Synthesis and Biological Evaluation of 5-Substituted Derivatives of the Potent Antiherpes Agent (north)-Methanocarbathymine

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The conformationally locked nucleoside, (north)-methanocarbathymine (**1a**), is a potent and selective anti-herpes agent effective against herpes simplex type 1 (HSV1) and type 2 (HSV2) viruses. Hereby, we report on the synthesis and biological evaluation of a small set of 5-substituted pyrimidine nucleosides belonging to the same class of bicyclo[3.1.0]hexane nucleosides. Both the 5-bromovinyl (**4**) and the 5-bromo analogue (**3**) appeared to be exclusive substrates of HSV1 thymidine kinase (TK), contrasting with the 5-iodo analogue (**2**), which was significantly phosphorylated by the human cytosolic TK. The binding affinity constant and catalytic turnover for HSV1 TK were measured to assess the influence of the substitution on these parameters. In the plaque reduction and cytotoxicity assays, the 5-bromo analogue (**3**) showed good activity against HSV1 and HSV2 with less general toxicity than **1a**. Against varicella-zoster virus (VZV), the north-locked 5-bromovinyl analogue (**4**) proved to be as potent as its conformationally unlocked 2′-deoxyriboside equivalent BVDU. The three compounds were also tested in vitro as prodrugs used in a gene therapy context on three osteosarcoma cell lines, either deficient in TK (TK$^-$), nontransduced, or stably transduced with HSV1 TK. The 5-iodo compound (**2**, CC_{50} 25 ± 7 μM) was more efficient than ganciclovir (GCV, CC_{50} 75 ± 35 μM) in inhibiting growth of HSV1-TK transfected cells and less inhibitory than GCV toward TK$^-$ cells, whereas compound **3** inhibited transfected and nontransfected cell lines in a relatively similar dose-dependent manner.

Introduction

On the basis of the broader substrate specificity of viral-encoded thymidine kinase (TK), nucleoside-like prodrugs have been successfully used for years in antiviral therapy.[1,2] After specific phosphorylation by viral-encoded TKs and subsequent phosphorylation by less specific cytosolic kinases, the triphosphate forms of such nucleoside analogues act as competitive substrates of the DNA polymerases. Once incorporated into DNA, these alternate substrates lead to elongation stops, reduced integrity, or instability of the newly synthesized DNA.[1,3−6] Classically, nucleoside analogues such as 5-bromovinyl-2′-deoxyuridine (BVDU), 5-iododeoxyuridine (IdU), acyclovir (ACV), and ganciclovir (GCV) have been used in the treatment of herpes simplex virus type 1 (HSV1), type 2 (HSV2), cytomegalovirus (CMV), and other herpesvirus infections.[7−10] More recently, gene transduction strategies based on the concomitant use of HSV1 TK and nucleoside analogues have been proposed against cancer cells[11−13] and for controlling graft versus host disease (GvHD) reactions after allogeneic bone-marrow transplantation (allo-BMT).[14−16] In the case of antiviral therapy against HSV1 and HSV2 especially, such nucleoside analogues have been used successfully for years. However, in the case of other herpesvirus infections and gene therapy trials, the very same analogues used at higher concentrations demonstrated various limitations in terms of specific activation level by viral TKs, efficiency, and safety.[6,17,18] For example, the high level of GCV needed for tumor regression during gene-therapy of cancer cells leads to important side effects such as hematological toxicity (neutropenia) and bone marrow depletion.[6] In the case of allo-BMT, the infusion of HSV1 TK gene-modified T-cells was proposed as a way to improve the chances of successful engraftment, thus allowing the control of eventual GvHD reaction outcomes by the subsequent administration of GCV.[16,19] Unfortunately, CMV infections, which occur frequently after allo-BMT, are also treated by GCV. Thus, a curative administration of GCV would simultaneously lead to the depletion of the newly infused T-cells. Thereby, antiviral therapy of repellent infections as well as nascent HSV1 TK-based gene therapy applications have increasingly shown urgent needs for new and more potent molecules that would overcome the limitations of the HSV1 TK/nucleoside analogues paradigm. So far, several HSV1 TK mutants with improved specificity toward a given substrate have been engineered.[20−25] Concomitantly, new prodrugs with lower toxicity profiles, higher specificity for viral TK activation, and DNA polymerase inhibition abilities have been developed.[26−29]

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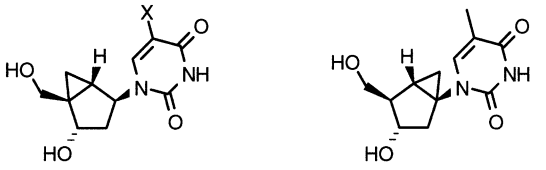


1a, X = CH_3
2, X = I
3, X = Br
4, X = CH=CH-Br

1 b

Figure 1. Structures of the bicyclo[3.1.0]hexane nucleosides. **1a, 2**−**4:** (north)-methanocarbathymine derivatives. **1b:** (south)-methanocarbathymine.

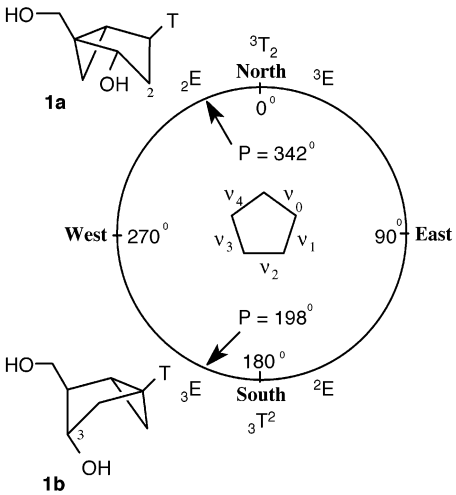


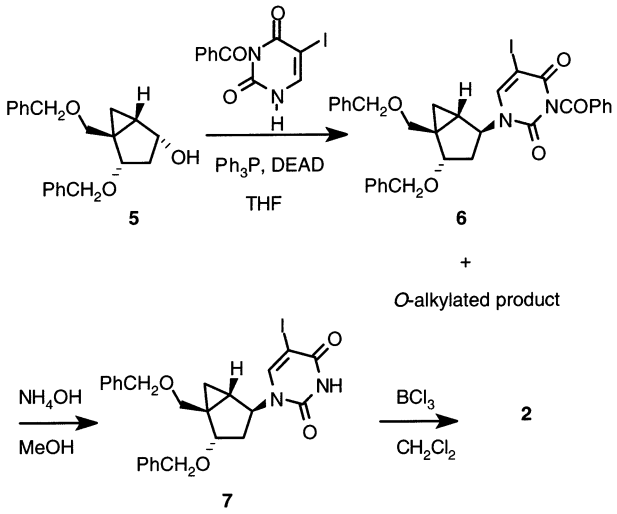
Figure 2. Fixed location of bicyclo[3.1.0]hexane nucleosides (T = thymine) in the pseudorotational cycle.

(north)-Methanocarbocyclic thymine [**1a**, (N)-MCT, Figure 1] is a recently discovered, effective anti-herpes virus agent that was shown to be 30 times more potent than ACV against HSV1 and HSV2 in an in vitro plaque reduction assay.[26,30] Since the 5-substituent in pyrimidine nucleosides is a modulator of antiherpes activity[31] such as in the very effective antiviral compound BVDU, we decided to explore a small set of substituents (X= Br, I, and CH=CH−Br) on this new class of locked carbocyclic nucleosides built on a bicyclo[3.1.0]hexane template.

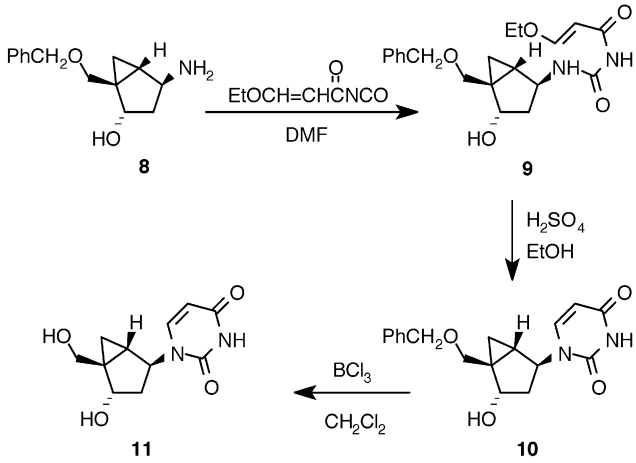
The series was limited exclusively to the conformationally locked north analogues, since the structurally related but conformationally opposite (south)-methanocarbocyclic thymine [**1b**, (S)-MCT], locked in the $_3E$ envelope conformation of the southern hemisphere, was found to be totally devoid of antiviral activity.[26] We have suggested that the difference between (N)-MCT and (S)-MCT might be related to their antipodal pseudosugar conformation (Figure 2) imposed by the rigid bicyclo-[3.1.0]hexane system.[26] Indeed, in the case of (N)-MCT, the conformation of the pseudosugar mimics that of an envelope ($_2E$) 2′-deoxysugar locked in the northern hemisphere of the pseudorotational cycle, whereas in (S)-MCT the pseudosugar mimics a 2′-deoxysugar locked in the ($_3E$) envelope conformation of the southern hemisphere.

In this study, we report the synthesis of the new compounds, their kinetic parameters as substrates for cytosolic (hTK1) and herpes (HSV1 TK) thymidine kinases. The antiviral activity of the same compounds was measured by the cytopatogenic effect (CPE) and viral plaque reduction (VPR) assays against HSV1, HSV2, vaccinia, pox, and varicella-zoster virus. Finally, cell growth inhibition assays on three osteosarcoma cell lines, either stably transduced with HSV1 TK (143B-TK$^+$-HSV1-WT), TK deficient (143B-TK-), or nontransduced (MG-63-hTK1), are presented.

Scheme 1



Scheme 2



Chemistry

Initially, a convergent approach starting from the already known compound **5**[32] was attempted by direct coupling with 5-iodo-3-benzoyl-1,3-dihydropyrimidine-2,4-dione under Mitsunobu conditions. To obtain this modified nucleobase, the method reported for the benzoylation of uridine and thymine was adapted for the synthesis of 5-halo-N^3-benzoylpyrimidines.[33] Such a convergent approach was plagued with low yields and the concomitant formation of the *O*-alkylated product (Scheme 1). However, despite this difficulty, we were able to isolate the desired *N*-alkylated product **6** and complete its conversion to the final target, 5-iodo derivative (**2**), after two deprotection steps. In view of the low yields obtained under Mitsunobu conditions, a linear approach starting from the known carbocyclic amine **8**[34] was initiated (Scheme 2). Under similar reaction conditions as those reported for the synthesis of **1a**,[34] in situ generated (2*E*)-ethoxy-1-oxoprop-2-enisocyanate was reacted with amine **8** to give the

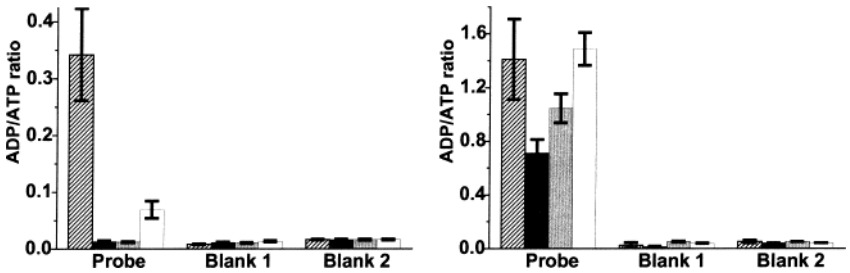
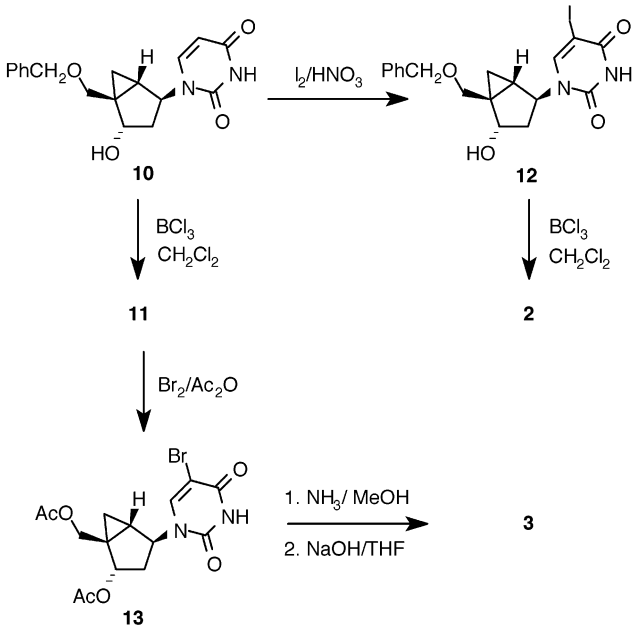
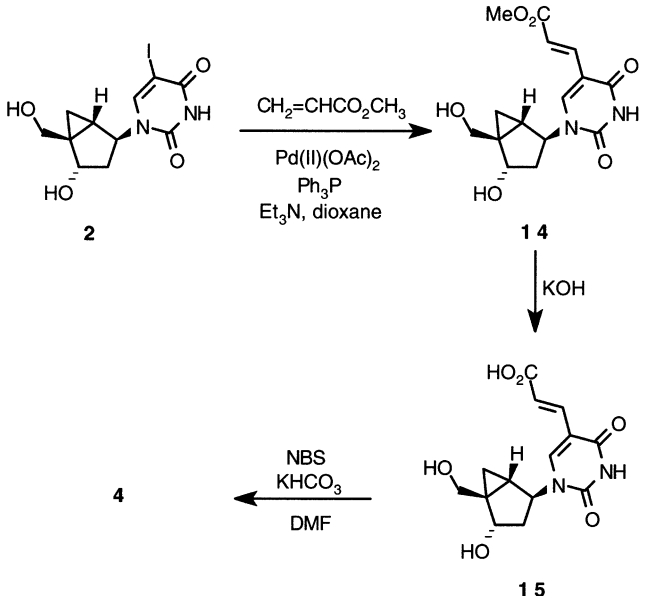


Figure 3. In vitro phosphorylation profiles of compounds (**2**−**4**) and thymidine performed by hTK1 (A) and HSV1 TK (B). Only 5-iodo (**2**) exhibited a significant phosphorylation level in the presence of hTK1, whereas all three compounds were significant substrates of HSV1 TK. Color codes for the nucleobases are dashed for thymine, black for 5-bromovinyl (**4**), gray for 5-bromo (**3**), and white for 5-iodo (**2**). Blank 1: mock experiment without TK. Blank 2: mock experiment without substrate.

Scheme 3



Scheme 4



corresponding acyclic intermediate **9**, which underwent smooth acid-catalyzed cyclization to the uridine analogue **10** (Scheme 2). Removal of the benzyl protecting group with boron trichloride afforded the already known conformationally locked (north)-methanocarba-2′-deoxyuridine analogue **11**.[26]

As shown in Scheme 3, both **10** and **11** are convenient starting materials for the synthesis of the 5-halo compounds. Therefore, starting with compound **10**, direct iodination provided intermediate **12**, which after removal of the benzyl-protecting group afforded the 5-iodo analogue **2**. This compound proved to be identical to the sample obtained via the direct Mitsunobu coupling in Scheme 1. In a similar fashion, compound **11** could be effectively brominated, and following base-catalyzed hydrolysis the diacetate intermediate (**13**) was converted to the 5-bromo analogue **3**.

To obtain the BVDU analogue **4**, the procedure reported by Herdewijn and co-workers[35] for the synthesis of plain, carbocyclic (±)-BVDU was followed (Scheme 4). Related procedures for the synthesis of carbocyclic (+)- and (−)-BVDU have also been reported.[36−38] Thus, reaction of **2** with methylacrylate in the presence of palladium(II) acetate, triphenyl phosphine, and triethylamine afforded the 5-(2-carbomethoxyvinyl) intermediate **14**, which after hydrolysis and treatment with *N*-bromosuccinimide (NBS) gave the conformationally locked version of carbocyclic BVDU (**4**).

Results and Discussion

Enzymatic Activity. The ADP/ATP ratios reflecting the phosphorylation levels of methanocarba analogues (**2**−**4**) by HSV1 TK or hTK1 are summarized in Figure 3. Significant HSV1 TK phosphorylation levels were observed for all methanocarba analogues. However, none of these appeared to be significant substrates for the ensuing thymidylate kinase activity that HSV1 TK may present with certain substrates,[39] since no trace of diphosphorylated compounds was detected. In the presence of hTK1, 5-iodo (**2**) was significantly phosphorylated, whereas 5-bromo (**3**) and 5-bromovinyl (**4**) were not significant substrates for this enzyme.

Since K_m is equal to K_i in the case of HSV1 TK, as previously demonstrated,[40] both values can be referred to as the binding affinity constant. The inhibition constant values ($K_i \pm$ SD) obtained for compounds (**2**−**4**) were compared to the previously published values of their corresponding deoxyribosyl and carbocyclic counterparts (Table 1). Since the catalytic turnover values (k_{cat}) for the deoxyribosyl and carbocyclic counterparts for HSV1 TK have not been reported, it is not possible to evaluate the impact of the restricted carbocyclic ring on catalysis. Nevertheless, the 5-bromovinyl (**4**) has a k_{cat} value of 0.07 s^{-1} that is lower than those for ACV

Table 1. Comparison of the Inhibition Constant and Catalytic Turnover of Methanocarba, Carbocyclic, and Deoxyribosyl Analogues of Thymine for HSV1 TK

HSV 1 TK		methanocarba analogues				carbocyclic analogues[c]		deoxyribosyl analogues[c]	
	Thy[a]	(N)-MCT (**1a**)[d]	BrV (**4**)[b]	Br (**3**)[b]	I (**2**)[b]	C-BVdU[e]	C-IdU[e]	BVdU	IdU
K_i (μM)	0.2 ± 0.05	16.1 ± 7.6	62.9 ± 6.1	69.8 ± 6.5	85.8 ± 13.5	0.22	0.21	0.1	0.09
k_{cat} (s^{-1})	0.35 ± 0.01	0.16 ± 0.04	0.07 ± 0.01	0.15 ± 0.04	0.14 ± 0.04	ND	ND	ND	ND

[a] Published as K_m value.[47] [b] This work (values are the mean of at least four separate experiments ± SD). [c] Published in ref 43 (for clarity reasons, SD values were omitted). [d] Published in ref 41 [e] The values of both racemate are reported (i.e. (±)-C-BVdU and (±)-CIdU); ND, not determined.

Table 2. Antiviral Activity against Herpes Viruses HSV-1 and HSV-2 in HFF[a] Cells[g]

	HSV-1 CPE			HSV-2 CPE			HSV-1 VPR			HSV-2 VPR		
compd	EC_{50}[b]	CC_{50}[c]	SI[d]	EC_{50}[b]	CC_{50}[c]	SI	EC_{50}[b]	CC_{50}[c]	SI	EC_{50}[b]	CC_{50}[c]	SI
1a[e]	0.12	>396	3300	0.35	>396	1131	0.040	>79	>1975	0.470	>79	>168
2	>275	>275	0	9.06	>275	30.3						
3	1.23	>315	>256	2.20	>315	>143	0.095	>315	>3315	0.378	>315	833
4	6.41	>291	>45	>291	>291	0						
ACV[f]	2.22			10.21			1.10			1.68		

[a] HFF = human skin fibroblast. [b] EC_{50} = inhibitory concentration (μM) required to reduce virus-induced cytopathogenic effect (CPE) or cause virus plaque reduction (VPR) by 50%. [c] CC_{50} = cytotoxic concentration that produces 50% inhibition of cell growth. [d] SI = selectivity index (CC_{50}/EC_{50}). [e] Adapted from ref 26. [f] ACV = acyclovir. [g] The presented values were assessed by the NIAID according to Kern et al.[44]

Table 3. Antiviral Activity against Vaccinia Virus (VV) and Cowpox Virus (CV) in HFF[a] Cells[e]

	VV CPE			VV VPR			CV CPE			CV VPR		
compd	EC_{50}[b]	CC_{50}[c]	SI[d]	EC_{50}[b]	CC_{50}[c]	SI	EC_{50}[b]	CC_{50}[c]	SI	EC_{50}[b]	CC_{50}[c]	SI
3	2.01	>315	>156	8.19	>315	>38	36.26	>315	>8	10.72	>315	>29

[a] HFF = human skin fibroblast. [b] EC_{50} = inhibitory concentration (μM) required to reduce virus-induced cytopathogenic effect (CPE) or cause virus plaque reduction (VPR) by 50%. [c] CC_{50} = cytotoxic concentration that produces 50% inhibition of cell growth. [d] SI = selectivity index (CC_{50}/EC_{50}). [e] The presented values were assessed by the NIAID according to Kern et al.[44]

and GCV (both 0.10 s^{-1}),[41] whereas the turnover constant values for compounds **3** and **2** are higher (k_{cat} 0.15 and 0.14 s^{-1}, respectively). A different contribution of the nucleobase in the dipole-charge interaction influencing the catalytic turnover may also be involved,[42] although structural rearrangements of the active site are probably induced upon binding of such ligands. In the case of (N)-MCT, the restricted ring induces a change in the inhibition constant value, in agreement with an altered binding mode of the sugar moiety with respect to the flexible substrate, thymidine (Thy).[25] On the other hand, the presence of a simple carbocyclic ring does not modify the K_i values, as the values for C-BVdU and C-IdU remained comparable to that of thymidine.[43] In summary, we believe that the higher inhibition constants determined for all 5-substituted methanocarba analogues reflect the combined effect of a restricted carbocyclic ring and a bulky substitution at position 5, which hinders accessibility to the active site based on steric grounds and most likely requires structural rearrangement within the active site to be accommodated.

Biological Activity. Antiviral Activity. The antiviral activity of these compounds was evaluated in two different laboratories against different strains of DNA viruses using previously published methodology.[26,44,45] All concentrations for EC_{50} and CC_{50} were converted from μg/mL to μM to best compare the results from the two laboratories. In the CPE assay, the 5-bromo analogue (**3**) was only ca. 2-fold more potent than ACV against HSV1 and HSV2, while relative to (N)-MCT (**1a**) it was somewhat weaker (Table 2). However, in the more meaningful VPR assay, the 5-bromo compound appeared to be much less cytotoxic and almost as potent as **1a** (Table 2). The 5-iodo (**2**) and 5-bromovinyl (**4**) analogues were not very effective against HSV1 and HSV2, which is in sharp contrast to the good in vitro antiviral activity reported for the cyclopentyl versions of these compounds.[37]

The recent interest in screening compounds against viruses that pose a threat from terrorist attacks prompted the National Institutes of Allergy and Infections Diseases (NIAID) to test these compounds against vaccinia and pox viruses according to method of Kern at al.[44] Against these viruses, only the 5-bromovinyl analogue (**3**, Table 3) showed modest activity.

At the Rega Institute, the compounds were evaluated against thymidine kinase positive (TK^+) and negative (TK^-) strains of VZV (Table 4) according to standard published methods.[45] Consistent with the expected required activation of these drugs by viral TK, the strains that do not express adequate levels of thymidine kinase were not sensitive to the drugs. On the other hand, against the TK^+ strains all the compounds were more potent than ACV, particularly the 5-bromovinyl analogue (**4**). It should be noted that the plain carbocyclic analogue of BVDU (C-BVDU) with a flexible cyclopentane ring— was reported to have good activity against VZV (EC_{50} = 0.02−0.04 μg/mL[35] or 0.06−0.12 μM), which makes it ca. 10-fold less potent than BVDU itself. From the results in Table 4, it seems that the presence of the bicyclo[3.1.0]hexane appears to be able to recover the loss in potency since the anti-VZV activity of compound **4** was virtually indistinguishable from that of BVDU.

Cellular Growth Inhibition. Cell growth inhibition activity was measured on three different types of osteosarcoma cells: either stably transfected with HSV1

Table 4. Antiviral Activity against Varicella-Zoster Virus (VZV)[f]

	antiviral activity (plaque reduction) EC_{50}[a] (μM)					
	TK^+ VZV		TK^- VZV		cytotoxicity (μM)	
compd	YS strain	OKA strain	07/1 strain	YS/R strain	MCC[b]	CC_{50}[c]
2	0.12	0.16	>5	3	>5	>200
3	0.20	0.20	>20	4	>50	>200
4	0.007	0.005	>5	>5	>5	>200
ACV[d]	2.4	2.4	30	19	>200	488
BVDU[e]	0.008	0.005	>150	>150	>150	>400

[a] EC_{50} = inhibitory concentration (μM) required to reduce virus plaques by 50%. [b] MCC = minimum cytotoxic concentration that causes microscopically detectable alterations of cell morphology. [c] CC_{50} = cytotoxic concentration that produces 50% inhibition of cell growth. [d] ACV = acyclovir. [e] BVDU = (*E*)-5-(2-bromovinyl)-2′-deoxyuridine. [f] The presented values were assessed by the Rega Institute according to Andrei et al.[45]

TK (143B-TK^+-HSV1-WT), or TK-negative (143B-TK^-), or nontransfected (MG-63-hTK1). Cell survival was determined using the tetrazolium-based (XTT) colorimetric assay performed after 4.5 days of incubation with five different concentrations of each compound. Three series of independent experiments were performed in triplicate with good reproducibility (Figure 4). The 5-bromovinyl (**4**) did not lead to a typical TK-mediated inhibition growth pattern and demonstrated increasing inhibition only at high concentration (>50 μM) in all three cell lines. Since growth inhibition appeared not to be linked to TK activity, an intrinsic toxicity of the compound at high concentration might be responsible for the observed cytotoxicity. The 5-bromo (**3**) analogue showed a dose-dependent reduction of cell growth in cell lines possessing TK activity. Doses required for 50% cell growth inhibition (CC_{50}) were 150 ± 80 μM in 143B-TK^+-HSV1-WT and 330 ± 30 μM in MG-63-hTK1. Interestingly, the inhibition profiles between transfected and nontransfected cells for the 5-bromo (**3**) were relatively close to each other. Although, the compound is not a substrate for hTK1 (Figure 3), it is possible that it interferes with thymidine monophosphate (TMP) biosynthesis within the cell. Thus, the cytosolic amount of thymidine monophosphate (TMP) would decrease, resulting in a reduced cell growth rate in nontransfected cells. The additional growth inhibition observed in HSV1 TK-transfected cells would thus be enhanced by the phosphorylated form of compound **3** that inhibits enzymes beyond the initial phosphorylation step.

Finally, 5-iodo (**2**) showed no significant cell growth inhibition in TK^- cells within the experimental range (CC_{50} > 500 μM). The CC_{50} values for 5-iodo (**2**) were 300 ± 30 μM in MG-63-hTK1 cells and 25 ± 7 μM in HSV1 TK-transfected cells with an ensuing measurable growth inhibition beyond 5 μM. By comparison, GCV showed nonspecific growth inhibition starting at 5 μM for TK^- cells (CC_{50} = 200 ± 100 μM) and MG-63-hTK1 cells (CC_{50} = 300 ± 100 μM), while a specific effect was observed against HSV1 TK-transfected cells (CC_{50} = 75 ± 35 μM). Thus, the 5-iodo analogue (**2**) appeared more potent and more selective than GCV in the inhibition of HSV1 TK-transfected cells. Moreover, the 5-iodo (**2**) was less inhibitory than GCV toward TK^- and nontransfected dividing cells. Remarkably, the CC_{50} values assessed in the nontransfected MG-63-hTK1 cells corroborated the ones measured in HFF cells by VPR or CPE assays (Table 2). In contrast, however, the CC_{50} values determined by the VPR or CPE assays were significantly different from those obtained in HSV1 TK stably transfected cells. The results from both CPE and VPR are from virally infected cells, and thus cytoplasmic production of the whole panel of viral enzymes, including viral TK and viral DNA polymerase, are involved. On the contrary, the 143B-TK^+-HSV1-WT cells feature only one viral enzyme, namely HSV1 TK. Thus, a difference in CC_{50} values can be easily explained by the presence or absence of viral DNA polymerase inhibition, depending on the assay.

In summary, we have developed methods for the synthesis of several 5-substituted uracil analogues built on a rigid bicyclo[3.1.0]hexane template. Due to the poor yield of the Mitsunobu coupling with uracil analogues, the method of choice was the linear approach starting from the corresponding carbocyclic amine **8**. The uracil analogue built from **8** was directly halogenated to provide analogues **2** and **3**, whereas the 5-bromovinyl (**4**) derivative was constructed from the 5-iodo analogue **2** following the same methodology used for the synthesis of BVDU. Moreover, these three nucleoside analogues, which are restricted in the north conformation and feature a bulky residue at position 5 of the nucleobase, are significant substrates of HSV1 TK. The antiviral activity of these compounds (**2**−**4**) reflects a different modulating role for the 5-substituent on the uracil ring when linked to the bicyclo[3.1.0]hexane template compared to equivalent 2′-deoxyriboside analogues and plain cyclopentyl nucleosides. Furthermore, modulation of the specific phosphorylation by virus-encoded TKs upon substitution in position 5 of the thymine ring was observed.

Experimental Section

Materials. All chemical reagents were commercially available. Reported melting points were determined on a Fisher-Johnson melting point apparatus and are uncorrected. Column chromatography was performed on silica gel 60, 230−240 mesh (E. Merck), and analytical TLC was performed on Analtech Uniplates silica gel GF. Routine IR and ^1H, ^{13}C, and ^{19}F NMR spectra were recordered using standard methods. Specific rotations were measured in a Perking-Elmer model 241 polarimeter. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Pyruvate kinase and L-lactate dehydrogenase purified from rabbit muscle and their respective substrates phosphoenol-pyruvate and NADH were purchased from Boehringer Mannheim; 1,4-dithio-DL-threitol (DTT), thymidine, and ATP were from Fluka. ACV and GCV were purchased from Glaxo-Wellcome and Roche, respectively. [*methyl*-^3H]-thymine was obtained from Amersham. Cell cultures mediums and reagents were obtained from Life Technology. 5-Bromodeoxyuridine (5-BrdU), XTT (2,3-bis[methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolin-5-carboxyanilide), and Menadion (vitamin K3) were obtained from Sigma. The following osteosarcoma cells were used for cell growth inhibition assays: 143B-TK^- cells (ATCC No. CRL 8303), MG-63-hTK1 cells (ATCC No. CRL-1427), and HSV1 TK stably transduced 143B-TK^+cells (ATCC No. CRL-8304) (143B-TK^+-HSV1-WT).

Assessment of Phosphorylation Profile. Both enzymes HSV1 TK and hTK1 were expressed and purified as previously published.[25,46] Fully active HSV 1 TK and hTK1 featuring wild-type kinetic properties were obtained and showed purity higher than 95%, when stained after SDS−PAGE and measured by densitometry. Phosphorylation of thymidine and the methanocarba analogues was monitored by reverse-phase ion-pair high-performance liquid chromatography (HPLC) as

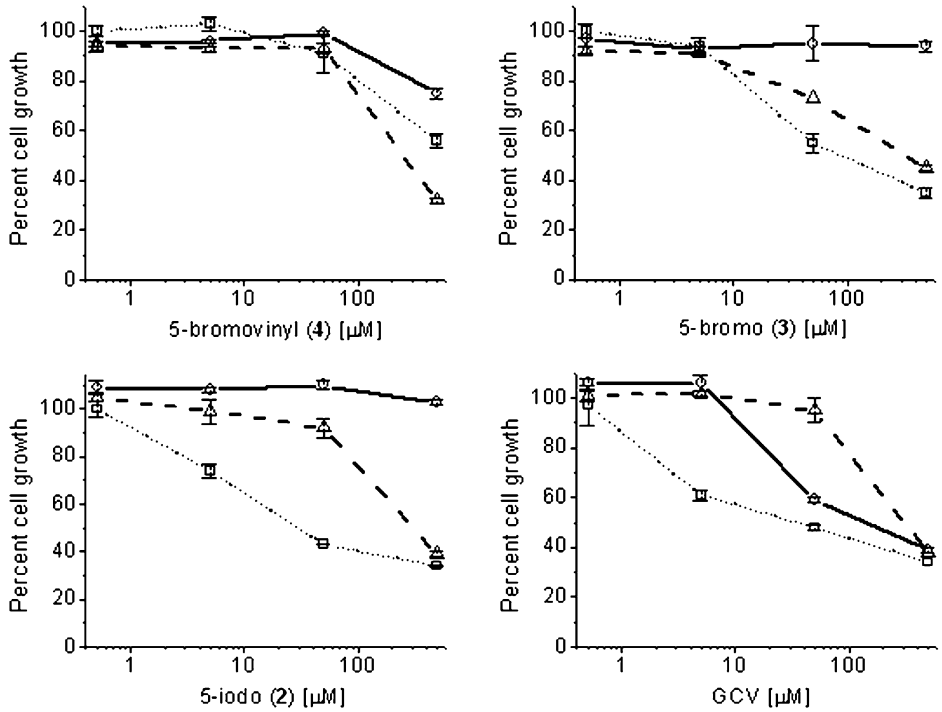


Figure 4. Cell growth profiles obtained after incubation with compounds (**2**−**4**), plus GCV as control. Plots are representative of three independent series of assays performed in triplicate with concentration of compounds ranging from 0.5 to 500 μM. (Cell lines are 143B-TK$^+$-HSV1-WT, □ and dotted line; 143B-TK$^-$, ○ and full line; and MG-63-hTK1, △ and dashed line).

described previously.[47] Briefly, 2 mM of substrate was incubated 60 min at 37 °C with respectively 4 μg of HSV1 TK or 4 μg of hTK1 in the presence of 5 mM ATP and 5 mM of Mg^{2+}. Blank reactions without enzyme or without substrate were run concurrently to account for background ATP hydrolysis. The detection limit for phosphorylated substrates was 20 nmol.[47]

Inhibition Constant Assessment. Conversion of [*methyl*-^{3}H]-T to its monophosphate in the presence of various concentrations of methanocarba analogue was performed as previously described.[48,49] Reactions were carried out in a final volume of 30 μL containing 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, and 2.5 mg/mL bovine serum albumin. Substrate and enzyme concentrations were chosen in order to respect Michaelis−Menten conditions for initial velocity measurements. The concentration of methanocarba analogue was varied, and measured slopes were drawn on a reciprocal plot (Lineweaver−Burk). Inhibition constants (apparent K_i) arose from data using three concentrations of test compounds and were analyzed using the double reciprocal plot method (Lineweaver−Burk's plot) as previously described.[50] The presented values are the results of six independent assays.

Catalytic Turnover Constant Assessment. Reaction mixtures with a final volume of 75 μL containing 50 mM Tris pH 7.5, 1 mM DTT, 0.21 mM phosphoenol-pyruvate, 2.5 mM $MgCl_2$, 5 mM ATP, 0.18 mM NADH, 0.8 μg pyruvate kinase, 0.5 μg L-lactate dehydrogenase, and 1.0 mM of substrate were incubated at 37 °C. Two minutes later, 1.5−4.6 μg of HSV-1 TK was added in order to initiate the reaction. The changes in absorbance driven by TK-dependent reaction were monitored during 10 min at 37 °C using a Cary 50 spectrophotometer (Varian). As previously published, a direct relationship exists between the decrease of absorbance over time of nucleoside analogues and their respective catalytic turnover constants (k_{cat}).[41] The data presented are the result of five independent series of measurements performed in triplicate. Control experiments were performed concurrently to account for spontaneous hydrolysis of ATP under the experimental conditions.

Cell Growth Inhibition Assays. The three cell lines were cultivated in Minimum essential medium (MEM) supplemented with 10% fetal calf serum and appropriate antibiotics. The adherent 143B-TK$^-$ osteosarcoma cells were additionally given 0.1 mg/mL 5-BrdU for TK$^-$ cell-selection. In the case of MG-63-hTK1 osteosarcoma cells, MEM was enriched with 1 mM sodium pyruvate and 1× MEM supplements. The same supplementation plus 1× HAT was used for 143B-TK$^+$-HSV1-WT osteosarcoma cells stably transduced with HSV1 TK wild type. Cells were incubated at 37 °C with 5% CO_2, subcultured by trypsinisation, and resuspended in fresh medium every third or fourth day. The respective cell growth inhibition rates induced by the compounds (**2**−**4**) and GCV were determined using the tetrazolium-based XTT colorimetric assay.[51,52] Single-cell suspensions obtained by trypsinization were seeded at 2 × 10^3 cells/well for both 143B-TK$^+$-HSV1-WT and 143B-TK$^-$ lines and 1.5 × 10^3 cells/well for MG-63-hTK1 line in 96-well tissue culture plates. Various concentrations of nucleoside analogues in the appropriate buffers were added up to a final volume of 200 μL per well and incubated 4.5 days at 37 °C with 5% CO_2. Then, 50 μL of prewarmed (37 °C) XTT (1.0 mg/mL) in RPMI 1640 medium supplemented with 100 μM Menadion and 25 mM HEPES pH 7.5 was added to each well. After 9 h incubation at 37 °C, the absorbance was read on a microplate reader (Versamax, Molecular Devices) at 450 nm with a reference at 750 nm. The percentage of cell growth was compared to the control wells containing cells, medium, and XTT and calculated according to the following equation: % cell growth = 100 × [OD drug-treated/OD control]. The reported values are the results of three independent series of measurements performed in triplicate.

General Synthesis of Monobenzoylated Pyrimidines. A stirred suspension of 24 mmol of 5-X-uracil (X = Br, I) in pyridine (24 mL) and acetonitrile (60 mL) maintained under an argon atmosphere was treated dropwise with benzoyl chloride (6 mL, 52 mmol) and stirring was continued for 4 days. Volatiles were evaporated under vacuum and reconcentrated three times from toluene (100 mL). The residue was treated with 0.25 M K_2CO_3 (90 mL) and dioxane (90 mL) and stirred at room temperature for 2 h. Dioxane was removed under vacuum and the remaining suspension was diluted with water (100 mL). The obtained solids were recrystallized from 95% ethanol to give the desired N^3-benzoyl analogues in yields ranging from 77%−83%.

5-Bromo-3-benzoyl-1,3-dihydropyrimidine-2,4-dione: 77% yield, mp 187−189 °C. Anal. ($C_{11}H_7BrN_2O_3$) C, H, N.

5-Iodo-3-benzoyl-1,3-dihydropyrimidine-2,4-dione: 83% yield, mp 203−205 °C. Anal. ($C_{11}H_7IN_2O_3$) C, H, N. Anal. Calcd for $C_{11}H_7IN_2O_3$: C, 38.62; H, 2.06; N, 8.19. Found: C, 38.84; H, 2.07; N, 8.13.

(1*S*,2*S*,4*S*,5*R*)-5-Iodo-3-(phenylcarbonyl)-1-{4-phenylmethoxy)-5-[(phenylmethoxy)methyl]-bicyclo[3.1.0]hex-2-yl}-1,3-dihydropyrimidine-2,4-dione (6). A solution of diethyl azodicarboxylate (DEAD, 0.53 mL, 3.31 mmol) in THF (20 mL) was added dropwise to a stirred solution of 5-iodo-3-(benzoyl)-1,3-dihydropyrimidine-2,4-dione (1.34 g, 3.92 mmol), **5**[32] (0.536 g, 1.65 mmol), and triphenylphosphine (0.866 g, 3.31 mmol) in THF (50 mL), all under an atmosphere of argon. After overnight stirring at room temperature, volatiles were removed under vacuum. A crude mixture of both *N*- (**6**) and *O*-alkylated products was obtained after silica gel flash chromatography using a step gradient (hexanes → 25% EtOAc/hexanes). The *O*-alkylated product (0.074 g, 7%, TLC, $R_f = 0.78$, 40% EtOAc/hexanes) was obtained as a slightly contaminated solid after a second flash column chromatography on silica gel using a similar step gradient (hexanes → 20% EtOAc in hexanes). Pure **6** (0.117 g, 11%) was obtained as a white glass as the gradient was increased from 25% → 30% EtOAc/hexanes: ^{1}H NMR ($CDCl_3$) δ 9.00 (s, 1 H), 7.30−8.10 (m, 15 H), 5.07 (d, 1 H, J = 6.8 Hz), 4.62−4.84 (m, 3 H), 4.50 (AB q, 2 H, J = 11.7 Hz), 4.20 (AB d, 1 H, J = 9.7 Hz), 3.15 (AB d, 1 H, J = 9.7 Hz), 2.05−2.23 (m, 1 H), 1.77−1.93 (m, 1 H), 1.40−1.50 (m, 1 H), 1.10 (irregular t, 1 H), 0.86 (irregular t, 1 H). Anal. ($C_{32}H_{29}IN_2O_5$) C, H, N.

(1*S*,2*S*,4*S*,5*R*)-5-Iodo-1-{4-phenylmethoxy)-5-[(phenylmethoxy)methyl]bicyclo[3.1.0]hex-2-yl}-1,3-dihydropyrimidine-2,4-dione (7). A solution of **6** (0.110 g, 0.17 mmol) in methanol (14 mL) was treated with concentrated NH_4OH (1 mL) and stirred for 1 h at room temperature. Volatiles were removed under vacuum and the white powder obtained was purified by silica gel flash chromatography using a step gradient (25% EtOAc/hexanes → 50% EtOAc in hexanes) to give **7** (0.080 g, 87%) as a white glassy solid: ^{1}H NMR ($CDCl_3$) δ 8.81 (s, 1 H), 8.29 (br s, 1 H), 7.25−7.50 (m, 10 H), 5.06 (d, 1 H, J = 6.6 Hz), 4.73 (AB q, 2 H, J = 12.2 Hz), 4.62 (irregular t, 1 H), 4.48 (AB q, 2 H, J = 11.9 Hz), 4.17 (AB d, 1 H, J = 10.0 Hz), 3.12 (AB d, 1 H, J = 10.0 Hz), 2.05−2.15 (m, 1 H), 1.77−1.93 (m, 1 H), 1.37 (m, 1 H), 1.10 (m, 1 H), 0.82 (irregular t, 1 H). Anal. ($C_{25}H_{25}IN_2O_4$) C, H, N.

(1*S*,2*S*,4*S*,5*R*)-1-[4-Hydroxy-5-(hydroxymethyl)bicyclo-[3.1.0]hex-2-yl]-5-iodo-1,3-dihydropyrimidine-2,4-dione (2). Method A. A solution of **7** (0.031 g, 0.057 mmol) in CH_2Cl_2 (10 mL) was stirred at −78 °C (acetone/dry ice) and treated with BCl_3 (1.2 mL, 1 M solution in CH_2Cl_2) for 1 h. Methanol was added (3 mL) and the reaction mixture was concentrated under vacuum and reconcentrated three times from MeOH (5 mL). The residue was purified by silica gel flash chromatography using a step gradient ($CHCl_3$ → 5% MeOH/$CHCl_3$). The solid obtained was recrystallized from MeOH/$CHCl_3$ to give **2** (0.012 g, 57%) as a white solid: mp 226−227 °C; $[\alpha]^{25}_D = -3.0$ (*c* 0.1, MeOH); ^{1}H NMR ($CDCl_3$ + D_2O) δ 8.53 (s, 1 H), 4.72 (d, 1 H, J = 6.8 Hz), 4.55 (irregular t, 1 H), 4.08 (AB d, 1 H, J = 11.5 Hz), 3.07 (AB d, 1 H, J = 11.5 Hz), 1.82 (dd, 1 H, $J \approx$ 14.7, 8.0 Hz), 1.50−1.55 (m, 1 H), 1.32 (irregular dd, 1 H), 0.82 (irregular t, 1 H), 0.63 (irregular dd, 1 H); ^{13}C NMR ($CDCl_3$) δ 153.13, 143.21, 138.93, 61.39, 61.05, 53.91, 48.55, 30.33, 29.22, 16.61, 2.34; FAB MS *m*/*z* (relative intensity) 365 (MH^+, 100), 239 (b + 2H, 45). Anal. ($C_{11}H_{13}IN_2O_4 \cdot H_2O$) C, H, N.

Method B. A solution of **12** (0.072 g, 0.16 mmol) in CH_2Cl_2 (10 mL) was stirred at −78 °C (acetone/dry ice) and treated with BCl_3 (1.6 mL, 1 M solution in CH_2Cl_2) for 1 h. After a similar workup and purification procedure, **2** (0.028 g, 50%) was obtained as a white solid, mp 229−230 °C, which was spectroscopically identical to the solid obtained under method A.

(1*S*,2*S*,4*S*,5*R*)-*N*-((2*E*)-Ethoxyprop-2-enoyl)({4-hydroxy-5-[(phenylmethoxy)methyl]bicyclo[3.1.0]hex-2-yl}amino)-carboxamide (9). 3-Ethoxypropenoyl chloride (2.14 g, 18 mmol) in benzene (50 mL) was added dropwise to a vigorously stirred suspension of AgNCO (6.0 g, 36 mmol) that was predried at 100 °C for 2 h. The resulting mixture was refluxed under argon for 45 min and cooled to room temperature. A 40 mL aliquot of the organic supernatant was then added dropwise to a solution of carbocyclic amine **8**[34] (2.08 g, 9 mmol) in DMF (50 mL), which was precooled to 0 °C (ice/salt) under a blanket of argon. The reaction mixture was stirred overnight and allowed to reach room temperature. Volatiles were removed under vacuum to provide **9** (3.19 g, 95%) as a glassy yellow solid. An analytically pure sample was obtained after silica gel flash chromatography using 50% EtOAc/hexanes followed by 100% EtOAc as eluants: ^{1}H NMR ($CDCl_3$) δ 9.64 (br s, 1 H), 8.85 (br d, 1 H, J = 7.0 Hz), 7.69 (d, 1 H, J = 12.2 Hz), 7.40 (m, 5 H), 5.41 (d, 1 H, J = 12.4 Hz), 4.83 (t, 1 H, J = 8.4 Hz), 4.64 (AB q, 2 H, J = 11.9 Hz), 4.29 (t, 1 H, $J \approx$ 6.7 H z), 4.02 (q, 2 H, J = 7.1 Hz), 3.92 (AB d, 1 H, J = 9.7 Hz), 3.51 (AB d, 1 H, J = 9.7 Hz), 2.36 (br s, 1 H), 2.11 (irregular q, 1 H), 1.60 (m, 1 H), 1.43 (t, 3 H, J = 7.1 Hz), 1,03 (irregular t, 1 H), 0.96 (m, 1 H), 0.70 (dd, 1 H, $J \approx$ 7.9, 5.9 Hz). Anal. ($C_{20}H_{26}N_2O_5 \cdot 0.25H_2O$) C, H, N.

(1*S*,2*S*,4*S*,5*R*)-1-{4-Hydroxy-5-[(phenylmethoxy)methyl]-bicyclo[3.1.0]hex-2-yl}-1,3-dihydropyrimidine-2,4-dione (10). A solution of **9** (3.19 g, 85 mmol) in 95% EtOH (100 mL) was treated with 1 M H_2SO_4 (100 mL) and heated to reflux for 1 h. Ethanol was removed under vacuum and the aqueous solution was neutralized with 2 N NaOH to pH 7 followed by extraction in chloroform (3 × 100 mL). The combined organic extracts were washed with saturated $NaHCO_3$, dried ($MgSO_4$), and concentrated under vacuum. Purification by silica gel flash chromatography with EtOAc afforded **10** (2.02 g, 73%) as a colorless foam: $[\alpha]^{25}_D$ = +59.0 (*c* 0.16, MeOH); ^{1}H NMR ($CDCl_3$) δ 8.35 (br s, 1 H), 8.04 (d, 1H, J = 8.0 Hz), 7.41 (m, 5 H), 5.46 (dd, 1 H, J = 8.0, 1.9 Hz), 5.07 (d, 1H, J = 7.1 Hz), 4.92 (t, 1 H, $J \approx$ 8.5 Hz), 4.63 (AB q, 2 H, J = 10.9 Hz), 4.19 (AB d, 1 H, J = 10.0 Hz), 3.37 (AB d, 1 H, J = 10.0 Hz), 2.06 (m, 1 H), 1.81 (m, 1 H), 1.45 (irregular dd, 1 H), 1.05 (m, 1 H), 0.85 (m, 1 H). Anal. ($C_{18}H_{20}N_2O_4 \cdot 0.25H_2O$) C, H, N.

(1*S*,2*S*,4*S*,5*R*)-1-{4-Hydroxy-5-[(phenylmethoxy)methyl]-bicyclo[3.1.0]hex-2-yl}-5-iodo-1,3-dihydropyrimidine-2,4-dione (12). A stirred solution of **10** (0.164 g, 0.5 mmol), iodine (0.254 g, 1 mmol), and 1 N HNO_3 (0.5 mL) in dioxane (5 mL) was heated to 100 °C for 1 h. Volatiles were removed under vacuum and the residue was reconcentrated thrice with ethanol (5 mL) and thrice with chloroform (5 mL). The solid obtained was purified by silica gel flash chromatography using a step gradient (50% EtOAc/hexanes → 100% EtOAc) to give **12** (0.191 g, 84%) as an off-white solid: mp 85−88 °C; ^{1}H NMR ($CDCl_3$) δ 8.68 (s, 1 H), 8.41 (br s, 1 H), 5.04 (d, 1 H, J = 6.8 Hz), 4.91 (t, 1 H, $J \approx$ 8.5 Hz), 4.77 (AB q, 2 H, J = 12.5 Hz), 4.08 (AB d, 1 H, J = 10.2 Hz), 3.22 (AB d, 1 H, J = 10.2 Hz), 2.06 (m, 1 H), 1.79 (m, 1 H), 1.43 (irregular dd, 1 H), 1.04 (irregular dd, 1 H), 0.80 (m, 1 H). Anal. ($C_{18}H_{19}IN_2O_4 \cdot 0.5H_2O$) C, H, N.

(1*R*,2*S*,4*S*,5*S*)-[2-Acetyloxy-4-(5-bromo-2,4-dioxo(1,3-dihydropyrimidinyl))bicyclo[3.1.0]hexyl]methyl Acetate (13). Under a blanket of argon, compound **11**[26] (0.073 g, 0.31 mmol) was dissolved in acetic anhydride (2 mL) with gentle warming. After cooling to room temperature, bromine (0.02 mL, 0.33 mmol) was added slowly and the solution was stirred at room temperature for 30 min. The solution was stored in the refrigerator overnight (ca. 4 °C), and the volatiles were removed under reduced pressure. The residue was reconcentrated three times from toluene (5 mL), and treatment of the orange residue with water gave 0.179 g of crude product, which was purified by silica gel flash chromatography using a step gradient (hexanes → 50% EtOAc/hexanes → EtOAc) to give **13** (0.117 g, 94%) as a reasonably pure colorless foam; ^{1}H NMR ($CDCl_3$) δ 8.72 (br s, 1 H), 8.03 (s, 1 H), 5.63 (t, 1 H, $J \approx$ 8.4 Hz), 5.15 (d, 1 H, J = 7.6 Hz), 4.79 (AB d, 1 H, J = 12.4 Hz), 3.75 (AB d, 1 H, J = 12.4 Hz), 2.39 (m, 1 H), 2.33 (s, 3 H), 2.16 (s, 3 H), 1.86 (m, 1 H), 1.60 (m, 1 H), 1.12 (m, 1 H), 1.02 (m, 1 H). 0.63 (irregular dd, 1 H). This product was used immediately in the following step.

(1*S*,2*S*,4*S*,5*R*)-5-Bromo-1-[4-hydroxy-5-(hydroxymethyl)-bicyclo[3.1.0]hex-2-yl]-1,3-dihydropyrimidine-2,4-dione (3). A solution of **13** (0.111 g, 0.27 mmol) in saturated methanolic ammonia (3 mL) was kept at room temperature overnight and then heated at 50 °C for 24 h. Volatiles were removed under vacuum, and the solid obtained (TLC, R_f = 0.32, 15% MeOH/$CHCl_3$) was found by ^{1}H NMR to still contain one acetate group. The solid was then dissolved in THF (2.5 mL) and treated with 1 N NaOH (0.2 mL), and the solution was stirred at room temperature for 4 h. Acetic acid (20 μL) was added, and volatiles were removed under vacuum. The crude solid was purified by reverse phase C-18 column chromatography using a step gradient (H_2O → 5% MeOH/H_2O → 10% MeOH/H_2O), and following a second chromatography on silica gel with a step gradient ($CHCl_3$ → 10% MeOH/$CHCl_3$), **3** (0.026 g, 30%) was obtained as a white solid: mp 218−219 °C; $[\alpha]^{25}_D$ = 18.2 (*c* 0.11, MeOH); ^{1}H NMR ($CDCl_3$ + D_2O) δ 8.55 (s, 1 H), 4.74 (d, 1 H, *J* = 6.6 Hz), 4.57 (t, 1 H, *J* ≈ 8.4 Hz), 4.11 (AB d, 1 H, *J* = 11.2 Hz), 3.07 (AB d, 1 H, *J* = 11.2 Hz), 1.86 (dd, 1 H, *J* = 14.4, 7.8 Hz), 1.58 (m, 1 H), 1.35 (m, 1 H), 0.83 (irregular t, 1 H), 0.65 (irregular dd, 1 H); ^{13}C NMR ($CDCl_3$) δ 154.70, 151.75, 142.70, 87.64, 61.27, 53.85, 48.78, 30.07, 29.06, 16.52, 2.36; FAB MS *m*/*z* (relative intensity) 317/319 (MH^+, 100/87), 191 (b + 2H, 30). Anal. ($C_{11}H_{13}BrN_2O_4$) C, H, N.

(1*S*,2*S*,4*S*,5*R*)-Ethyl(2*E*)-3-{1-[4-Hydroxy-5-(hydroxymethyl)-bicyclo[3.1.0]hex-2-yl]-2,4-dioxo(1,3-dihydropyrimidine-5-yl)}prop-2-enoate (14). In a vial fitted with a Teflon-lined cap, a solution of palladium(II) acetate (5.5 mg, 25 mmol) and triphenylphosphine (0.013 g, 50 mmol) in dioxane (1 mL) was allowed to stand for 10 min during which time the solution turned red. Immediately after, triethylamine (0.055 mL, 0.4 mmol) and methyl acrylate (0.110 mL, 1.24 mmol), followed by **2** (0.089 g, 0.25 mmol), were added. After adding additional dioxane (2 mL), the vial was sealed and heated at 78 °C for 4 h. A similar reaction was set up separately, and the two combined reactions were concentrated under reduced pressure. Initial purification by silica gel flash chromatography using a step gradient ($CHCl_3$ → 5% MeOH/$CHCl_3$ → 10% MeOH/$CHCl_3$) gave unreacted **2** (0.054 g) along with product-containing fractions that were evaporated and triturated with EtOAc to give 14 (0.076 g, 48%) as white crystals: mp 240−241 °C; TLC, R_f = 0.54, 15% MeOH/$CHCl_3$; $[\alpha]^{25}_D$ = −2.4 (*c* 2.3, MeOH); ^{1}H NMR ($CDCl_3$ + D_2O) δ 8.57 (s, 1 H), 7.33 (d, 1 H, *J* = 16.0 Hz), 6.86 (d, 1 H, *J* = 16.0 Hz), 4.78 (d, 1 H, *J* = 6.8 Hz), 4.61 (t, 1 H, *J* ≈ 8.4 Hz), 4.14 (AB d, 1 H, *J* = 11.5 Hz), 3.70 (s, 3 H), 3.10 (AB d, 1 H, *J* = 11.5 Hz), 1.89 (dd, 1 H, *J* = 14.3, 8.05), 1.58 (m, 1 H), 1.38 (irregular dd, 1 H), 0.58 (irregular t, 1 H), 0.63 (irregular dd, 1 H). Anal. ($C_{15}H_{18}IN_2O_6$·0.25H_2O) C, H, N.

(1*S*,2*S*,4*S*,5*R*)-(2*E*)-3-{1-[4-Hydroxy-5-(hydroxymethyl)-bicyclo[3.1.0]hex-2-yl]-2,4-dioxo(1,3-dihydropyrimidine-5-yl)}prop-2-enoic Acid (15). A solution of **14** (0.165 g, 0.51 mmol) in 1.8 *N* KOH (2 mL) was stirred overnight at room temperature. The solution was then acidified with concentrated HCl to pH 2 and the precipitate formed was collected by filtration. The filtrate was reduced to dryness and the solid residue was triturated with MeOH. The combined solids were dissolved in hot MeOH, the solution was filtered, and the filtrate was allowed to stand at room temperature. Since no crystals formed, the methanolic solution was concentrated to give **15** (0.137 g, 87%) as a white solid: mp 240 °C dec; ^{1}H NMR ($CDCl_3$ + D_2O) δ 8.53 (s, 1 H), 7.26 (d, 1 H, *J* = 15.8 Hz), 6.79 (d, 1 H, *J* = 15.8 Hz), 4.78 (d, 1 H, *J* = 6.6 Hz), 4.61 (irregular t, 1 H), 4.14 (AB d, 1 H, *J* = 11.5 Hz), 3.15 (AB d, 1 H, *J* = 11.5 Hz), 1.87 (irregular dd, 1 H), 1.59 (m, 1 H), 1.37 (irregular dd, 1 H), 0.85 (irregular t, 1 H), 0.63 (irregular dd, 1 H). This product was used directly in the next step.

(1*S*,2*S*,4*S*,5*R*)-5-((1*E*)-2-Bromovinyl)-1-[4-hydroxy-5-(hydroxymethyl)bicyclo[3.1.0]hex-2-yl]-1,3-dihydropyrimidine-2,4-dione (4). To a mixture of **15** (0.126 g. 0.41 mmol) in DMF (2.5 mL) was added $KHCO_3$ (0.118 g, 1.18 mmol) followed by slow addition of NBS (0.072 g, 0.41 mmol) dissolved in DMF (1 mL). After 2.5 h of stirring, insoluble materials were removed by filtration and the filtrate was concentrated under vacuum. The crude product was purified by silica gel flash column chromatography using a step gradient ($CHCl_3$ → 5% MeOH/$CHCl_3$ → 10% MeOH/$CHCl_3$). A second chromatographic purification on silica gel (CH_2Cl_2 → 5% *i*-propanol/CH_2Cl_2 → 10% *i*-propanol/CH_2Cl_2) and a final chromatography on a C-18 reverse phase column using a step gradient (H_2O → 20% MeOH/H_2O) were required to obtain **4** (0.035 g, 25% yield) as an off-white solid: mp 120−122 °C; $[\alpha]^{25}_D$ = −23.0 (*c* 0.13, MeOH); ^{1}H NMR ($CDCl_3$ + D_2O) δ 8.29 (s, 1 H), 7.24 (d, 1 H, *J* = 13.6 Hz), 6.76 (d, 1 H, *J* = 13.6 Hz), 4.76 (d, 1 H, *J* = 6.8 Hz), 4.61 (t, 1 H, *J* ≈ 8.4 Hz), 4.14 (AB d, 1 H, *J* = 11.5 Hz), 3.11 (AB d, 1 H, *J* = 11.5 Hz), 1.85 (dd, 1 H *J* ≈ 14.7, 8.2 Hz), 1.58 (m, 1 H), 1.35 (irregular dd, 1 H), 0.84 (irregular t, 1 H), 0.65 (irregular dd, 1 H); FAB MS *m*/*z* (relative intensity) 343/345 (MH^+, 100/95), 217 (b + 2H, 35). Anal. ($C_{13}H_{15}BrN_2O_4$·0.5H_2O) C, H, N.

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List of Abbreviations

acyclovir, ACV; allogeneic bone-marrow transplantation, allo-BMT; cytomegalovirus, CMV; cytopatogenic effect, CPE; ganciclovir, GCV; graft versus host disease, GvHD; hypoxanthine, aminopterin, thymidine medium, HAT; herpes simplex type 1, HSV1; herpes simplex type 2, HSV2; human cytosolic thymidine kinase, hTK1; Minimum essential medium, MEM; National Institutes of Allergy and Infectious Diseases, NIAID; (north)-methanocarbocyclic thymine, (N)-MCT; (south)-methanocarbocyclic thymine, (S)-MCT; thymidine kinase, TK; thymidine monophosphate, TMP; thymidine, Thy; varicella-zoster virus, VZV; viral plaque reduction, VPR; 2,3-bis[methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolin-5-carboxanilide, XTT; (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, BVDU; 5-iodo-deoxyuridine, IdU

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