Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1731–1738

BIOORGANIC & MEDICINAL CHEMISTRY

5-(Trifluoromethyl)-β-L-2′-deoxyuridine, the L-Enantiomer of Trifluorothymidine: Stereospecific Synthesis and Antiherpetic Evaluations

Raul Salvetti,[a] Arnaud Marchand,[d] Massimo Pregnolato,[a] Annalisa Verri,[b] Silvio Spadari,[b] Federico Focher,[b] Martin Briant,[c] Jean-Pierre Sommadossi,[c] Christophe Mathé[d,*] and Gilles Gosselin[d,e]

[a]*Dipartimento di Chimica Farmaceutica, Università degli Studi, I-27100 Pavia, Italy*
[b]*Istituto di Genetica Biochimica ed Evoluzionistica, CNR, I-27100 Pavia, Italy*
[c]*Novirio-Pharmaceuticals, Inc., Cambridge, MA, USA*
[d]*Laboratoire de Chimie Organique Biomoléculaire de Synthèse, U.M.R. 5625 CNRS-UM II, Université Montpellier II, 34095 Montpellier, France*
[e]*Laboratoire Coopératif Novirio-CNRS-Université Montpellier II, 34095 Montpellier, France*

Received 9 January 2001; revised 27 February 2001; accepted 2 March 2001

Abstract—As a part of our ongoing work on β-L-nucleoside analogues as potential antiviral drugs, we have synthesized 5-(trifluoromethyl)-β-L-2′-deoxyuridine (L-TFT), the hitherto unknown L-enantiomer of trifluorothymidine (CF_3dUrd, TFT). We have also studied the effect of L-TFT on human and herpes simplex virus (HSV) type 1 and 2 thymidine kinases, and human thymidine phosphorylase, as well as its anti-HSV-1 and anti-HSV-2 activities in cell cultures. L-TFT has been found: (i) to inhibit HSV-1 TK with activity comparable to TFT, with no effect on human TK, (ii) to be phosphorylated by the viral enzyme with similar efficiency to TFT, (iii) to be resistant, in contrast to TFT, to hydrolysis by human thymidine phosphorylase. Unfortunately, when evaluated in cell cultures, L-TFT did not show any anti-HSV-1 and anti-HSV-2 activities.

Introduction

Infections by herpes viruses are among the most common and easily transmitted human viral diseases. Numerous distinct herpes viruses have been identified and the treatment by therapeutic agents of diseases caused by some of them, such as herpes simplex virus (HSV) has produced clinical benefit. Among these drugs, 5-(trifluoromethyl)-β-D-2′-deoxyuridine [CF_3dUrd, trifluorothymidine (TFT)][1] has been approved by the Food and Drug Administration since 1980 for use in the topical treatment of primary keratoconjonctivitis and epithelial keratitis.[2] The mode of action of TFT is based on its intracellular conversion to TFT monophosphate by both cellular and virus-encoded thymidine kinase. Further phosphorylation steps by nucleotide kinases gives TFT triphosphate which is preferentially incorporated into viral DNA.[3] TFT, as well as its phosphorylated metabolites, can also inhibit viral DNA biosynthesis by acting as competitive inhibitors of virus-encoded thymidine kinase[4] and HSV DNA polymerase[2] with respect to thymidine and thymidine triphosphate. TFT shows also a concomitant cytotoxicity in uninfected cells probably related to the inhibition of cellular enzymes including human thymidine kinase, thymidylate synthase as well as DNA polymerases.[5,6]

In the last years, L-nucleoside analogues, the mirror images of the natural D-nucleosides, have drawn considerable attention as potential antiviral drugs.[7] The possibility that L-nucleoside derivatives may be more efficient than the corresponding D-enantiomers, owing to their powerful antiviral activity and favorable toxicity profile, has been demonstrated for some of them.[8] On the basis of these findings, we thought it was of interest to synthesize the hitherto unknown L-enantiomer (5-CF_3-β-L-dUrd **6**, L-TFT) of TFT, in order to study its effect on human and herpes simplex virus type

*Corresponding author. Tel.: +33-4-6714-4776; fax: +33-4-6704-2029; e-mail: cmathe@univ-montp2.fr

0968-0896/01/$ - see front matter
PII: S0968-0896(01)00062-1

1 and 2 thymidine kinases, human thymidine phosphorylase (TK, HSV-1 TK, HSV-2 TK and TP, respectively) as well as its anti-HSV-1 and HSV-2 activities in cell cultures, comparatively to TFT.

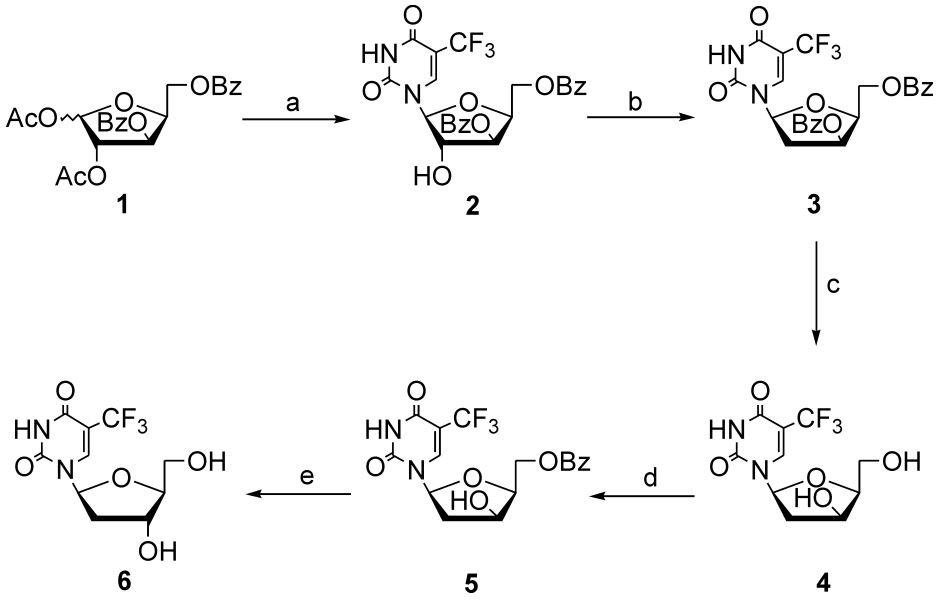
Results and Discussion

Chemistry

Several methodologies have been described in the literature for the preparation of 2′-deoxynucleoside analogues.[9] For our purpose, we decided to synthesize in a stereospecific manner the target molecule **6** by condensing a suitable protected L-pentofuranose sugar with the commercially available 5-(trifluoromethyl)uracil. In accord with Baker's rule,[10,11] and owing to 2-*O*-acyl participation during condensation, we selected as starting sugar 1,2-di-*O*-acetyl-3,5-di-*O*-benzoyl-L-*xylo*-furanose **1** (Scheme 1). This compound was readily prepared from L-xylose following a synthetic pathway previously described by Gosselin et al.[12] The glycosylation reaction of silylated 5-(trifluoromethyl)uracil with **1** was carried out under Vörbruggen conditions, in anhydrous 1,2-dichloroethane using (trimethylsilyl) trifluoromethane sulfonate (TMSOTf) as a catalyst.[13] From the crude coupling product, regioselective 2′-*O*-deacetylation with hydrazine hydrate gave 1-(3,5-di-*O*-benzoyl-β-L-*xylo*-furanosyl)-5-(trifluoromethyl)uracil **2** in 82% overall yield after silica gel column chromatography. Compound **2** was then reacted with *O*-phenyl chloro(thio)formate (PhOC(S)Cl) and 4-dimethylaminopyridine (DMAP) in anhydrous 1,2-dichloroethane to give the corresponding 2′-*O*-[phenoxy(thiocarbonyl)] intermediate, which was subsequently deoxygenated with tris(trimethylsilyl)silane in dry dioxane in the presence of α,α′-azoisobutyronitrile (AIBN), thus affording the 2′-deoxy-β-L-*threo* derivative **3** as a white foam in 92% overall yield. Deprotection of **3** with solid sodium methoxide in dry methanol provided the unprotected 2′-deoxynucleoside **4** as a crystalline solid in 67% after purification. In order to prepare the target compound **6**, the 2′-deoxy-β-L-*threo*-pentofuranonucleoside **4** was selectively converted into its 5′-*O*-benzoyl derivative **5**. Inversion of configuration at C-3′ was achieved via Mitsunobu conditions[14] by using benzoic acid as incoming nucleophile. The reaction of **5** with diethyl azodicarboxylate (DEAD), benzoic acid and triphenylphosphine (PPh_3) at 0 °C, followed by debenzoylation with methanolic ammonia provided, after purification on silica gel column, the desired enantiomer L-TFT **6** as a crystalline white solid in 23% overall yield.

Biological studies

L-TFT specifically inhibits herpes simplex virus thymidine kinases. L-TFT and its D-counterpart (TFT) have been tested against both herpes simplex virus (HSV-1 and HSV-2) and human thymidine kinases. Interestingly, this study demonstrated that the L-enantiomer inhibits HSV-1 TK with an IC_{50} value of 0.5 μM with no effect against human TK at concentrations up to 200 μM, the highest concentration tested (Fig. 1A). Such a result is particularly interesting if we compare it with the values obtained for TFT, the clinically used Viroptic®. In fact, TFT inhibits not only herpes virus TK (HSV-1 TK, $IC_{50} = 0.25$ μM; HSV-2 TK, $IC_{50} = 2$ μM) but also human TK ($IC_{50} = 2$ μM) (Fig. 1B). Table 1, where a comprehensive view of the effect of both enantiomer on HSV-1 and HSV-2 TK's is presented, clearly shows that TFT and L-TFT preferentially inhibit HSV-1 TK. L-TFT was further studied in order to better understand its mechanism of



Scheme 1. Reagents and conditions: (a) (i) silylated 5-trifluoromethyluracil, TMSOTf, $(CH_2Cl)_2$, rt; (ii) H_2NNH_2. H_2O, acetic acid, pyridine, rt; (b) (i) PhOC(S)Cl, DMAP, $(CH_2Cl)_2$, rt; (ii) $(Me_3Si)_3SiH$, AIBN, dioxane, reflux; (c) NaOMe, MeOH, rt; (d) BzCl, pyridine, 0 °C; (e) (i) DEAD, benzoic acid, PPh_3, THF, 0 °C; (ii) MeOH–NH_3, rt.

action. Experiments in which the inhibitor was tested at different substrate (thymidine) concentrations demonstrated that L-TFT inhibits HSV-1 TK with a competitive or partially mixed-type mechanism of action with a Ki value of 0.5 μM (Fig. 2). The kinetic behavior suggests a specific interaction of L-TFT with the active site of the viral enzyme.

L-TFT is phosphorylated by HSV-1 thymidine kinase. To understand whether L-TFT is a non-substrate inhibitor of viral TK or, like its D-counterpart, a substrate-inhibitor, we incubated HSV-1 TK with L-TFT (or its D-enantiomer, as control) in the presence of [γ-^{32}P]-ATP. Resolution of the reaction products was performed by HPLC as described in Experimental. Figure 3 shows the comparison of the chromatograms of the products of reaction in which both enantiomers are tested as substrate. For each enantiomer a specific peak of radioactivity, corresponding to the monophosphate form, follows the elution of ATP. No peak of labeled monophosphate is present when the reaction mixture is immediately heated at 80 °C, centrifuged and injected in HPLC (data not shown). The slight differences in the elution time of D- and L-TFTMP in the two elutions are mirrored by differences in ATP elution, thus they do not depend upon enantiomeric properties of the compounds. This result demonstrates that, in vitro, HSV-1 TK is able to phosphorylate L-TFT as well as D-TFT.

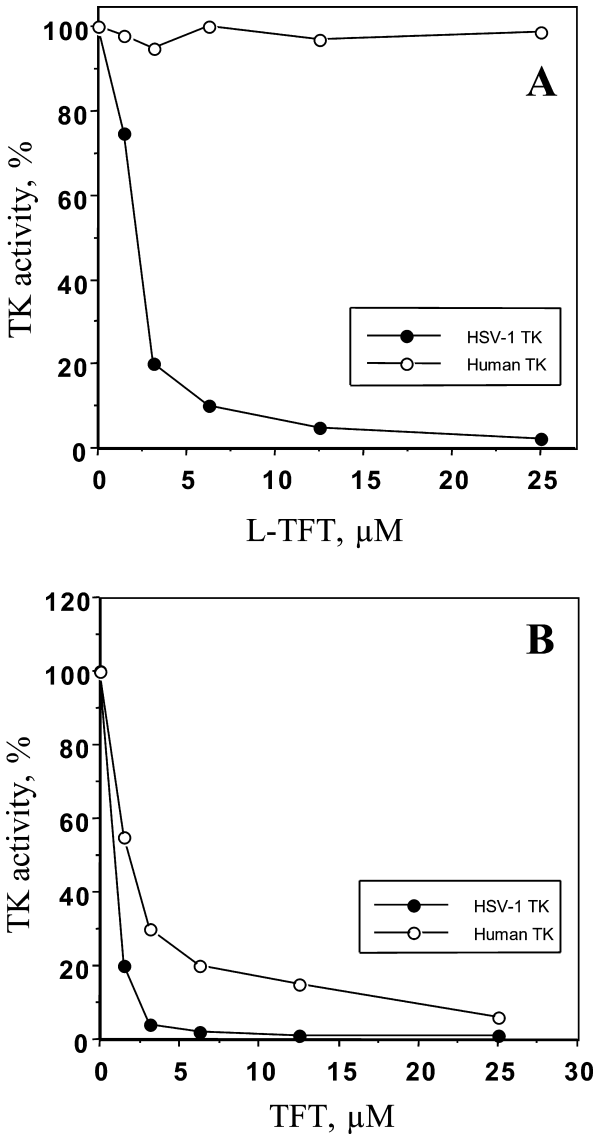


Figure 1. Effect of different concentrations of L-TFT (panel A) or TFT (panel B) on HSV-1 (●) and human (○) thymidine kinase activities.

Table 1. Effect of TFT and L-TFT nucleoside analogues on herpes virus thymidine kinases

Compound	HSV-1 TK IC_{50} (μM)	HSV-2 TK IC_{50} (μM)
TFT	0.25	2.0
L-TFT	0.5	14.5

Under our chromatographic conditions, we can only quantify the presence of ATP and nucleoside monophosphate since the labeled nucleoside diphosphate eventually formed by the thymidylate kinase activity associated with the HSV-1 TK is not completely separated from the peak of labeled ATP. Therefore, we have no information whether L-TFT is further converted to diphosphate by the viral thymidylate kinase activity.

L-TFT is resistant to phosphorolysis by human thymidine phosphorylase. The antiviral activity of several nucleoside analogues is often limited by their intracellular rapid degradation by thymidine phosphorylase (TP). Thus, in an attempt to avoid this degradation and consequently to improve the drug activity, modified nucleosides have been synthesized; in particular, compound in which the D-deoxyribose moiety has been replaced by the L-enantiomer.[7] In order to verify if L-TFT, like L-thymidine and its derivatives,[7] is resistant to human TP degradation, we incubated both enantiomer

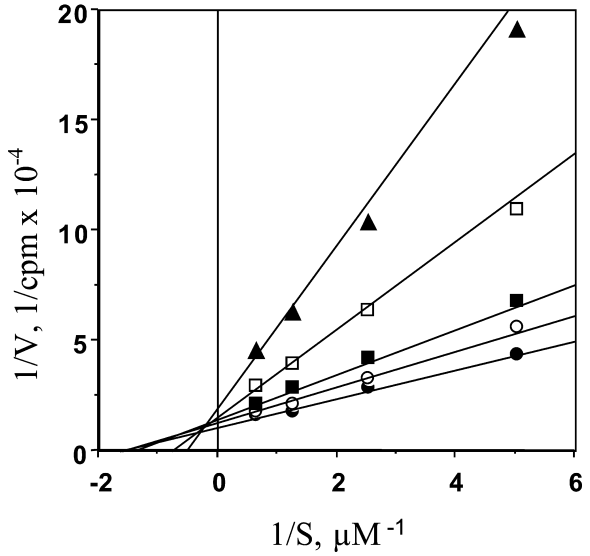


Figure 2. Lineweaver–Burk plot of the effect of L-TFT on the activity of HSV-1 TK in the presence of increasing concentrations of the substrate [^{3}H]-dThd. L-TFT concentrations: 0 μM (●), 0.25 μM (○), 0.5 μM (■), 1 μM (□) and 2 μM (▲).

of TFT with recombinant human TP[15] as described in Experimental, the reaction being followed for 0, 10, 20 and 30 min. Interestingly, L-TFT, contrary to its D-enantiomer, is completely resistant to degradation by human TP (Fig. 4).

Effect of L-TFT on [^{3}H]-thymidine incorporation in HeLa TK$^-$/HSV-1 TK+ cells. In order to verify the ability of L-TFT to inhibit also in vivo thymidine phosphorylation by HSV-1 TK, we have evaluated if L-TFT was able to reduce the [^{3}H]-dThd incorporation into DNA in intact cells. For this study we used HeLa cells TK$^-$/HSV-1 TK$^+$ (HeLa 5a) in which the human TK is replaced by HSV-1 TK and [^{3}H]-dThd incorporation into DNA is strictly dependent upon viral enzyme activity, as demonstrated by the use of specific non-substrate[16] and substrate inhibitors[7] of viral TK. Cells were incubated in the presence of [^{3}H]-dThd and increasing concentrations of L-enantiomer (or D-enantiomer, as control). As expected, TFT strongly affected [^{3}H]-dThd incorporation in HeLa 5a cells, whereas L-enantiomer was inactive (Fig. 5). This fact suggests that L-TFT is probably unable to cross the cell membrane[17] and, indirectly, that an enantioselective active transport is responsible for the cellular uptake of TFT. We assumed that, if present in the cytoplasm, L-TFT would have competed with [^{3}H]-dThd for the enzymatic phosphorylation, thus reducing the amount of [^{3}H]-dThd incorporated into DNA during the DNA synthesis.

Antiviral assays

The antiviral activities of L-TFT, comparatively to TFT, against HSV-1 and HSV-2, were determined by plaque reduction (PR) assays in human foreskin fibroblast (HFF) cells. The results are summarized in Table 2. From these data, it appears that L-TFT does not demonstrate any antiherpetic activity in cell cultures, in contrast to its D-counterpart (TFT).

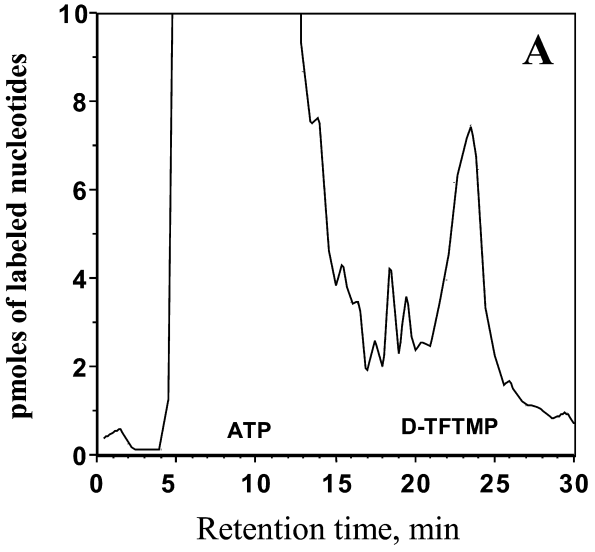


Figure 3. Phosphorylation of TFT and L-TFT by HSV-1 TK. HPLC elution profiles of the reaction products of TFT (panel A) and L-TFT (panel B) after 60 min incubation with HSV-1 TK and [γ^{32}P]-ATP.

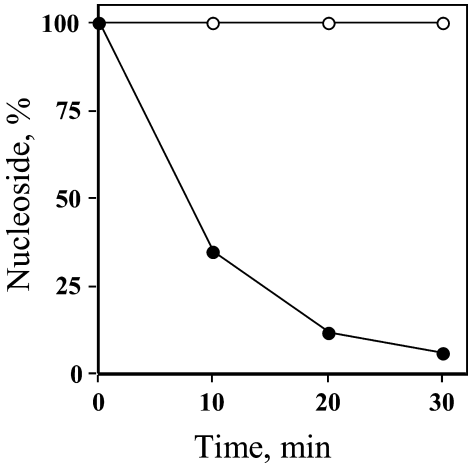


Figure 4. Activity of human thymidine phosphorylase on TFT (●) and L-TFT (○).

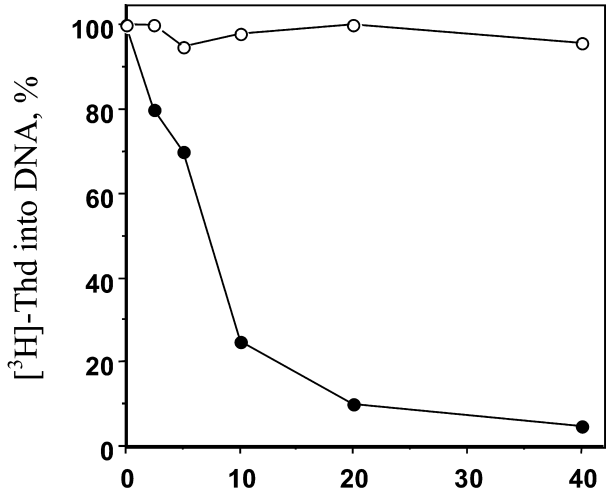


Figure 5. Effect of different concentrations of TFT (●) or L-TFT (○) on DNA synthesis evaluated as [^{3}H]-dThd incorporation in HeLa cells TK$^-$/HSV-1 TK$^+$. 100% corresponds to 3 pmol of acid precipitable [^{3}H]-dTMP after 20 min incubation at 37 °C. Each point is the average of three determinations.

Table 2. Antiviral activities of TFT (Trifluorothymidine) and L-TFT

Compound	Virus (cells)[a]	EC_{50} (μg/mL)[b]	CC_{50} (μg/mL)[c]	SI[d]
TFT	HSV-1	2.2	100	45.5
	HSV-2	10.5	100	9.5
L-TFT	HSV-1	> 50	> 50	—
	HSV-2	> 50	> 50	—

[a]HSV-1 and HSV-2 were propagated in human foreskin fibroblasts (HFF) and virus titer determined by PR assays.
[b]EC_{50} (50% effective concentration) or compound concentration required to reduce viral plaque formation by 50% compared to the untreated control.
[c]CC_{50} (50% cytotoxic concentration) was defined as the compound concentration required to reduce the cell number by 50%.
[d]SI, selectivity index (CC_{50}/EC_{50}).

Conclusion

In this work, we have synthesized in a stereospecific manner 5-(trifluoromethyl)-β-L-2′-deoxyuridine (L-TFT **6**) the hitherto unknown L-enantiomer of TFT, in order to study: i) its affinity for human, HSV-1 and HSV-2 thymidine kinases; (ii) its susceptibility to be phosphorylated by the aforementioned thymidine kinases and to be hydrolyzed by human thymidine phosphorylase; (iii) its effect on [^{3}H]-dThd incorporation in HeLa cells TK^-/HSV-1 TK^+. Our results have demonstrated that L-TFT specifically inhibits HSV thymidine kinases with no effect against human thymidine kinase. The best inhibition was observed with HSV-1 TK with an IC_{50} value comparable to the one obtained with TFT. The mechanism of inhibition for L-TFT is purely competitive. HPLC analysis of the reaction products showed that HSV-1 TK was able to phosphorylate L-TFT to the corresponding monophosphate with efficiency comparable to TFT. In contrast to its D-counterpart, L-TFT was fully resistant to degradation by human thymidine phosphorylase and had no effect on [^{3}H]-dThd incorporation into DNA in intact cells. Anti-HSV-1 and HSV-2 activities of L-TFT were subsequently evaluated in HFF cells using PR assays. Unfortunately, the L-enantiomer of TFT did not show any antiherpetic activity in cell cultures. This lack of antiviral activity could be attributed to a deficiency of cellular uptake, of the anabolic pathway from the starting nucleoside to the 5′-triphosphate form, or the lack of interaction of the L-triphosphate with the viral DNA polymerase. However that may be, the present findings do not preclude the pursuit of studies on β-L-nucleoside analogues as potential antiviral drugs. Experiments related to these topics are currently in progress in our laboratory.

Experimental

Chemistry

Evaporation of solvents was carried out on a rotary evaporator under reduced pressure. Melting points were determined in open capillary tubes on a Gallenkamp MFB-595-010 M apparatus and are uncorrected. UV spectra were recorded on an Uvikon 931 (Kontron) spectrophotometer. ^{1}H NMR spectra were recorded at 400 MHz, ^{13}C NMR spectra at 100 MHz and ^{19}F NMR at 235 MHz in $(CD_3)_2SO$ at room temperature with a Brüker DRX 400. Chemical shifts (δ) are quoted in parts per million (ppm) referenced to the residual solvent peak $(CD_3)CD_2HSO$ being set at δ-$_H$ 2.49 and δ-$_C$ 39.5 relative to tetramethylsilane (TMS). ^{19}F chemical shits are reported using trichlorofluoromethane as external reference. Deuterium exchange and COSY experiments were performed in order to confirm proton assignments. Coupling constants, *J*, are reported in Hertz. 2-D ^{1}H–^{13}C heteronuclear COSY were recorded for the attribution of ^{13}C signals. FAB mass spectra were recorded in the positive-ion or negative-ion mode on a JEOL SX 102. The matrix was a glycerol and thioglycerol mixture (G–T: 50:50, v/v). Specific rotations were measured on a Perkin-Elmer Model 241 spectropolarimeter (path length 1 cm), and are given in units of 10^{-1} cm^2 g^{-1}. Elemental analyses were carried out by the Service de Microanalyses du CNRS, Division de Vernaison (France). Thin layer chromatography was performed on precoated aluminium sheets of Silica Gel 60 F_{254} (Merck, Art. 5554), visualization of products being accomplished by UV absorbance followed by charring with 10% ethanolic sulphuric acid and heating. Column chromatography was carried out on Silica Gel 60 (Merck, Art. 9385). Commercial reagents and solvents analytical grade were used unless otherwise stated. [^{3}H-methyl]-thymidine (25 Ci/mmol) and [γ^{32}P]-ATP (3000 Ci/mmol) were from Amersham (Arlington Heights, IL). 5-(trifluoromethyl)-β-D-2′-deoxyuridine (TFT) (batch 45749000) was purchased from Instel Chimios S.A. (France).

1-(3,5-Di-*O*-benzoyl-β-L-*xylo*-furanosyl)-5-trifluoromethyluracil 2. A mixture of 5-trifluoromethyluracil (6.3 g, 35 mmol), hexamethyldisilazane (200 mL) and a catalytic amount of ammonium sulphate was refluxed under argon for 18 h. The clear solution was concentrated to dryness under reduced pressure. The oily residue was dissolved in dry CH_2Cl_2 (170 mL) and a solution of **1**[12] (17 g, 38.5 mmol) in CH_2Cl_2 (170 mL) and TMSOTf (14 mL, 77.6 mmol) were added. The reaction was stirred for 1 h under argon and diluted with $CHCl_3$ (250 mL). The solution was washed with saturated $NaHCO_3$ (2×400 mL), water (2×200 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. The resulting crude material was dissolved in a mixture of pyridine and acetic acid (4:1, v/v, 250 mL), then hydrazine hydrate (98%, 5.2 mL, 105 mmol) was added. After 15 h stirring, acetone (100 mL) was added and stirring was continued for 2 h. The solvents were removed and the residue was dissolved in CH_2Cl_2 (300 mL). The organic layer was washed with saturated $NaHCO_3$ (2×200 mL) and water (100 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. Silica gel column chromatography of the residue using as eluent a stepwise gradient of MeOH (0–2%) in CH_2Cl_2 afforded compound **2** as a white foam (14.9 g, 82% overall yield from **1**): $[\alpha]_D^{20}$ – 83 (*c* 1.00 in DMSO); UV λ_{max} (EtOH) 264 nm (ε 10100), 231 nm (ε 26200); ^{1}H NMR (DMSO-d_6) δ 4.52 (1H, d, J = 3.9 Hz, 2′-H), 4.65 (1H, m, 5′-H), 4.87 (2H, m, 4′-H and 5″-H), 5.42 (1H, m, 3′-H), 5.73 (1H, s, 1′-H), 6.47 (1H, d, 2′-OH), 7.4–7.9 (10H, m,

2×C_6H_5), 8.20 (1H, s, 6-H), 11.92 (1H, s, 3-NH); ^{13}C NMR (DMSO-d_6) δ 62.7 (5′-C), 78.1 (3′-C), 78.4 (2′-C), 80.6 (4′-C), 93.0 (1′-C), 103.3 (5-C, q, J = 31.8 Hz), 123.5 (CF_3, q, J = 269.5 Hz), 129.5–134.7 (C-Arom), 141.6 (6-C, q, J = 5.9 Hz), 150.3 (2-C), 159.8 (4-C), 165.3 (CO), 166.4 (CO); ^{19}F NMR (DMSO-d_6) δ −61.6 (CF_3); m/z (FAB > 0) 521 $(M+H)^+$, 341 $(S)^+$, 181 $(BH_2)^+$; m/z (FAB < 0) 1039 $(2M-H)^-$, 519 $(M-H)^-$, 179 $(B)^-$. Anal. calcd for $C_{24}H_{19}F_3N_2O_8$/0.5 H_2O: C, 54.45; H, 3.81; N, 5.29; F, 10.77. Found: C, 54.46; H, 3.80; N, 5.57; F, 10.34.

1-(3,5-Di-*O*-benzoyl-2-deoxy-β-L-*threo*-pentofuranosyl)-5-trifluoromethyluracil 3. To a stirred solution of **2** (17.7 g, 34 mmol) in dry CH_2Cl_2 (200 mL) were added successively DMAP (12.4 g, 102 mmol) and phenoxy (thiocarbonyl) chloride (6.90 mL, 51 mmol). After 30 min, the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (200 mL) and the organic layer was washed with 0.5 N HCl (2×200 mL), brine (300 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. The resulting crude material was dissolved in dry dioxane (200 mL) AIBN (2.24 g, 13.6 mmol) and tris(trimethylsilyl)silane (13.6 mL, 44.1 mmol) were added. The resultant solution was heated under reflux for 30 min. After cooling to room temperature, the solvent was removed under reduced pressure. Chromatography of the residue on a silica gel column using as eluent a stepwise gradient of MeOH (0–2%) in CH_2Cl_2 afforded compound **3** as a white foam (15.8 g, 92%): $[\alpha]_D^{20}$ −120 (*c* 1.03 in DMSO); UV λ_{max} (EtOH) 263 nm (ε 11900), 231 nm (e 33200); ^{1}H NMR (DMSO-d_6) 2.50 (1H, m, 2′-H), 2.92 (1H, m, 2″-H), 4.65 (2H, m, 4′-H and 5′-H), 4.80 (1H, m, 5″-H), 5.69 (1H, m, 3′-H), 6.12 (1H, dd, $J_{1'-2'}$ = 1.7 Hz, $J_{1'-2''}$ = 7.6 Hz), 7.4–7.9 (10H, m, 2×C_6H_5), 8.20 (1H, s, 6-H), 11.89 (1H, s, 3-NH); ^{13}C NMR (DMSO-d_6) δ 39.2 (2′-C), 62.9 (5′-C), 74.0 (3′-C), 81.6 (4′-C), 86.5 (1′-C), 103.4 (5-C, q, J = 32.0 Hz), 123.5 (CF_3, q, J = 267.0 Hz), 129.5–134.6 (C-Arom), 141.6 (6-C, q, J = 6.0 Hz), 150.3 (2-C), 159.9 (4-C), 165.5 (CO), 166.3 (CO); ^{19}F NMR (DMSO-d_6) δ −62.3 (CF_3); m/z (FAB > 0) 505 $(M+H)^+$, 325 $(S)^+$, 181 $(BH_2)^+$; m/z (FAB < 0) 1511 $(3M-H)^-$, 1007 $(2M-H)^-$, 503 $(M-H)^-$, 179 $(B)^-$. Anal. calcd for $C_{24}H_{19}F_3N_2O_7$: C, 57.14; H, 3.79; N, 5.55; F, 11.30. Found: C, 56.96; H, 3.85; N, 5.64; F, 11.05.

1-(2-Deoxy-β-L-*threo*-pentofuranosyl)-5-trifluoromethyluracil 4. To a stirred solution of **3** (28.0 g, 55.5 mmol) in dry MeOH (555 mL) was added solid sodium methoxide (4.5 g, 83.3 mmol). The resulting solution was stirred for 20 min, neutralized by addition of 1 N HCl (83 mL) and evaporated to dryness. The residue was subjected to a silica gel column chromatography, with a stepwise gradient of MeOH (0–7%) in CH_2Cl_2 to afford compound **4** (11 g, 67%) which was crystallized from a chloroform/acetone mixture: mp 178–179 °C; $[\alpha]_D^{20}$ −3 (*c* 1.01 in DMSO); UV λ_{max} (EtOH) 263 nm (ε 9900); ^{1}H NMR (DMSO-d_6) 2.0 (1H, d, $J_{2'-2''}$ = 14.7 Hz, 2′-H), 2.52 (1H, m, 2″-H), 3.69 (2H, m, 5′-H and 5″-H), 3.90 (1H, m, 4′-H), 4.22 (1H, m, 3′-H), 4.79 (1H, t, J = 5.5 Hz, 5′-OH), 5.30 (1H, d, J = 3.0 Hz, 3′-OH), 6.03 (1H, d, $J_{1'-2''}$ = 6.9 Hz, 1′-H), 8.51 (1H, s, 6-H), 11.88 (1H, s, 3-NH); ^{13}C NMR (DMSO-d_6) δ 41.7 (2′-C), 60.3 (5′-C), 69.2 (3′-C), 86.2 (1′-C), 87.0 (4′-C), 102.8 (5-C, q, J = 31.0 Hz), 123.7 (CF_3, q, J = 267.0 Hz), 143.9 (6-C, q, J = 6.0 Hz), 150.5 (2-C), 159.9 (4-C); ^{19}F NMR (DMSO-d_6) δ −62.3 (CF_3); m/z (FAB > 0) 889 $(3M+H)^+$, 593 $(2M+H)^+$, 297 $(M+H)^+$, 181 $(BH_2)^+$, 117 $(S)^+$; m/z (FAB < 0) 887 $(3M-H)^-$, 591 $(2M-H)^-$, 295 $(M-H)^-$, 179 $(B)^-$. Anal. calcd for $C_{10}H_{11}F_3N_2O_5$: C, 40.54; H, 3.74; N, 9.46. Found: C, 40.63; H, 3.81; N, 9.51.

1-(5-*O*-Benzoyl-2-deoxy-β-L-*threo*-pentofuranosyl)-5-trifluoromethyluracil 5. Benzoyl chloride (0.94 mL, 8.10 mmol) in dry pyridine (14 mL) was added dropwise to a stirred solution of compound **4** (2.0 g, 6.75 mmol) in dry pyridine (55 mL) at 0 °C. After addition was completed, water (10 mL) was added and the solvents were removed. The residue was dissolved in CH_2Cl_2 (50 mL) and the organic layer was washed with saturated $NaHCO_3$ (2×50 mL), water (50 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by silica gel column chromatography using a stepwise gradient of MeOH (0–4%) in CH_2Cl_2 to afford compound **5** as a white foam (2.47 g, 86%): $[\alpha]_D^{20}$ −62 (*c* 0.39 in DMSO); UV λ_{max} (EtOH) 262 nm (ε 10600), 229 (ε 14400); ^{1}H NMR (DMSO-d_6) 2.07 (1H, d, $J_{2'-2''}$ = 14.9 Hz, 2′-H), 2.62 (1H, m, 2″-H), 4.27 (1H, m, 4′-H), 4.38 (1H, m, 3′-H), 4.55 (2H, m, 5′-H and 5″-H), 5.68 (1H, d, J = 3.1 Hz, 3′-OH), 6.03 (1H, d, $J_{1'-2'}$ = 7.0 Hz, 1′-H), 7.5–8.0 (5H, m, C_6H_5), 8.57 (1H, s, 6-H), 11.88 (1H, s, 3-NH); ^{13}C NMR (DMSO-d_6) δ 41.5 (2′-C), 64.3 (5′-C), 69.5 (3′-C), 83.5 (4′-C), 86.3 (1′-C), 103.2 (5-C, q, J = 32.0 Hz), 123.6 (CF_3, q, J = 267.0 Hz), 129.6–134.4 (C-Arom), 143.8 (6-C, q, J = 6.0 Hz), 150.6 (2-C), 159.9 (4-C), 166.5 (CO); ^{19}F NMR (DMSO-d_6) δ −62.4 (CF_3); m/z (FAB > 0) 401 $(M+H)^+$, 221 $(S)^+$, 181 $(BH_2)^+$; m/z (FAB < 0) 399 $(M-H)^-$, 179 $(B)^-$. Anal. calcd for $C_{17}H_{15}F_3N_2O_6$/0.5 H_2O: C, 49.88; H, 3.94; N, 6.84; F, 13.92. Found: C, 50.17; H, 4.08; N, 7.01; F, 13.46.

1-(2-Deoxy-β-L-*erythro*-pentofuranosyl)-5-trifluoromethyluracil 6. DEAD (0.94 mL, 6 mmol) was added to a stirred solution of compound **5** (0.80 g, 2 mmol), PPh_3 (1.57 g, 6 mmol) and benzoic acid (0.73 g, 6 mmol) in dry tetrahydrofuran (THF) (40 mL) at 0 °C under argon. The resulting solution was stirred for 1 h at 0 °C. Solvent was removed and the residue was subjected to silica gel column chromatography using as eluent a stepwise gradient of MeOH (0–2%) in CH_2Cl_2. The appropriate fractions were pooled and directly treated with methanolic ammonia (previously saturated at −10 °C and tightly stoppered; 50 mL) for 18 h at room temperature. Evaporation to dryness and column chromatography on silica gel using a stepwise gradient of MeOH (0–7%) in CH_2Cl_2 afforded compound **6** (136 mg, 23%) which was crystallized from a chloroform/acetone mixture: mp 180–181 °C (lit.,[18] 186–189 °C for the D-enantiomer); $[\alpha]_D^{20}$ −41 (*c* 0.97 in DMSO) {lit.,[19] for the D-enantiomer, $[\alpha]_D^{20}$ + 47.3 (*c* 1.00 in water)}; UV λ_{max} (EtOH) 263 nm (ε 9700); ^{1}H NMR (DMSO-d_6) 2.20 (2H, m, 2′-H and 2″-H), 3.58 (1H, ddd, $J_{5'-4'}$ = 2.9 Hz, $J_{5'-5''}$ = 11.9 Hz, 5′-H), 3.66 (1H, ddd, 5″-H), 3.81 (1H, dd, $J_{4'-3'}$ = 6.5 Hz,

4′-H), 4.43 (1H, m, 3′-H), 5.21 (1H, t, $J=4.4$ Hz, 5′-OH), 5.26 (1H, d, $J=4.4$ Hz, 3′-OH), 6.07 (1H, t, $J=6.0$ Hz, 1′-H), 8.72 (1H, s, 6-H), 11.83 (1H, s, 3-NH); ^{13}C NMR (DMSO-d_6) δ 41.5 (2′-C), 61.1 (5′-C), 70.2 (3′-C), 86.3 (1′-C), 88.5 (4′-C), 103.3 (5-C, q, $J=31.9$ Hz), 123.9 (CF_3, q, $J=268.8$ Hz), 143.1 (6-C, q, $J=6.1$ Hz), 150.4 (2-C), 159.9 (4-C); ^{19}F NMR (DMSO-d_6) δ −61.3 (CF_3); m/z (FAB > 0) 297 $(M+H)^+$, 181 $(BH_2)^+$, 117 $(S)^+$; m/z (FAB < 0) 295 $(M-H)^-$, 179 $(B)^-$. UV, NMR and MS spectra were superimposable on those obtained from a commercially available TFT sample. Anal. calcd for $C_{10}H_{11}F_3N_2O_5$: C, 40.54; H, 3.74; N, 9.46; F, 19.24. Found: C, 40.52; H, 3.67; N, 9.54; F, 19.03.

Biological studies

Thymidine kinase assays. HSV-1 and HSV-2 TK were purified and assayed as previously described:[16,20] briefly, enzyme was incubated at 37 °C for 30 min in a mixture (25 μL) containing 30 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] K^+, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM dithiothreitol (DTT), and 0.4 μM (HSV-1) or 0.6 μM (HSV-2) [^{3}H-methyl]-dThd (2200 cpm/pmol). Human cytosolic TK, purified from HeLa cells,[16] was assayed in the same reaction conditions and 0.5 μM [^{3}H-methyl]-dThd (2200 cpm/pmol). The reaction was terminated by spotting 20 μL of the incubation mixture on a 25-mm DEAE paper disk (DE-81 paper, Whatman). The disk was washed with an excess of 1 mM ammonium formate (pH 3.6) in order to remove unconverted nucleoside, then with ethanol. [^{3}H]-TMP was estimated by scintillation counting in 1 mL of Betamax™ (ICN-Biomedicals).

When [γ-^{32}P]-ATP was used in TK assay, enzyme was incubated in the mixture described above containing 1 mM [γ-^{32}P]-ATP (400 cpm/pmol), 2 mM $MgCl_2$ and 40 μM of TFT or L-TFT. After 1 h at 37 °C, to each sample 1.5 μL of EDTA 0.5 M were added to stop the reaction. Samples were heated 5 min at 80 °C and centrifuged at 10,000 rpm 10 min. 20 μL of the supernatant was injected in HPLC.

The reverse-phase method employing the Bio-Rad 100 MAPS preparative system was used. Nucleosides and nucleotides were resolved on a Superspher 100 RP18 column (Merck) at room temperature in the following conditions: injection volume, 20 μL; detection, UV 260 nm; eluents, buffer A (20 mM KH_2PO_4, pH 5.6), buffer B (20 mM KH_2PO_4, pH 5.6, 60% methanol). Gradient conditions: 0–5 min, 0% buffer B; 15 min, 50% buffer B; 16–28 min, 100% buffer B. Flow rate: 0.5 mL/min. 56 fractions (fraction volume: 250 μL) were collected and counted in a β-counter.

Nucleoside degradation by human thymidine phosphorylase and HPLC analysis of the reaction products. A mixture (25 μL) containing 0.1 mM thymidine or 0.1 mM of TFT or L-TFT, 50 mM sodium arsenate, pH 6.2 and an amount of recombinant thymidine phosphorylase (TP)[15] to give linear reaction rate was incubated at 37 °C for 0, 10, 20 and 30 min and then heated 5 min at 90 °C. The tubes were then centrifuged at 10,000 rpm for 5 min, the supernatant (20 μL) was injected in HPLC and analyzed following the gradient conditions described above. The relative peak areas of nucleoside (thymidine, TFT or L-TFT) and base (eventually produced) were used to determine the enzymatic activity of TP.

Cells. The cells used in this study were HeLa 5a (HeLa TK^- transformed to the TK^+ phenotype with a functional copy of the HSV-1 TK gene). The cells were obtained from Professor G. Della Valle (University of Bologna, Italy). The cells were maintained at 37 °C in Dulbecco's modified essential medium (DMEM) containing 10% foetal calf serum (FCS).

In vivo incorporation of [^{3}H]-thymidine in HeLa cells. HeLa 5a cells were grown in DMEM with foetal calf serum at 37 °C. Exponentially growing cells were trypsinized, resuspended in DMEM without calf serum at the concentration of 6×10^5/mL and 100 μL aliquots were incubated for 30 min at 37 °C in test tubes. Finally, to each tube, [^{3}H]-Thd (2.4 μCi) and different concentrations of the nucleoside analogue were added. Incubation was then continued for 0, 10, 20 and 30 min. At each time, 0.08 mL samples of culture were spotted on 25 mm GF/C (Whatman) filters. The filters were washed three times in 5% (v/v) TCA for 5-10 min and twice in ethanol. They were then dried and the acid insoluble radioactivity was estimated by scintillation counting of the filters in 1 mL of scintillating mixture Betamax (ICN-Biomedicals).

Antiherpetic activities in cell cultures

Media and virus strains. The medium utilized was Earle's minimal essential medium (MEM) containing Earle's balanced salt solution supplemented with either 2 or 10% fetal bovine serum (FBS), 100 U of penicillin per mL, 25 μg of gentamicin per mL and 2 mM L-glutamine. HSV-1 (strain E-377) and HSV-2 (strain MS) were used in the virus inhibition assays.

Cell cultures. The routine growth and passage of human foreskin fibroblat (HFF) cells were performed using MEM supplemented with 10% FBS.

Plaque reduction assay for HSV-1 and HSV-2. 2 days prior to use, HFF cells were plated into six-well plates and incubated at 37 °C with 5% CO_2 and 90% humidity. On the date of assay, the drug was made up in MEM with 2% FBS and then serially diluted 1:5 in MEM using six concentrations of drug. The initial starting concentration was usually 100 μg/mL down to 0.03 μg/mL. The virus used for infection was diluted in MEM containing 10% FBS to a desired concentration which will give 20–30 plaques per well. The media was then aspirated from the wells and 0.2 mL of virus added to each well in triplicate with 0.2 mL of media being added to drug toxicity wells. The plates are then incubated for 1 h with shaking every 15 min. After the incubation period, MEM containing the various drug concentrations was added to appropriate wells in duplicate and in a volume 2.0 mL. Pooled human globulin

obtained from Baxter Health Care Corp. was diluted 1:500 and added to the media that the drug was diluted in to prevent extra-cellular spread of HSV. The cultures were then incubated for 3 days. At the end of the incubation period, 1.0 ml of 0.1% crystal violet in 20% methanol was added to each well and incubated for 10 min. The liquid was then aspirated off, monolayers were washed, and plaques were enumerated using a stereomicroscope.

Acknowledgements

These investigations were supported by Grants from A.N.R.S., Agence Nationale de Recherche sur le SIDA (France), CNR PF Biotecnologie and EC TMR-ERBMRXCT 970125 (Italy) and Novirio Pharmaceuticals, Inc., Cambridge, MA (USA). A.M. is particularly grateful to the Ministère de l'Education Nationale, de la Recherche et de la Technologie (France) for a doctoral fellowship. A.V. was supported by a fellowship provided by Fondazione A. Buzzati-Traverso.

References

1. Heidelberger, C.; Parsons, D.; Remy, D. C. *J. Am. Chem. Soc*. **1962**, *84*, 3597.
2. Carmine, A. A.; Brogden, R. N.; Heel, R. C.; Speight, T. M.; Avery, G. S. *Drugs* **1982**, *23*, 329.
3. Prusoff, W. H.; Zucker, M.; Mancini, W. R.; Otto, M. J.; Lin, T.-S.; Lee, J.-J. *Antiviral Res*. **1985**, *S1*, 1.
4. Wigdahl, B. L.; Parkhurst, J. R. *Antimicrob. Agents Chemother*. **1978**, *14*, 470.
5. Fischer, P. H.; Prusoff, W. H. In *Chemotherapy of Viral Infections, Handbook of Experimental Pharmacology*; Came, P. E.; Caliguiri, L. A., Eds.; Springer-Verlag: Berlin, 1982, p 95–116.
6. Shannon, W. M. In *Antiviral Agents and Viral Diseases of Man*; Galasso, G. J., Ed.; Raven Press: New York, 1984, p 55–121.
7. Spadari, S.; Maga, G.; Verri, A.; Focher, F. *Exp. Opin. Invest. Drugs* **1998**, *7*, 1285.
8. Wang, P.; Hong, J. H.; Cooperwood, J. S.; Chu, C. K. *Antiviral Res*. **1998**, *40*, 19.
9. Robins, M. J.; Wilson, J. S.; Hansske, F. *J. Am. Chem. Soc*. **1983**, *105*, 4059, and references therein.
10. Baker, B. R.; Joseph, J. P.; Schaub, R. E.; Williams, J. H. *J. Org. Chem*. **1954**, *19*, 1786.
11. Baker, B. R.; Schaub, R. E.; Kissman, H. M. *J. Am. Chem. Soc*. **1955**, *77*, 5911.
12. Gosselin, G.; Bergogne, M.-C.; Imbach, J.-L. *J. Heterocyclic Chem*. **1993**, *30*, 1229.
13. Vorbrüggen, A.; Krolikiewicz, K.; Bennua, B. *Chem. Ber*. **1981**, *114*, 1234.
14. Hughes, D. L. *Org. React*. **1992**, *42*, 335.
15. Focher, F.; Spadari, S.; Ubiali, D.; Pregnolato, M.; Zhi, C.; Gambino, J.; Wright, G. *J. Med. Chem*. **2000**, *43*, 2601.
16. Focher, F.; Hildebrand, C.; Freese, S.; Ciarrocchi, G.; Noonan, T.; Sangalli, S.; Brown, N. C.; Spadari, S.; Wright, G. *J. Med. Chem*. **1988**, *31*, 1496.
17. Upston, J. M.; Gero, A. M. *Biochim. Biophys. Acta* **1995**, *1236*, 249.
18. Heidelberger, C.; Parsons, D. G.; Remy, D. C. *J. Med. Chem*. **1964**, *7*, 1.
19. Kawakami, H.; Ebata, T.; Koseki, K.; Matsushita, H.; Naoi, Y.; Itoh, K.; Mizutani, N. *Heterocycles* **1990**, *31*, 569.
20. Focher, F.; Sangalli, S.; Ciarrocchi, G.; Pedraly-Noy, G.; Sandoli, D.; Rebuzzini, A.; Wright, G.; Brown, N. C.; Spadari, S. *J. Chemother*. **1989**, *1*, 1107.