.[3,4] Uracil, thymine, cytosine, adenine, and guanine nucleosides **1** possessing an isoxazolidine moiety have been recently synthesized, as racemic mixtures, to investigate their pharmacological activities;[5] in particular (±)-AdT has been reported to inhibit HIV replication in C 8166, with an activity inversely related to the multeplicity of infection used[5c] (Figure 1). To our knowledge, however, no data about the toxicity of this compound have so far appeared in the literature.

However, the consideration that both enantiomeric purity and absolute configuration are key factors in determining the physiological activity of these molecules suggests the synthesis of enantiomerically pure *N,O*-nucleosides in order to investigate their biological features.

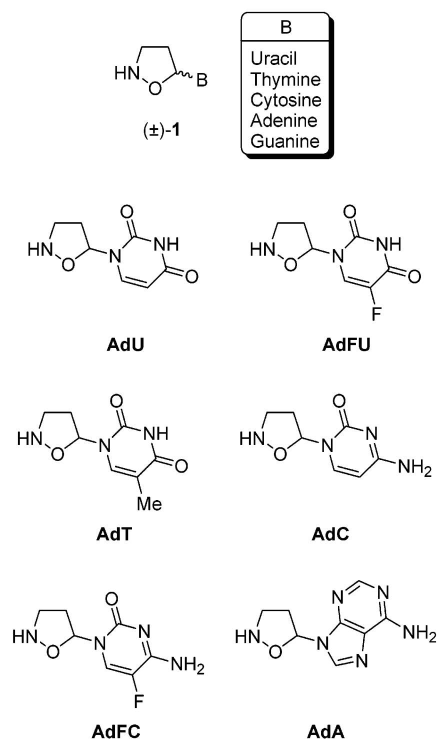

Figure 1. Isoxazolidinyl nucleobases.

As part of our continuing efforts to develop novel strategies for preparing heterocyclic nucleosides, we have focused our attention on the applicability of 1,3-dipolar cycloadditions of nitrones to the synthesis of isoxazolidinyl nucleosides **1**, unsubstituted at the nitrogen atom, in an enantiomeric pure form. Nitrones containing a chiral auxiliary on the nitrogen atom have been selected as the most convenient precursors: our preliminary results have described the synthesis of enantiomerically pure AdT and AdFU.[6] Accordingly, we report here the extension of the synthetic approach[7] to the asymmetric synthesis of purine and pyrimidine *N,O*-nucleosides AdU, AdC, AdFC, and AdA (Figure 1).

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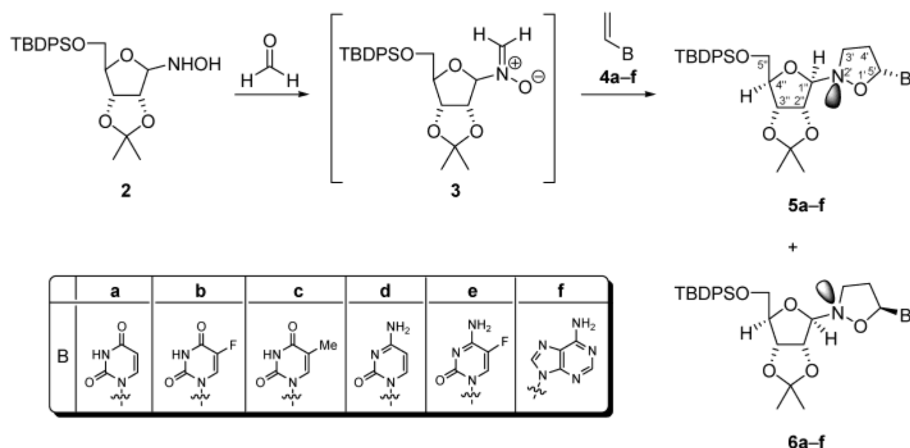
Scheme 1



In particular, we exploit in this paper the use of a series of vinyl nucleobases[8] as dipolarophiles for a one-pot reaction pathway toward enantiomerically pure 4′-aza-2′,3′-dideoxyfuranosyl nucleosides. This synthetic approach has been compared with the two-step methodology based on the Vorbrüggen nucleosidation reaction.

The obtained compounds have been tested for an evaluation of their toxicity and as inductors of apoptosis.

Results and Discussion

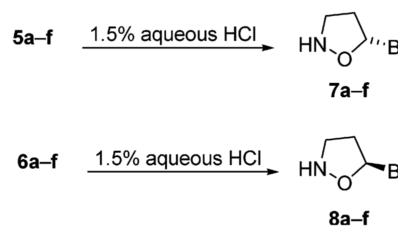
Chemistry. The chiral nitrone **3**, as a not isolated intermediate, was prepared from D-ribose in four steps.[7a] Thus, D-ribose was converted into the 5-*O*-*tert*-butyldiphenylsilyl-2,3-*O*-isopropylidene-D-ribofuranose, protected at the CH_2OH group, and reacted with hydroxylamine to give the ribosyl hydroxylamine **2**; further reaction of **2** with formaldehyde and vinyl bases **4**, performed in $CHCl_3$ at 60 °C for 12 h, afforded regioselectively, through unisolated intermediate nitrone **3**, a mixture of two homochiral isoxazolidines **5** and **6**, epimeric at $C_{5'}$, in a relative ratio 1.5:1 (40% yield) (Scheme 1).

Purification by HPLC chromatography allowed the isolation of pure adducts **5a**−**f** and **6a**−**f**. All structures described were determined by ^1H and ^{13}C NMR analysis. In particular, the stereochemistry of the obtained adducts was assessed by NOE measurements: in fact, the positive NOE effect observed for protons 4″ when irradiating 1″, indicates a cis relationship between these protons. These data confirm that the sugar moiety has a β configuration in all the nucleosides.

Mixtures of diastereomeric invertomers could be observed for compounds **5** and **6**. Variable temperature NMR measurements, performed until −80 °C, show the presence of only one set of resonances, suggesting the existence of only one isomer or a nitrogen inversion sufficiently fast to impart time-averaged properties to the observed compounds.

PM3[9] and AM1[10] quantomechanical calculations indicate that the $N_{2'}$−$C_{5'}$ trans isomers are more stable than the cis derivatives. Thus, for compound **5c**, chosen as a model compound, an energy difference of 4.3 kcal/mol was calculated in favor of the trans isomer: this barrier sufficiently explains the fact that experimentally only one invertomer was formed. The calculated energy barrier for the nitrogen inversion is 13.9 kcal/mol: a value of 16.2 kcal/mol is reported for similar systems.[11]

Scheme 2



On the basis of the marked preference for the trans form, the obtained anomers **5** and **6** can be considered as analogues of α-nucleosides. Thus, the difference of configuration of their $C_{5'}$ atoms is compensated by the nitrogen inversion, and both anomers possess the same trans disposition of their $N_{2'}$ and $C_{5'}$ substituents.

The relative configuration at $N_{2'}$ and $C_{5'}$ for nucleosides **5** and **6** has been tentatively assigned as reported in Scheme 1, according to quantomechanical calculations. The major stereoisomers **5a**−**f** possess the configuration (5′*R*) which is more stable than the configuration (5′*S*) of about 0.8 kcal/mol: this value is in agreement with the observed α/β ratio.

The synthetic approach toward homochiral *N,O*-nucleosides **7** and **8** has been completed by selective cleavage of the sugar moiety, performed by treatment with 1.5% aqueous HCl (Scheme 2). Thus, both anomers **7a**−**f** and **8a**−**f** have been obtained with a global yield of 30% and 10%, respectively, starting from the nitrone **3**.

We have recently reported a different synthetic approach to enantiomerically pure isoxazolidinyl nucleosides **7b**,**c** and **8b**,**c**, based on the 1,3-dipolar cycloaddition of the transient nitrone **3** with vinyl acetate, followed by the Vorbrüggen nucleosidation with silylated nucleobases.[6]

We have here compared two procedures. Ribosyl hydroxylamine **2** has been reacted with formaldehyde and vinyl acetate to afford a mixture of two homochiral isoxazolidines **9** and **10**, epimeric at C_5, in a relative ratio 1.5:1 (90% yield).[6] The subsequent coupling with silylated nucleobases **11a**−**f**, in the presence of TMSOTf at 0 °C, occurred with a 80% yield and led to nucleosides **5** and **6** (≈1.4:1 ratio), which have been separated by

Scheme 3

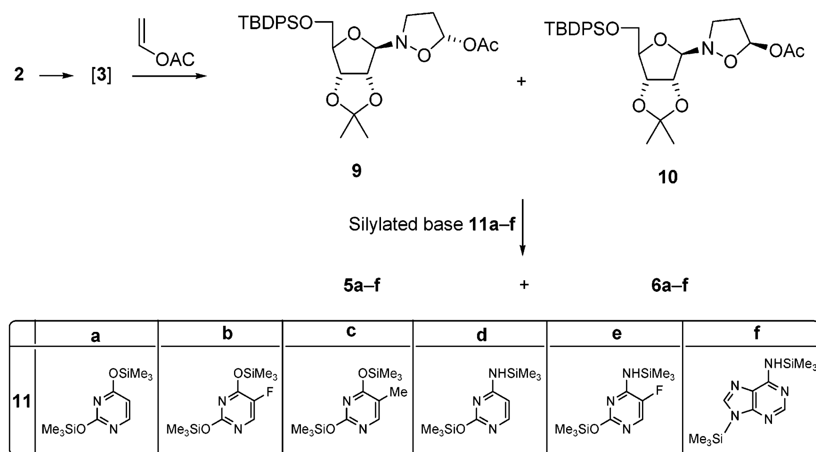


Table 1. Evaluation of Cytotoxicity of (−)-AdFU in Lymphoid and Monocytoid Cell Lines[a]

	24 h		48 h		72 h	
cell line	CC_{50} (μM)[b]	AC_{50} (μM)[b]	CC_{50} (μM)	AC_{50} (μM)	CC_{50} (μM)	AC_{50} (μM)
Molt-3[c]	>1000	329	806	96	366	13
U937	>1000	>1000	>1000	123	687	3
HL-60	>1000	>1000	>1000	>1000	N.D.[d]	N.D.
THP-1	>1000	>1000	N.D.	N.D.	N.D.	N.D.

[a] Cells were exposed in optimal culture conditions to concentrations of AdFU ranging from 8 μM to 512 μM or control medium for the times indicated. [b] Cytotoxic concentration 50 (CC_{50}) and apoptotic concentration 50 (AC_{50}) are the concentrations of the drug required to cause 50% toxicity, detected by trypan blue exclusion test, and 50% apoptosis, detected by microscopy analysis, respectively. Each value is determined from two or more experiments. [c] Values determined using 5-fluorouracil, in Molt-3 cells, were as follows: 24 h, CC_{50} > 1000, AC_{50} = 28; 48 h, CC_{50} = 718, AC_{50} = 16; 72 h, CC_{50} = 14, AC_{50} = 15. [d] Not done.

flash chromatography and then by HPLC (Scheme 3). This reaction pathway showed a global yield of 72%.

Cytotoxicity Assays. One of the major limitations in the use of nucleoside analogues as antiviral drugs is their toxicity. However, compounds exhibiting reasonably low levels of cytotoxicity, preferentially directed toward the cell types targeted by viral agents causing chronic infection, could exploit this characteristic in order to improve their specific antiviral activity also by eliminating infected cells. For this reason, we tested the cytotoxicity of the new synthesized compounds using a conventional viability assay, such as the trypan blue exclusion test, in cell lines of lymphoid and monocytoid origin.

Moreover, considering the pivotal role of apoptosis as a mechanism, triggered by cellular signals as well as by a variety of drugs, for the controlled removal of dead cells, we assayed the ability of (−)-AdFU **8b**, chosen as a model compound, to specifically induce apoptosis, detected by microscopy analysis. Results, expressed as cytotoxic concentration 50 (CC_{50}, concentration that causes 50% toxicity by trypan blue) and apoptotic concentration 50 (AC_{50}, concentration required to cause apoptosis in 50% of treated cells), respectively, are reported in Table 1.

No significant toxicity, when evaluated using the classical trypan blue test, was detected until 3 days of treatment in the lymphoid (Molt-3) and monocytoid (U937) cell lines assayed. However, (−)-AdFU was shown to specifically induce remarkable levels of apoptosis, in Molt-3 and U937 cells. In particular, the lymphoid cell line was more sensitive to AdFU-induced apoptosis than the monocytoid cells. In some experiments, induction of apoptosis in Molt-3 and U937 cells was confirmed by flow cytometry analysis following propidium iodide staining. Two other monocytoid cell lines tested showed no sensitivity to AdFU-induced cytotoxicity.

The kinetic of the cytotoxic activity of (−)-AdFU clearly suggests that apoptosis is the exclusive form of cell death induced by this drug in lymphoid and monocytoid cells. Actually, even cytotoxity detected by the trypan blue test at 72 h can be reasonably attributable to late apoptosis, rather than to other forms of cell death.

Moreover, values determined using the free base 5-fluorouracil in Molt-3 cells in parallel, for comparative purposes, revealed a different profile of potency and kinetic of cell death induction, suggesting that (−)-ADFU activity is not due simply to release of 5-FU by the (−)-ADFU molecule.

On the basis of these results, we have further investigated the capacity of (−)-AdFU to modulate apoptosis in Molt-3 lymphoid cells. First, interaction with Fas-induced apoptosis was investigated. As shown in Figure 2, we found that (−)-AdFU had remarkable Fas-potentiating activity. Infact, (−)-AdFU at concentrations which induced low levels of apoptosis was able to enhance apoptotic cell death triggered by anti-Fas added to the cells at suboptimal concentrations.

To obtain further information on the mechanisms regulating AdFU-induced apoptosis, the possible involvement of the caspase cascade was investigated using two approaches. The first approach consisted of adding synthetic, cell-permeable, noncleavable peptide analogues of caspase substrates to AdFU-treated cultures to irreversibly inhibit protease activity. Z-VAD-FMK, pan-caspase, Z-DEVD-FMK, caspase-3 specific, and Z-LEHD-FMK, caspase-9 specific, inhibitors all significantly reduced apoptosis of infected cultures in comparison with control cultures (data not shown). Moreover, the level of caspase-8 enzymatic activity was determined, using a colorimetric assay able to specifically detect substrate cleavage. Comparison at 24 h of

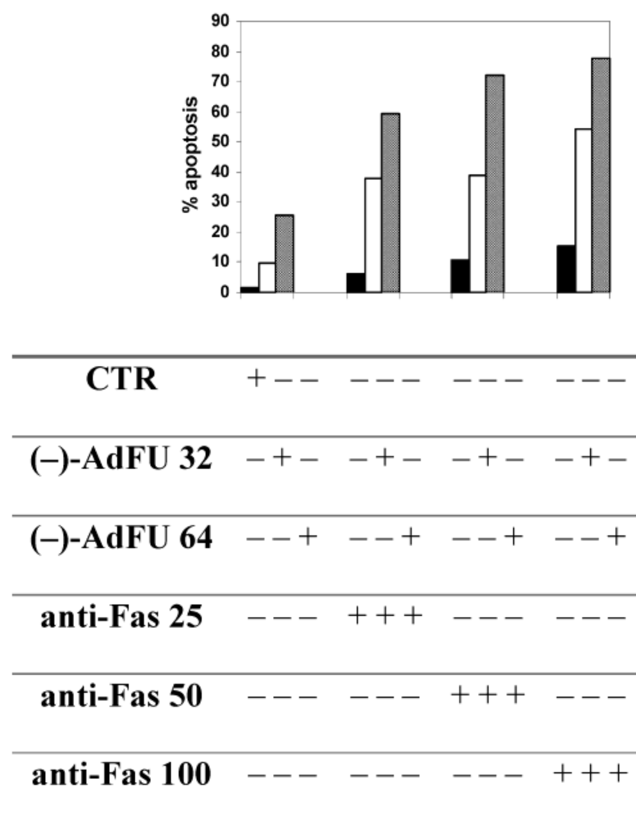


Figure 2. Apoptosis modulation by (−)-AdFU in Molt-3 lymphoid cells. Combination treatment of cell with (−)-AdFU plus anti-Fas (CH11); Molt-3 cells were incubated with 25, 50, or 100 ng/mL anti-Fas, either alone or immediately after cells had been exposed to (−)-AdFU at the concentrations 32 or 64 μM.

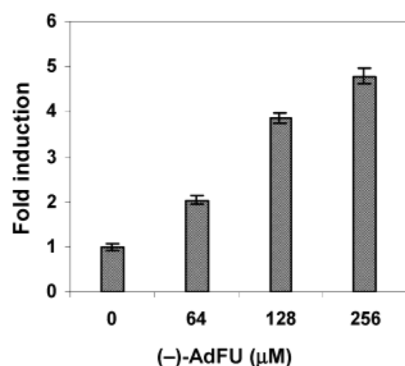


Figure 3. Dose-dependent activity of caspase-8 by (−)-AdFU; results are expressed as fold induction of caspase-8 activity from samples of (−)-AdFU-treated cells at the concentrations 64, 128, or 256 μM with respect to control cells exposed to medium alone and cultured without (−)-AdFU. Mean values ± SD obtained from three replicate determinations are reported

the absorbance from samples of (−)-AdFU-treated cells with samples from untreated controls showed a significant, dose-dependent increase in protease activity following the treatment (Figure 3). Thus, the involvement of the caspase-cascade, including apical caspases, was clearly demonstrated in (−)-AdFU-induced apoptosis.

Conclusions

Enantiomers of 4′-aza-2′,3′-dideoxynucleosides have been prepared by two different synthetic approaches. The two-step procedure, based on the 1,3-dipolar cycloaddition of the chiral nitrone **4** with vinyl acetate and the subsequent Vorbrüggen nucleosidation, leads to improved yields.

The cytotoxicity and the apoptotic activity of the obtained compounds have been investigated. The obtained data indicate that AdFU, while presenting low levels of cytotoxicity assessed by a conventional assay to detect viability, such as the trypan blue exclusion test, is, conversely, a good inductor of cell death by apoptosis on lymphoid and, less efficiently, monocytoid cells. More important, AdFU seems to act as a strong potentiator of Fas-induced cell death, opening new perspectives in future investigations on its possible use as a therapeutic agent.

Experimental Section

General. Melting points were determined with a Kofler apparatus and are uncorrected. Elemental analyses were performed with a Perkin-Elmer elemental analyzer. NMR spectra were recorded on a Varian instrument at 200 or 500 MHz (^{1}H) and at 50 or 125 MHz (^{13}C) using deuteriochloroform or deuterated methanol as solvent; chemical shifts are given in ppm from TMS as internal standard. Thin-layer chromatographic separations were performed on Merck silica gel 60-F_{254} precoated aluminum plates. Preparative separations were carried out by column and flash chromatography using Merck silica gel 0.063−0.200 mm and 0.035−0.070 mm, respectively, with chloroform−methanol mixtures as eluents. HPLC purifications were made with a preparative column (Microsorb Dynamax 100 Å, 21.4 × 250 mm). The purity of all homochiral compounds has been tested with a Nucleosil Chiral-2, 4 × 250 mm column with mixtures of *n*-hexane−2-propanol as eluents. The identification of samples from different experiments was secured by mixed mps and superimposable NMR spectra.

Starting Materials. Vinyl bases **4a**−**f** were prepared by literature methods.[8] Formaldehyde, vinyl acetate, uracil, 5-fluorouracil, thymine, cytosine, 5-fluorocytosine, adenine, and D-ribose were purchased from Aldrich Co. All solvents were dried according to literature methods.

Method A. General Procedure for 1,3-Dipolar Cycloaddition Reactions between Vinyl Bases 4a−f and Vasella-Type Nitrone 3. A suspension containing vinylbase **4a**−**f** (1 equiv), ribosyl hydroxylamine **2** (1 equiv), and formaldehyde (1 equiv) in chloroform (10 mL) was heated in a sealed vessel at 60 °C under stirring, until the hydroxylamine was consumed (7−8 h); after this time, 0.5 equiv of hydroxylamine and formaldehyde were added, and the mixture was left to react for additional 4 h. Removal of the solvent in a vacuum affords a crude material which was purified by flash chromatography to give a mixture of homochiral isoxazolidines **5a**−**f** and **6a**−**f**, which after separation by HPLC (2-propanol/*n*-hexane) show physical and spectral data listed below.

Method B. General Procedure for the Reaction between Silylated Bases 11a−f and Isoxazolidines 9, 10. A suspension of bases **11a**−**f** (0.62 mmol) in dry acetonitrile (3 mL) was treated with bis(trimethylsilyl)acetamide (BSA) (2.48 mmol; 1.24 mmol for **11f**) and refluxed for 15 min under stirring; for **11f**, BSA was added at rt, and the suspension was stirred for 24 h at rt. To the clear solution obtained were added dropwise a solution of the epimeric isoxazolidines **9**, **10**[6] (0.52 mmol; 0.31 mmol for **11f**) in dry acetonitrile (3 mL) and trimethylsilyl triflate (TMSOTf) (0.78 mmol; 0.06 mmol for **11f**), and the reaction mixture was refluxed for 1 h; for **11f**, TMSOTf was added at rt, and the solution was stirred for 6 h at rt. After being cooled at 0 °C, the solution was neutralized by careful addition of aqueous 5% sodium bicarbonate and then concentrated in vacuo. After addition of dichloromethane (8 mL), the organic phase was separated, washed with water (2 × 10 mL), dried over sodium sulfate, filtered, and evaporated to dryness. The residue was purified by flash chroma-

tography and then by HPLC (2-propanol/*n*-hexane) to give the homochiral isoxazolidines **5a**−**f** and **6a**−**f**.

(5′*R*)-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-Butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-1*H*-pyrimidine-2,4-dione (5a). Yield 24% (method A), 42% (method B); $[\alpha]^{25}_{D}$ = +11.66 (*c* 0.6, $CHCl_3$); mp 82−84 °C, white solid from ethyl acetate. ^1H NMR (200 MHz, $CDCl_3$): δ 1.07 (s, 9H), 1.35 (s, 3H), 1.53 (s, 3H), 2.17−2.22 (m, 1H, $H_{4'b}$), 2.76−2.80 (m, 1H, $H_{4'a}$), 3.04 (dddd, 1H, J = 1.5, 6.5, 8.5, 10.0 Hz, $H_{3'a}$), 3.16 (dddd, 1H, J = 2.0, 5.5, 8.5, 10.5 Hz, $H_{3'b}$), 3.71 (m, 2H, $H_{5''a}$, $H_{5''b}$), 4.31 (dt, J = 1.5, 6 Hz, $H_{4''}$), 4.62 (d, 1H, J = 1.5 Hz, $H_{1''}$), 4.69−4.72 (m, 2H, $H_{2''}$, $H_{3''}$), 5.59 (d, J = 8.5 Hz, H_5), 6.22 (dd, 1H, J = 3.7 Hz, $H_{5'}$), 7.37−7.44 (m, 5H, aromatic protons), 7.45 (d, J = 8.5 Hz, H_6), 7.47−7.67 (m, 5H, aromatic protons), 8.66 (bs, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 25.13, 26.80, 26.85, 36.34, 48.67, 64.23, 81.67, 83.02, 84.30, 86.37, 100.02, 102.22, 113.30, 127.77, 127.81, 129.93, 132.99, 133.06, 135.50, 135.54, 139.71, 150.00, 162.94. Anal. ($C_{31}H_{39}N_3O_7Si$) C, H, N.

(5′*S*)-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-Butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-1*H*-pyrimidine-2,4-dione (6a). Yield 16% (method A), 30% (method B); $[\alpha]^{25}_{D}$ = −29.9 (*c* 0.6, $CHCl_3$); mp 68−70 °C, white solid from ethyl acetate. ^1H NMR (200 MHz, $CDCl_3$): δ 1.07 (s, 9H), 1.34 (s, 3H), 1.54 (s, 3H), 2.19−2.25 (m, 1H, $H_{4'b}$), 2.73−2.80 (m, 1H, $H_{4'a}$), 3.14−3.17 (m, 2H, $H_{3'a}$, $H_{3'b}$), 3.70−3.74 (m, 2H, $H_{5''a}$, $H_{5''b}$), 4.26 (dt, J = 3.0, 6.0 Hz, $H_{4''}$), 4.58 (dd, 1H, J = 4, 6.0 Hz, $H_{3''}$), 4.68 (dd, 1H, J = 2.5, 6.5 Hz, $H_{2''}$), 4.81 (d, 1H, J = 2.5 Hz, $H_{1''}$), 5.61 (d, 1H, J = 8.5 Hz, H_5), 5.94 (dd, J = 3.0, 7.5 Hz, $H_{5'}$), 7.36−7.46 (m, 5H, aromatic protons), 7.59 (d, 1H, J = 8.5 Hz, H_6), 7.65−7.68 (m, 5H, aromatic protons), 8.54 (bs, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 25.38, 26.78, 27.17, 37.10, 48.15, 64.08, 81.23, 82.77, 84.33, 85.98, 99.02, 102.16, 113.38, 127.75, 127.77, 129.85, 129.85, 129.91, 133.13, 133.20, 135.56, 139.99, 149.91, 163.00. Anal. ($C_{31}H_{39}N_3O_7Si$) C, H, N.

(5′*R*)-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-Butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-5-fluoro-1*H*-pyrimidine-2,4-dione (5b). Yield 23% (method A), 46% (method B).[6] Physical and spectroscopic data are identical to these previously reported.[6]

(5′*S*)-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-Butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-5-fluoro-1*H*-pyrimidine-2,4-dione (6b). Yield 17% (method A), 26% (method B).[6] Physical and spectroscopic data are identical to these previously reported.[6]

(5′*R*)-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-Butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-5-methyl-1*H*-pyrimidine-2,4-dione (5c). Yield 25% (method A), 42% (method B).[6] Physical and spectroscopic data are identical to these previously reported.[6]

(5′*S*)-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-Butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-5-methyl-1*H*-pyrimidine-2,4-dione (6c). Yield 15% (method A), 30% (method B).[6] Physical and spectroscopic data are identical to these previously reported.[6]

(5′*R*)-4-Amino-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo-[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-1*H*-pyrimidin-2-one (5d). Yield 22% (method A), 44% (method B); $[\alpha]^{25}_{D}$ = −14.6 (*c* 1.02, $CHCl_3$); mp 80−82 °C, yellow solid from ethyl acetate. ^1H NMR (200 MHz, $CDCl_3$): δ 1.06 (s, 9H), 1.35 (s, 3H), 1.53 (s, 3H), 2.23 (dddd, 1H, J = 1.5, 2.5, 7.0, 13.5 Hz, $H_{4'a}$), 2.79 (dq, 1H, J = 6.0, 7.0 Hz, $H_{4'b}$), 3.07 (m, 2H, $H_{3'a}$, $H_{3'b}$), 3.72 (ddd, 2H, J = 6.0, 7.0, 11.0, Hz, $H_{5''}$), 4.28 (dt, 1H, J = 1.5, 5.5 Hz, $H_{4''}$), 4.65 (d, 1H, J = 2.0 Hz, $H_{1''}$), 4.71 (m, 2H, $H_{2''}$ and $H_{3''}$), 5.53 (d, 1H, J = 7.0 Hz, H_5), 6.21 (dd, 1H, J = 2.5, 7.0 Hz, $H_{5'}$), 7.36−7.44 (m, 5H, aromatic protons), 7.56 (d, 1H, J = 7.0 Hz, H_6), 7.64−7.66 (m, 5H, aromatic protons); ^{13}C NMR (50 MHz, $CDCl_3$): δ 19.21, 25.17, 26.79, 36.63, 47.81, 64.29, 81.45, 82.89, 85.60, 86.20, 93.42, 99.69, 113.25, 127.76, 129.86, 133.08, 133.13, 135.27, 141.14, 155.67, 165.60. Anal. ($C_{31}H_{40}N_4O_6Si$) C, H, N.

(5′*S*)-4-Amino-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo-[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-1*H*-pyrimidin-2-one (6d). Yield 18% (method A), 28% (method B); $[\alpha]^{25}_{D}$ = +4.96 (*c* 0.3, $CHCl_3$); pale yellow oil. ^1H NMR (200 MHz, $CDCl_3$): δ 1.06 (s, 9H), 1.35 (s, 3H), 1.54 (s, 3H), 2.24 (dddd, 1H, J = 3.4, 5.7, 8.5, 13.5 Hz, $H_{4'a}$), 2.82 (m, 1H, $H_{4'b}$), 3.09 (m, 2H, $H_{3'}$), 3.70 (dd, 1H, J = 7.0, 10.5 Hz, $H_{5''a}$), 3.74 (dd, 1H, J = 5.5, 10.5 Hz, $H_{5''b}$), 4.27 (ddd, 1H, J = 2.5, 6.5, 7.0 Hz, $H_{4''}$), 4.60 (dd, 1H, J = 2.5, 6.5 Hz, $H_{3''}$), 4.67 (dd, 1H, J = 2.5, 6.0 Hz, $H_{2''}$), 4.79 (d, 1H, J = 2.5 Hz, $H_{1''}$), 5.49 (d, 1H, J = 7.0 Hz, H_5), 5.97 (dd, 1H, J = 3.0, 7.0 Hz, $H_{5'}$), 7.37−7.44 (m, 5H, aromatic protons and H_6); ^{13}C NMR (50 MHz, $CDCl_3$): δ 19.22, 25.40, 26.81, 27.15, 29.68, 37.56, 47.99, 64.02, 81.51, 82.77, 85.52, 85.82, 93.16, 99.54, 113.28, 127.77, 129.86, 135.61, 141.38, 155.42, 165.43. Anal. ($C_{31}H_{40}N_4O_6Si$) C, H, N.

(5′*R*)-4-Amino-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo-[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-5-fluoro-1*H*-pyrimidin-2-one (5e). Yield 37%; $[\alpha]^{25}_{D}$ = +117.65 (*c* 1.4, $CHCl_3$); amorphous solid. ^1H NMR (200 MHz, $CDCl_3$): δ 1.06 (s, 9H), 1.35 (s, 3H), 1.53 (s, 3H), 2.21−2.26 (m, 1H, $H_{4'b}$), 2.75−2.82 (m, 1H, $H_{4'a}$), 3.01−3.12 (m, 2H, $H_{3'a}$, $H_{3'b}$), 3.72 (ddd, 2H, J = 6, 11 Hz, $H_{5''a}$, $H_{5''b}$), 4.28 (t, 1H, J = 6 Hz, $H_{4''}$), 4.61 (s, 1H, $H_{1''}$), 4.71 (s, 2H, $H_{2''}$, $H_{3''}$), 6.14 (dd, 1H, J = 2.5, 8 Hz, $H_{5'}$), 7.37−7.39 (m, 5H, aromatics H),7.41 (d, 1H, J = 7.5 Hz, H_6), 7.58−7.71 (m, 5H, aromatics H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 25.13, 25.32, 26.78, 36.54, 47.74, 64.17, 81.41, 82.90, 85.63, 85.21, 113.29, 127.75, 129.87, 135.54, 138.71, 153.74, 157.82, 158.10. Anal. ($C_{31}H_{39}FN_4O_6Si$) C, H, N.

(5′*S*)-4-Amino-1-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo-[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-5-fluoro-1*H*-pyrimidin-2-one (6e). Yield 18% (method A), 34% (method B); $[\alpha]^{25}_{D}$ = −47.3 (*c* 2.5, $CHCl_3$); amorphous solid. ^1H NMR (200 MHz, $CDCl_3$): δ 1.06 (s, 9H), 1.35 (s, 3H), 1.54 (s, 3H), 2.23−2.28 (m, 1H, $H_{4'b}$), 3.07−3.17 (m, 1H, $H_{4'a}$), 3.67−3.76 (m, 2H, $H_{5''a}$, $H_{5''b}$), 4.20−4.27 (m, 1H, $H_{4''}$), 4.59 (dd, 1H, J = 3, 6 Hz, $H_{3''}$), 4.69 (dd, 1H, J = 2.5, 6 Hz, $H_{2''}$), 4.81 (d, 1H, J = 2.5 Hz, $H_{1''}$), 5.93 (dd, 1H, J = 4.5, 8 Hz, $H_{5'}$), 7.36−7.44 (m, 5H, aromatics H), 7.61−7.66 (m, 5H, aromatics H), 7.72 (d, 1H, J = 6 Hz, H_6); ^{13}C NMR (50 MHz, $CDCl_3$): δ 25.42, 26.82, 27.20, 26.68, 37.40, 47.74, 64.07, 81.39, 82.71, 85.54, 85.82, 99.22, 113.36, 127.74, 129.85, 133.19, 135.57, 153.62, 157.48, 157.74. Anal. ($C_{31}H_{39}FN_4O_6Si$) C, H, N.

(5′*R*)-9-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-Butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-9*H*-purin-6-ylamine (5f). Yield 29%; $[\alpha]^{25}_{D}$ = +83.7 (*c* 0.9, $CHCl_3$); amorphous solid. ^1H NMR (200 MHz, $CDCl_3$): δ 0.92 (s, 9H), 1.34 (s, 3H), 1.53 (s, 3H), 2.60−2.75 (m, 1H, $H_{4'b}$), 2.88−3.03 (m, 1H, $H_{4'a}$), 3.23−3.35 (m, 1H, $H_{3'a}$), 3.37−3.51 (m, 1H, $H_{3'b}$), 3.65−3.77 (m, 2H, $H_{5''a}$, $H_{5''b}$), 4.23 (dt, J = 1.5, 4.5 Hz, $H_{4''}$), 4.70 (d, 1H, J = 2 Hz, $H_{1''}$), 4.60−4.73 (m, 2H, $H_{2''}$, $H_{3''}$), 5.97 (bs, 2H, NH_2), 6.47 (dd, J = 2.4, 7.2 Hz, $H_{5'}$), 7.39−7.46 (m, 5H, aromatics H), 7.47−7.68 (m, 5H, aromatics H), 8.07 (s, 1H, H_2), 8.34 (s, 1H, H_8); ^{13}C NMR (50 MHz, $CDCl_3$): δ 25.08, 25.80, 26.82, 35.68, 48.35, 63.52, 80.82, 82.95, 83.05, 86.51, 99.70, 113.26, 127.77, 129.93, 132.99,145,22, 153.48, 155.47. Anal. ($C_{32}H_{40}N_6O_5Si$) C, H, N.

(5′*S*)-9-{2-[(3a*R*,4*R*,6*R*,6a*S*)-6-(*tert*-Butyl-diphenyl-silanyloxymethyl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxol-4-yl]-isoxazolidin-5-yl}-9*H*-purin-6-ylamine (6f). Yield 25%; $[\alpha]^{25}_{D}$ = −49.5 (*c* 1.1, $CHCl_3$); amorphous solid. ^1H NMR (200 MHz, $CDCl_3$): δ 0.90 (s, 9H), 1.32 (s, 3H), 1.54 (s, 3H), 2.63−2.73 (m, 1H, $H_{4'b}$), 2.93−3.02 (m, 1H, $H_{4'a}$), 3.24−3.34 (m, 2H, $H_{3'a}$), 3.37−3.55 (m, 1H, $H_{3'b}$), 4.13−4.26 (m, 2H, $H_{5''a}$, $H_{5''b}$), 4.21 (dt, J = 2, 5 Hz, $H_{4''}$), 4.43−4.68 (m, 2H, $H_{2''}$, $H_{3''}$), 4.85 (d, 1H, J = 2.6 Hz, $H_{1''}$), 6.24 (bs, 2H, NH_2), 6.43 (dd, J = 2.6, 7.4 Hz, $H_{5'}$), 7.36−7.46 (m, 5H, aromatics H), 7.65−7.68 (m, 5H, aromatics H), 8.02 (s, 1H, H_2), 8.29 (s, 1H,

H_8); ^{13}C NMR (50 MHz, $CDCl_3$): δ 25.34, 26.79, 27.17, 36.60, 48.67, 63.50, 81.52, 82.84, 83.33, 85.99, 99.84, 113.04, 127.75, 129.85, 133.13, 145.43, 153.12, 155.51. Anal. ($C_{32}H_{40}N_6O_5Si$) C, H, N.

General Procedure for Hydrolysis of Homochiral Isoxazolidines 5a−f and 6a−f. Isoxazolidines **5a**−**f** and **6a**−**f** were dissolved in a 1.5% (weight by weight) HCl solution in EtOH (2.5 mL), and the reaction mixture was stirred at room temperature for 3 h. The solution was brought to pH 10 by adding aqueous 10% sodium carbonate and extracted with dichloromethane (2 × 10 mL). The organic phase, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was purified by radial chromatography (chloroform/methanol 9:1) to furnish homochiral *N,O*-nucleosides **7a**−**f** and **8a**−**f**.

(5′*R*)-1-Isoxazolidin-5-yl-1*H*-pyrimidine-2,4-dione (7a). Yield 91%; $[\alpha]^{25}_D$ = +97.5 (*c* 0.19; CH_3OH); mp 183−185 °C, white solid from methanol. ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.62 (m, 1H, $H_{2'b}$), 3.12 (m, 1H, $H_{3'a}$), 3.87 (m, 1H, $H_{3'b}$), 5.82 (d, 1H, J = 7.5 Hz, H_5), 6.19 (dd, 1H, J = 3.1,7.9 Hz, $H_{5'}$), 7.41 (d, 1H, J = 7.5 Hz, H_6), 10.28 (bs, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 30.98, 47.34, 85.52, 100.80, 102.18, 151.06, 164.22. Anal. ($C_7H_9N_3O_3$) C, H, N.

(5′*S*)-1-Isoxazolidin-5-yl-1*H*-pyrimidine-2,4-dione (8a). Yield 92%; $[\alpha]^{25}_D$ = −84.7 (*c* 0.23; CH_3OH); mp 183−185 °C, white solid from methanol. Anal. ($C_7H_9N_3O_3$) C, H, N.

(5′*R*)-5-Fluoro-1-isoxazolidin-5-yl-1*H*-pyrimidine-2,4-dione (7b). Yield 90%; $[\alpha]^{25}_D$ = +52.4 (*c* 0.43; CH_3OH); mp 155−157 °C (lit.[6] 153−155 °C), white solid from methanol. Anal. ($C_7H_8FN_3O_3$) C, H, N. Physical and spectroscopic data are identical to these previously reported.[6]

(5′*S*)-5-Fluoro-1-isoxazolidin-5-yl-1*H*-pyrimidine-2,4-dione (8b). Yield 89%; $[\alpha]^{25}_D$ = −63.8 (*c* 0.17; CH_3OH); white solid from methanol, mp 155−157 °C (lit.[6] 153−155 °C). Anal. ($C_7H_8FN_3O_3$) C, H, N. Physical and spectroscopic data are identical to these previously reported.[6]

(5′*R*)-1-Isoxazolidin-5-yl-5-methyl-1*H*-pyrimidine-2,4-dione (7c). Yield 93%; $[\alpha]^{25}_D$ = −82.7 (*c* 0.21; CH_3OH); mp 201−203 °C, white solid from methanol (lit.[6] 202−204 °C). Anal. ($C_8H_{11}N_3O_3$) C, H, N. Physical and spectroscopic data are identical to these previously reported.[6]

(5′*S*)-1-Isoxazolidin-5-yl-5-methyl-1*H*-pyrimidine-2,4-dione (8c). Yield 91%; $[\alpha]^{25}_D$ = +94.3 (*c* 0.14; CH_3OH); mp 201−203 °C, white solid from methanol (lit.[6] 202−204 °C). Anal. ($C_8H_{11}N_3O_3$) C, H, N. Physical and spectroscopic data are identical to these previously reported.[6]

(5′*S*)-4-Amino-1-isoxazolidin-5-yl-1*H*-pyrimidin-2-one (7d). Yield 94%; $[\alpha]^{25}_D$ = −12.7 (*c* 0.3; CH_3OH); mp 190−194 °C, amorphous solid. ^{1}H NMR (200 MHz, CD_3OD): δ 2.39−2.54 (m, 1H, $H_{4'a}$), 2.57−2.71 (m, 1H, $H_{4'b}$), 3.11−3.34 (m, 2H, $H_{3'a,}$ $H_{3'b}$), 5.89 (d, 1H, J = 7.4 Hz, H_5), 5.98 (dd, J = 3.6, 7.2 Hz, $H_{5'}$), 7.63 (d, 1H, J = 7.4 Hz, H_6); ^{13}C NMR (50 MHz, CD_3OD): δ 37.42, 49.52, 90.96, 95.89, 143.75, 158.34, 167.37. Anal. ($C_7H_{10}N_4O_2$) C, H, N.

(5′*R*)-4-Amino-1-isoxazolidin-5-yl-1*H*-pyrimidin-2-one (8d). Yield 89%; $[\alpha]^{25}_D$ = +15.3 (*c* 0.55; CH_3OH); mp 190−194 °C, amorphous solid. ^{1}H NMR (200 MHz, CD_3OD): δ 2.42−2.52 (m, 1H, $H_{4'a}$), 2.56−2.73 (m, 1H, $H_{4'b}$), 3.19−3.30 (d, 1H, J = 7.4 Hz, H_5), 5.97 (m, 1H, $H_{5'}$), 7.63 (d, 1H J = 7.4 Hz, H_6); ^{13}C NMR (50 MHz, CD_3OD): δ 37.49, 49.28, 90.84, 95.87, 143.91, 158.38, 167.81. Anal. ($C_7H_{10}N_4O_2$) C, H, N.

(5′*R*)-4-Ammino-5-fluoro-1-isossazolidin-5-yl-1*H*-pyrimidin-2-one (7e). Yield 94%; $[\alpha]^{25}_D$ = +12.7 (*c* 0.3; CH_3OH); amorphous solid. ^{1}H NMR (200 MHz, CD_3OD): δ 2.42 (m, 1H, $H_{4'a}$), 2.64(m, 1H, $H_{4'b}$), 3.20 (m, 2H, H3′a, H3′b), 5.98 (dd, 1H, J = 3.5, 7 Hz, $H_{5'}$), 7.62 (d, 1H, J = 6 Hz, H_6). ^{13}C NMR (50 MHz, CD_3OD): δ 37.42, 46.42, 90.96, 128.21, 143.75, 158.34, 167.37. Anal. ($C_7H_9FN_4O_2$) C, H, N.

(5′*S*)-4-Ammino-5-fluoro-1-isossazolidin-5-yl-1*H*-pyrimidin-2-one (8e). Yield 89%; $[\alpha]^{25}_D$ = −15.3 (*c* 0.35; CH_3OH); amorphous solid. Anal. ($C_7H_9FN_4O_2$) C, H, N.

(5′*R*)-9-Isoxazolidin-5-yl-9*H*-purin-6-ylamine (7f). Yield 71%; $[\alpha]^{25}_D$ = +24.6 (*c* 1.4, $CHCl_3$); amorphous solid. Anal. ($C_8H_{10}N_6O$) C, H, N. Physical and spectroscopic data are identical to these previously reported.

(5′*S*)-9-Isoxazolidin-5-yl-9*H*-purin-6-ylamine (8f). Yield 69%; $[\alpha]^{25}_D$ = −21.4 (*c* 1.4, $CHCl_3$); amorphous solid. Anal. ($C_8H_{10}N_6O$) C, H, N. Physical and spectroscopic data are identical to these previously reported.

Evaluation of Toxicity and Apoptosis. Toxicity was evaluated by a standard viability assay, using the trypan blue exclusion test. Normally, apoptosis was evaluated by morphological analysis of the cells, performed following staining with acridine orange as previously described.[12] Briefly, over 600 cells, including those showing typical apoptotic characteristics, were counted using a fluorescence microscope. The identification of apoptotic cells was based on the presence of uniformly stained nuclei showing chromatin condensation and nuclear fragmentation. In some experiments, apoptosis was detected by flow cytometry analysis of isolated nuclei, following staining with propidium iodide, on a Becton Dickinson FACScan Analytic Flow Cytometer, as previously described.[13]

To evaluate Fas-induced apoptosis, anti-human Fas antibody (clone CH11, Upstate Biotechnology Inc., Lake Placid, NY) was added at various concentrations to the untreated or AdFU-treated cells in 96-well plates. Cultures were then incubated for 24 h at 37 °C.

Assay for Caspase-8 Activity and Caspase-Inhibition Experiments. Caspase-8 activity was assayed using the ApoAlert colorimetric assay kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. Enzyme activity was expressed as fold increase with respect to control cells. To inhibit protease activity, synthetic, cell-permeable, noncleavable peptide analogues of caspase substrates were added to infected and control cultures. Z-VAD-FMK, Z-DEVD-FMK and Z-LEHD-FMK (Calbiochem, San Diego, CA) irreversible inhibitors were utilized.

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