Nucleosides with a Twist. Can Fixed Forms of Sugar Ring Pucker Influence Biological Activity in Nucleosides and Oligonucleotides?†

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The sugar moiety of nucleosides in solution is known to exist in a rapid dynamic equilibrium between extreme Northern and Southern conformations as defined in the pseudorotational cycle. In the present work, we describe how the bicyclo[3.1.0]hexane template fixes the ring pucker of 2′-deoxy-methanocarba-nucleosides **1**−**5** and **12** to values corresponding to either one of these two extreme conformations that are typical of nucleosides. The syntheses of the fixed Northern conformers **1**−**5** were performed by Mitsunobu coupling of the heterocyclic bases with the chiral carbocyclic alcohol **6** [(1*R*,2*S*,4*R*,5*S*)-1-[(benzyloxy)methyl]-2-(*tert*-butyloxy)-4-hydroxybicyclo[3.1.0]hexane], while the synthesis of the Southern conformer, (S)-methanocarba-T (**12**), was reported earlier. Carbocyclic thymidine (carba-T, **13**) was used as a reference, flexible carbocyclic nucleoside. Antiviral evaluation of these compounds revealed a very potent antiherpetic activity associated with the Northern thymidine analogue **2**, which was more powerful than the reference standard acyclovir against both HSV-1 and HSV-2. (N)-Methanocarba-T (**2**) was further evaluated as a component of a short oligodeoxynucleotide (ODN) phosphorothioate (5′-CTTCATTTTTTCTTC-3′) where all thymidines were replaced by **2**. The expected thermodynamic stability resulting from the preorganization of the pseudosugar rings into a Northern conformation, typical of A-DNA, was evident by the increase in T_m of the corresponding DNA/RNA heteroduplex. However, the rigid A-tract ODN caused loss of RNase H recruitment. A detailed conformational analysis of (N)-methanocarba-T (**2**) and (S)-methanocarba-T (**12**), as representative examples of conformationally rigid pseudorotational antipodes, revealed that in addition to their different forms of ring pucker, (S)-methanocarba-T appears to be a rather stiff molecule with fewer low-energy conformational states available compared to (N)-methanocarba-T. The *syn*/*anti*-energy barrier for these nucleoside analogues is 5−6 kcal/mol higher than for common nucleosides.

Introduction

The sugar ring of nucleosides and nucleotides equilibrates in solution between two extreme forms of ring pucker neighboring a 2′-*exo*/3′-*endo* (Northern) conformation and the opposite 2′-*endo*/3′-*exo* (Southern) conformation.[1] In the pseudorotational cycle (Figure 1),[1,2] an absolute Northern conformation would correspond to a range of *P* (angle of pseudorotation) between 342° and 18° ($_2$E → ^{3}T$_2$ → ^{3}E), whereas an absolute Southern conformation would be defined in a range of *P* between 162° and 198° (^{2}E → ^{2}T$_3$ → $_3$E). Preference for any of these specific conformations in solution is determined by the interplay of important interactions resulting from anomeric and *gauche* effects.[1,3] In the solid state, however, only one of the two solution conformations is present, and its selection is additionally determined by crystal-packing forces.[1,4] Similarly, when a nucleoside or nucleotide binds to its target enzyme, only one form is expected to be present at the active site. While the energy gap between Northern and Southern conformations is in the neighborhood of 4 kcal/mol,[1] such a disparity can explain the difference between micromolar and nanomolar binding affinities.[5,6]

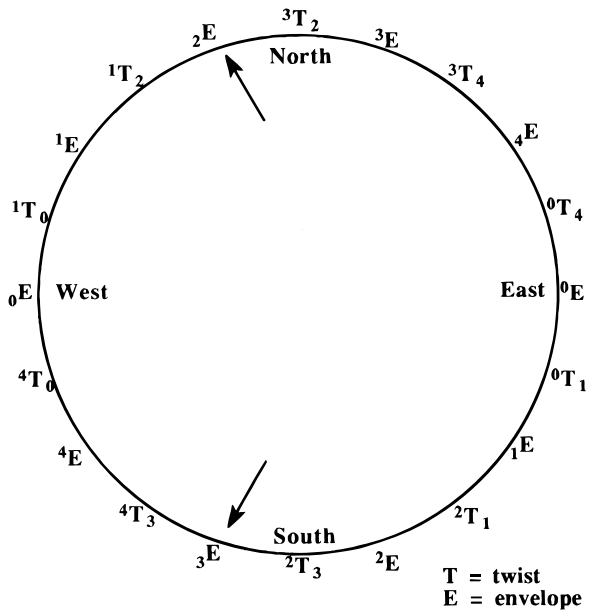

Figure 1. Pseudorotational cycle.

Independently, we and others have constructed conformationally rigid nucleosides on a bicyclo[3.1.0]hexane template whose value of *P* fits within the range of absolute Northern or Southern conformations.[7−12] This bicyclo[3.1.0]hexane template exists exclusively as a pseudoboat, and carbocyclic nucleosides built on it can adopt either a Northern or a Southern conformation

† Dedicated to Prof. Joseph H. Burckhalter on the occasion of his recent induction into the National Inventors Hall of Fame.

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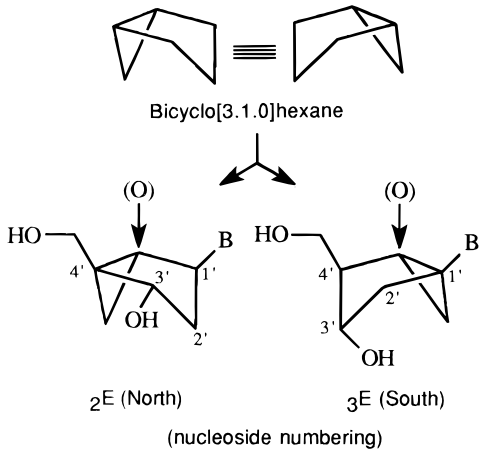
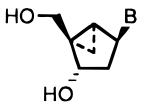
Scheme 1



depending on the relative disposition of substituents on the ring (Scheme 1). Thus, a Northern C2′-*exo* ($_2$E) envelope conformation is obtained when the cyclopropane ring appears fused between carbon C4′ and the carbon supplanting the ribofuranoside oxygen. Conversely, fusion of the cyclopropane ring between carbon C1′ and the carbon supplanting the ribofuranoside oxygen provides the Southern envelope conformation.

Encouraged by the excellent *in vitro* antiviral activity of the Northern bicyclo[3.1.0]hexane carbocyclic analogue of 2′-deoxyaristeromycin (**1**) against human cytomegalovirus (HCMV) and EBV,[12] we decided to complete the investigation of the remaining nucleoside bases connected to the same rigid pseudosugar template (**2**−**5**).

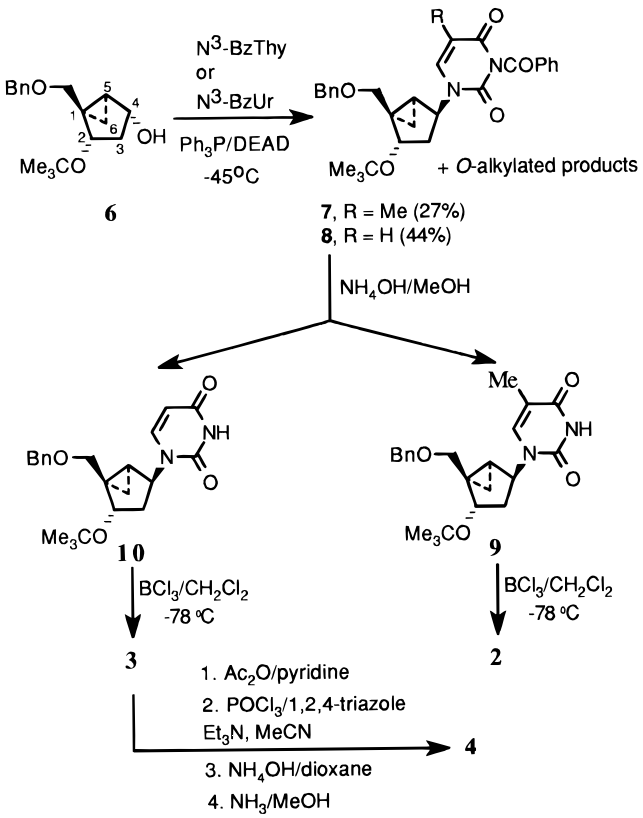


1, B = adenine [(N)-2'-deoxy-*methanocarba*-A]
2, B = thymine [(N)-*methanocarba*-T]
3, B = uracil [(N)-2'-deoxy-*methanocarba*-U]
4, B = cytosine [(N)-2'-deoxy-*methanocarba*-C]
5, B = guanine [(N)-2'-deoxy-*methanocarba*-G]

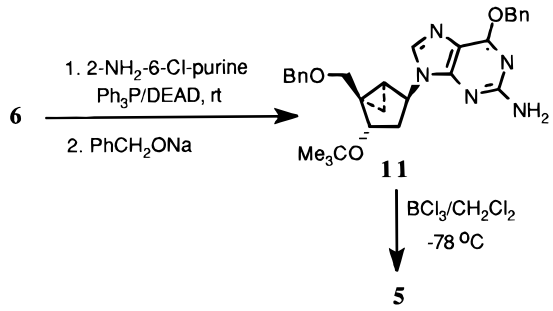
Synthesis

Our recently developed convergent method for the synthesis of **1**[12] was adapted to incorporate the rest of the heterocyclic bases onto the chiral carbocyclic alcohol **6** [(1*R*,2*S*,4*R*,5*S*)-1-[(benzyloxy)methyl]-2-(*tert*-butyloxy)-4-hydroxybicyclo[3.1.0]hexane] by a direct Mitsunobu coupling reaction.[13] For the pyrimidine derivatives, protected N^3-benzoylthymine[14] and N^3-benzoyluracil[14] were coupled according to Scheme 2. One of the drawbacks of this approach, as reported in other instances,[7,9] is that the *O*-alkylated products are often prevalent. In the case of **7**, the *O*-alkylated product predominated, whereas for the uracil analogue **8**, the situation was reversed. Base-catalyzed deprotection of the *N*-benzoyl group from intermediates **7** and **8** gave the penultimate intermediates **9** and **10**, respectively, and simultaneous removal of both *O*-benzyl and *O*-*tert*-butyl groups with BCl_3 provided the desired targets (N)-methanocarba-T (**2**) and (N)-2′-deoxy-methanocarba-U (**3**). (N)-2′-Deoxy-methanocarba-C (**4**) was prepared from (N)-2′-deoxy-methanocarba-U via formation of the triazole intermediate according to published methods.[15]

Scheme 2



Scheme 3



For the synthesis of (N)-2′-deoxy-methanocarba-G (**5**), coupling under Mitsunobu conditions (Scheme 3) proceeded with a comparable yield to that of the pyrimidines. As reported in a similar case, only the desired *N*-9 isomer (34%) was obtained with virtually no detection of the *N*-7 isomer.[7,9] The strategy of converting the 2-amino-6-chloro intermediate into the 6-*O*-benzyl derivative **11** was found advantageous to facilitate the one-step removal of all protective groups in the generation of the guanine base.[7,9]

Antiviral Activity

The compounds were tested against three types of DNA viruses, such as herpes simplex 1 and 2 (HSV-1 and HSV-2) and human cytomegalovirus (HCMV).[16] According to the cytopathogenic effect (CPE) inhibition assay (Table 1), a very potent antiherpetic activity associated with (N)-methanocarba-T (**2**) was discovered. In decreasing order of potency, (N)-2′-deoxy-methanocarba-C (**4**), (N)-2′-deoxy-methanocarba-G (**5**), and (N)-2′-deoxy-methanocarba-A (**1**) lagged behind. Anti-HCMV activity was significant only for (N)-2′-deoxy-methanocarba-A (**1**) as reported earlier.[12] Such a

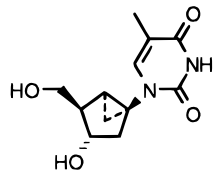
Table 1. Antiviral Activity of Rigid (N)-Methanocarbocyclic Nucleosides **1**−**5** According to the Cytopathogenic Effect (CPE) Inhibition Assay

compd	virus[a] (HFF cells)	EC_{50}[b] (μg/mL)	CC_{50}[c] (μg/mL)	SI[d]	control[e] (EC_{50}, μg/mL)
1	HSV-1	72.0	>100	>1.4	ACV (0.80)
	HSV-2	13.9	>100	>7.2	ACV (4.00)
	HCMV	3.1	>100	>32.2	GCV (0.30)
2	HSV-1	0.03	>100	>3333	ACV (0.40)
	HSV-2	0.09	>100	>1111	ACV (0.06)
	HCMV	>20	63.7	<3.2	GCV (0.30)
3	HSV-1	>100	>100	1	ACV (0.60)
	HSV-2	>100	>100	1	ACV (1.50)
	HCMV	>100	>100	1	GCV (0.40)
4	HSV-1	0.14	68	486	ACV (0.70)
	HSV-2	>20	96	<4.8	ACV (6.20)
	HCMV	>4.0	8.8	<2.2	GCV (0.02)
5	HSV-1	4.0	>100	>25	ACV (0.60)
	HSV-2	9.9	>100	<10.1	ACV (1.50)
	HCMV	>20	64.3	<3.2	GCV (0.40)

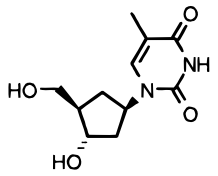
[a] HSV-1 = herpes simplex type 1; HSV-2 = herpes simplex type 2; HCMV = human cytomegalovirus; HFF = human foreskin fibroblasts. [b] EC_{50} = inhibitory concentration required to reduced virus-induced cytopathogenicity by 50%. [c] CC_{50} = cytotoxic concentration that produces 50% cell death. [d] SI = selectivity index (CC_{50}/EC_{50}). [e] ACV = acyclovir; GCV = gancyclovir.

striking selectivity for herpes viruses displayed by **2** prompted us to compare it to the Southern carbocyclic antipode (S)-methanocarba-T (**12**) whose synthesis we have recently reported.[11] In addition, we obtained a sample of (±)-carba-T (**13**) to use as a flexible carbocyclic nucleoside control (kindly supplied by Dr. Y. Fulmer Shealy, Southern Research Institute). (±)-Carba-T was reported to have *in vitro* antiherpetic activity in the CPE inhibition assay.[17,18] The EC_{50} values for (±)-carba-T measured by this method were 0.8 μg/mL (HSV-1) and 7.0 μg/mL (HSV-2) in primary rabbit kidney cells[17] and 24 μg/mL (HSV-1) and 57 μg/mL (HSV-2) in Vero cells.[18] Later, (+)-carba-T was synthesized and reported to have EC_{50} values of 0.2 μg/mL (HSV-1) and 2 μg/mL (HSV-2) in the same system.[19]

In view of the variability of the CPE method, we decided to compare these three compounds (**2**, **12**, and **13**) using the more accurate plaque reduction assay method with acyclovir as a positive control. Under these conditions, (N)-methanocarba-T (**2**) showed excellent and reproducible activity against HSV-1 and HSV-2, with a potency surpassing that of acyclovir (Table 2). This activity was better in the conventional postinfection treatment protocol (see the Experimental Section) than in a drug-pretreated plaque reduction assay. On the other hand, (S)-methanocarba-T (**12**), as well as (±)-carba-T (**13**), was devoid of antiherpetic activity according to this more reliable assay.



12 [(S)-*methanocarba*-T]



13 (*carba*-T)

Regarding cell cytotoxicity against host HFF cells, the three compounds were nontoxic (CC_{50} > 100 μg/mL) against stationary cells. However, the order of potency against rapidly dividing cells was (±)-carba-T (**13**) (CC_{50} = 0.65 μg/mL) > (N)-methanocarba-T (**2**) (CC_{50} = 32.9 μg/mL) > (S)-methanocarba-T (**12**) (CC_{50} > 100 μg/mL). Gancyclovir, which was used as a reference for cell cytotoxicity, showed an average CC_{50} of 40.0 μg/mL in rapidly dividing HFF cells.

Table 2. Antiherpetic Activity of Rigid (N)-Methanocarba-T (**2**), Rigid (*S*)-Methanocarba-T (**12**), and Carbocyclic Thymidine [(±)-Carba-T (**13**)] Measured by the Plaque Reduction Assay in HFF

compd	virus[a]	EC_{50}[b] (μg/mL)	CC_{50}[c] (μg/mL)	SI[d]	ACV[e] (EC_{50}, μg/mL)
2	HSV-1	0.01	>20	>2000	0.30
		0.08[f]	>20	>250	0.30[f]
	HSV-2	0.12	>20	>167	0.80
		0.43[f]	>20	>46.5	1.10[f]
12	HSV-1	>50	>50	1	0.15
		>20	>20	1	0.30
	HSV-2	>50	>50	1	0.60
		>20	>20	1	1.10
13	HSV-1	>10	>10	1	0.30
	HSV-2	>10	>10	1	0.80

[a] HSV-1 = herpes simplex type 1; HSV-2 = herpes simplex type 2. [b] EC_{50} = inhibitory concentration required to reduce the number of virus plaques by 50%. [c] CC_{50} = cytotoxic concentration that produces 50% of cell death. [d] SI = selectivity index (CC_{50}/EC_{50}). [e] ACV = acyclovir control. [f] These values correspond to a drug pretreated plaque reduction assay.

Antisense Activity

Standard double-stranded helices could exist in the classic B-DNA form, where all the sugars have a Southern conformation, or in the A-DNA form, with the sugars having a Northern conformation.[20] Since during formation of the DNA/RNA heteroduplex, the A-form—typical of RNA—is dominant,[21] it was reasoned that assisting the DNA half to preorganize into an A-conformation would increase the thermodynamic stability of the DNA/RNA heteroduplex. The single incorporation of (N)-methanocarba-T (**2**) and (S)-methanocarba-T (**12**) into small oligodeoxynucleotides (ODN) confirmed this assumption and showed that these pseudorotational antipodes have significant differences in modulating the thermodynamic stability of DNA/RNA heteroduplexes.[8,10] Altmann et al.[8] showed that substitution of (N)-methanocarba-T (**2**) for natural thymidine in DNA/RNA heteroduplexes increased the thermodynamic stability of the double helix, as indicated by a positive increase in the melting temperature (T_m), whereas (S)-methanocarba-T (**12**) induced a small destabilizing effect.[10] The increase in thermodynamic stability reported for two different (N)-methanocarba-T-containing ODNs versus the wild-type was between 0.8° and 2.1° for a single modified nucleotide; however, no data was reported for an ODN containing multiple (N)-methanocarba-Ts.[8] It is noteworthy that the destabilizing effect observed with multiple (S)-methanocarba-Ts did not appear to be additive, as its effect tapered off when the number of modified nucleotides was increased.[10] For this reason, it was of interest to investigate whether the stabilizing effect of multiple (N)-methanocarba-Ts was additive. In addition, notwithstanding that an increase in the thermodynamic stability of the DNA/RNA heteroduplex is desirable for antisense activity, no antisense data were reported for ODNs containing (N)-methanocarba-T.

To answer these questions, we synthesized a test sequence targeted to the coding region of the SV40 large T-antigen[22] (ODN test 1, Table 3) as the phosphorothio-

Table 3. Effect of (N)-Methanocarba-T Substitution on Heteroduplex Stability, RNase H Activity, and T-Antigen Inhibition with the Modified Phosphorothioate 5′-CTTCATTTTTTCTTC-3′

ODN	T analog[a]	C analog[a]	T_m[b] (°C)	RNAse H[c]	IC_{50}[d]
test 1	(N)-methano-carba-T	pC	57.5	−	no inhibition at 20 μM
control 1	T	pC	44.0	+	no inhibition at 20 μM
control 2	T	5-Me-C	<40	+	no inhibition at 20 μM
control 3	pU	pC	70.0	+	0.1 μM

[a] T and C positions are completely substituted with (N)-carba-T, thymidine (T), 5-propynyl-2′-deoxyuridine (pU), 5-propynyl-2′-deoxycytidine (pC), or 5-methyl-2′-deoxycytidine (5-Me-C). [b] T_m was measured using the complementary RNA strand in the buffer, 140 mM KCl/5 mM Na_2HPO_4/1 mM $MgCl_2$, pH 7.2. [c] The modified ODNs were hybridized to a 5′-end-labeled RNA and treated with HeLa cell nuclear extract (a source of human RNase H) to test for *in vitro* recruitment and activation of RNase H. [d] IC_{50} values for the inhibition of T-antigen expression in cell culture using the microinjection assay described in ref 22.

ate 5′-CTTCATTTTTTCTTC-3′, where all thymidines (T) were replaced by (N)-methanocarba-Ts. This and other control ODNs were evaluated by T_m analysis on the complementary RNA target (Table 3). The control ODNs were equivalent phosphorothioates with modifications designed to optimize antisense activity (Table 3).[22] The data showed that the increase in thermodynamic stability of the heteroduplex due to the presence of multiple (N)-methanocarba-T nucleotides was indeed additive, and the average stabilization per substitution was ca. 1.3 °C relative to thymidine. However, despite the increase in T_m, the ODN failed to inhibit T-antigen expression following cellular microinjection. The highest intracellular concentration tested was 20 μM. This contrasts the antisense effect (IC_{50} = 0.1 μM) of the 5-propynyl-2′-deoxyuridine-containing ODN (control 3). A probable reason for the lack of antisense activity for the (N)-methanocarba-T ODN is that it does not recruit RNase H cleavage of the targeted RNA strand upon hybridization (Table 3). This contrasts the RNase H recruitment properties of the three control ODNs. RNase H cleavage has been shown to be important for maximal antisense potency,[23,24] and RNase H recruitment has previously been shown to be very dependent on sugar pucker.[25] All modifications of a 2′-deoxyribose which favor the Northern conformation have in fact resulted in the loss of RNase H recruitment, except when the modified 2′-deoxynucleotides appear at the termini of the ODNs leaving a gap for efficient target RNA cleavage.[23,26]

Conformational Analysis

Based on the X-ray structures of **1**,[12] **2**,[8] and **12**,[10] the pseudorotational parameter *P* associated with ring puckering can be calculated[27] and compared to the values of *P* derived from the X-ray structures of prototypic nucleosides known to crystallize in the Northern and Southern hemispheres. Choosing a Northern 2′-deoxyribonucleoside conformer as a reference is difficult because compounds of this type tend to crystallize as Southern conformers due to the strong *gauche* interaction between the sugar oxygen (O4′) and the 3′-OH group. A reasonable approximation is uridine, a ribonucleoside known to crystallize in the Northern hemisphere.[28] The *P* value for uridine is 3.7°, close to a perfect $^3T_2$, whereas (N)-2′-deoxy-methanocarba-A (**1**) and (N)-methanocarba-T (**2**) prefer conformations closer to a $_2$E envelope (*P* = 342° and 343°, respectively). Such values of *P*, therefore, position these carbocyclic nucleosides in the absolute Northern range, neighboring our prototype nucleoside uridine. The remarkable similarity observed for the values of *P* in both X-ray structures of (N)-methanocarba-T (**2**) and (N)-2′-deoxy-methanocarba-A (**1**) is noteworthy and provides a strong support for the rigid nature of the bicyclo[3.1.0]hexane system. In the Southern hemisphere, the sugar puckerings of reference thymidine[29] (*P* = 188°) and (S)-methanocarba-T (**12**; *P* = 190°) are much closer, and both prefer near-$_3$E envelope conformations that differ only by 2°. These comparisons between rigid carbocyclic nucleosides and reference nucleosides validate the role of the cyclopropane ring in inducing the desired form of ring pucker in both hemispheres. Indeed, carba-T (**13**),[30] without a fused cyclopropane ring, has an unusual conformation in the crystal state close to a $_1$E (*P* = 118°) envelope. Such an uncommon conformation results from the lack of anomeric and *gauche* interactions that otherwise exist in thymidine but which are annulled by the absence of the furanose oxygen in carba-T.[3]

Table 4. Conformation Parameters and Energies (kcal/mol) Calculated from X-ray Structures and Molecular Modeling

X-ray structure	global minima (H-bond)	minima (*anti*)	H-bond	$E_{barrier}$ (*syn*/*anti*)
(N)-Methanocarba-T (**2**)				
$\chi = -147°$ *anti*	$\chi = 30°$ *syn*	$\chi = -150°$	$E = 3$	8
$\gamma = 67°$	$\gamma = 45°$	$\gamma = 60°$		
$P = 343°$	$P = 346°$	$P = 344°$		
$E = 67.23$	$E = 64.07$	$E = 67.09$		
(S)-Methanocarba-T (**12**)[a]				
$\chi = 59°$ *syn*	$\chi = 45°$ *syn*	$\chi = -135°$	$E = 4$	9
$\gamma = 56°$	$\gamma = 60°$	$\gamma = -60°$		
$P = 190°$	$P = 191°$	$P = 190°$		
$E = 82.92$	$E = 82.65$	$E = 86.62$		

[a] Molecule B for (S)-methanocarba-T: $\chi = 68°$ (*syn*), $\gamma = 51°$, $P = 190°$, $E = 83.44$ kcal/mol (molecule A was selected because its energy is closest to that of the global minimum).

Despite our knowledge concerning the immutability of the bicyclo[3.1.0]hexane moiety, as inferred from the X-ray structures[8,10,12] and variable temperature ^{1}H NMR measurements of carbocyclic nucleosides containing it,[7,9] we decided to investigate more thoroughly the behavior of Northern and Southern pseudorotational antipodes containing this rigid carbasugar template using the X-ray structures of (N)-methanocarba-T (**2**) and (S)-methanocarba-T (**12**) as starting conformations. The X-ray coordinates of each of the structures were exported into the CaChe Scientific Work System (Oxford Molecular) v. 4.0, and the corresponding energies were calculated (Table 4) after locking the geometry of the torsion angles χ (pseudoglycosyl bond)[27] and γ (exocyclic CH_2OH bond).[27] From these structures, a conformational analysis was performed for each compound by allowing the torsion angles χ and γ to rotate 360° in increments of 15°. This exhaustive search generated an optimized potential energy map for each compound from which the global energy minima conformations were identified (Table 4). In both instances, the global energy minima corresponded to conformations where

the torsion angle χ favors a *syn*-orientation that facilitates an intramolecular hydrogen bond between the C2 carbonyl and the hydroxyl of the CH_2OH group. As seen in Table 4, the X-ray structure of (S)-methanocarba-T (**12**)[10] showed a similar preference, and in terms of all conformational parameters and energy, it is very close to the calculated global minimum. On the other hand, the X-ray structure of (N)-methanocarba-T (**2**)[8] differed significantly from the global minimum since it prefers, instead, the *anti*-conformation. Energetically, the X-ray conformation is about 3 kcal/mol higher than the global minimum conformation. However, such energy difference corresponds almost entirely to hydrogen bonding. The hydrogen bond energy was estimated, in both cases, by measuring the depth of the lowest energy well from the surrounding low-energy conformations without hydrogen bonding. From the data in Table 4, one can also appreciate that the energy difference between the global minimum in the *syn*-configuration and the lowest possible *anti*-configuration for (N)-methanocarba-T is also 3 kcal/mol. For (S)-methanocarba-T the corresponding difference between *syn*- and *anti*-conformations approaches 4 kcal/mol, a difference that is also due to hydrogen bonding.

As anticipated, the differences in the values of *P* between X-ray and energy-minimized structures were minimal in the pseudorotational scale[1,2] for each pseudorotational antipode (Table 4). For (N)-methanocarba-T (**2**), the maximum variance of *P* was 3°, whereas for (S)-methanocarba-T (**12**) it was just 1° (Table 4) for either molecule, A or B, in the crystal asymmetric unit.[10] This means that the ring pucker remained fundamentally unchanged. In terms of χ and γ, however, significant differences were observed between the two pseudorotational antipodes themselves and conventional nucleosides. Despite the fact that in both cases energetically reasonable *syn*- or *anti*-conformations could be achieved, the rotational barrier for χ ($E_{syn/anti}$) was 5−6 kcal/mol higher than for thymidine (calculated under the same conditions) with (S)-methanocarba-T having a higher energy barrier than (N)-methanocarba-T (Table 4). These energy barriers correspond to values that exclude hydrogen bonding. On the other hand, the rotational barrier for γ was lower than for thymidine by about 2 and 1 kcal/mol, respectively, for (N)-methanocarba-T and (S)-methanocarba -T. The combination of higher energy barriers for (S)-methanocarba-T revealed in the potential energy map a much stiffer molecule with fewer low-energy conformational states available when compared to (N)-methanocarba-T. Such stiffness can be better appreciated by comparing the distribution of energy conformations for rotamers 5 kcal/mol above the global minimum (Figures 2 and 3). In the case of (N)-methanocarba-T, there is a wider distribution of rotamers at ca. 5 kcal/mol above the global minimum, whereas for (S)-methanocarba-T there are fewer rotamers available.

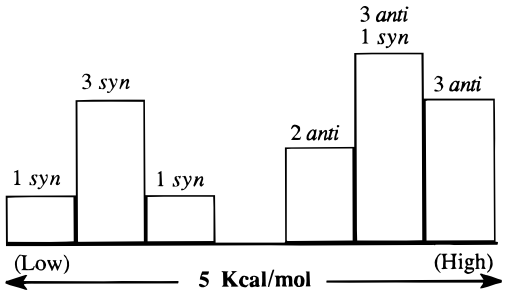


Figure 2. Distribution of 14 rotamers of (N)-methanocarba-T with energies up to 5 kcal/mol above the global minimum (the height of the bars is proportional to the number of rotamers).

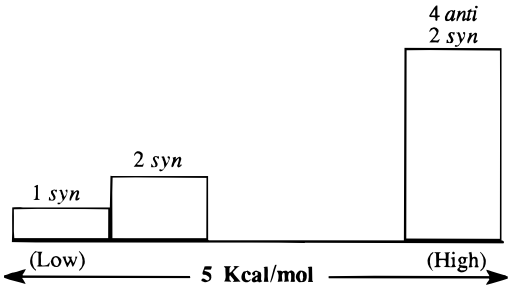


Figure 3. Energy distribution of nine rotamers of (S)-methanocarba-T with energies up to 5 kcal/mol above the global minimum (the height of the bars is proportional to the number of rotamers).

Discussion

The complete definition of the conformation of a nucleoside usually involves the determination of three groups of structural parameters: (a) the orientation about the glycosyl bond as *syn* or *anti*, which is more precisely defined by the value of the angle χ, (b) the orientation of the hydroxymethyl group determined by the value of the angle γ, and (c) the deviation from planarity of the sugar ring measured by the angle of pseudorotation *P*. For a nucleoside in solution, the values of these parameters are subject to changes that are dependent on the nature of the substituents present on the base and the sugar moiety. With the use of bicyclo[3.1.0]hexane as a pseudosugar template, we have been able to constrain pseudorotation to values of *P* that are normal for nucleosides existing in a Northern or Southern conformation as demonstrated for (N)-methanocarba-T (**2**) and (S)-methanocarba-T (**12**). The conformational analysis of these compounds further indicated that the base (torsion angle χ) as well as the hydroxymethyl group (torsion angle γ) can adopt conformations similar to those found in conventional nucleosides but with the difference that they are associated with a fixed ring pucker.

From energy considerations, rotation about the hydroxymethyl group (γ) was relatively free for both (N)-methanocarba-T (**2**) and (S)-methanocarba-T (**12**). However, rotation about the pseudoglycosyl bond (χ) was more restricted than for conventional nucleosides, even neglecting the contribution of the intramolecular hydrogen bond favored by the calculations *in vacuo*. The observed stiffness of χ and the differences in ring pucker between (N)-methanocarba-T and (S)-methanocarba-T could explain their distinct biological behavior. However, an additional element associated with the presence of the bicyclo[3.1.0]hexane itself could play a role. Indeed, the presence of the fused cyclopropane moiety has the potential for causing repulsive steric interactions by the presence of the extra "CH_2" below the plane of the ring. Although this property is common to both rigid pseudorotational antipodes, the distance between the "CH_2" groups from the superimposed pseudosugar structures of **2** and **12** is 1.74 Å (Figure 4). Therefore, this group appears to be shifted more toward the base in the case of (S)-methanocarba-T, and it too might contribute to its lack of biological activity.

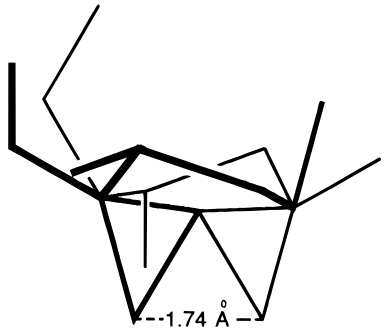


Figure 4. Superposition of Northern (bold lines) and Southern (thin lines) bicyclo[3.1.0]hexane templates.

Unfortunately, all these properties are inextricably linked and could not be dissected individually. The structural data suggest that there are indeed significant differences between the two conformationally distinct pseudorotational antipodes (N)- methanocarba-T (**2**) and (S)-methanocarba-T (**12**). However, any attempt to relate these differences to a specific biological activity still remains an elusive goal since antiviral activity and cell cytotoxicity are the end points of multienzymatic processes, and we presently do not know at which stage the rigid conformation plays a critical role. Nucleosides, as well as their carbocyclic counterparts, constitute prodrugs that must be activated to the requisite 5′-mono-, di-, or triphosphate anabolites by viral and/or cellular kinases, and these enzymes should be studied individually with each rigid conformer. The potent and selective antiviral activity of (N)-methanocarba-T would suggest that viral kinases are predominantly involved, but these aspects must be carefully studied in the future to reveal what critical step or steps are responsible for the observed differences. Nevertheless, the excellent antiviral activity of (N)-methanocarba-T is encouraging since it is expected to be more specific, given the fact that the compound must fit adequately only into receptor sites that accept its rigid pseudosugar ring structure. A comprehensive study involving the remainder of rigid Southern conformers bearing cytosine, guanine, and adenine bases is underway to identify a biological system where a rigid Southern conformation might be the preferred one.

Experimental Section

All chemical reagents were commercially available. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Column chromatography was performed on silica gel 60, 230−400 mesh (E. Merk), and analytical TLC was performed on Analtech Uniplates silica gel GF. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-250 instrument at 250 and 62.9 MHz, respectively. Spectra were referenced to the solvent in which they were run (7.24 ppm for $CDCl_3$). Following the norm for reporting NMR data in nucleosides, the identities of protons and carbons on the pseudosugar ring (carbocyclic moiety) are indicated by numbers with primes. Specific rotations were measured in a Perkin Elmer Model 241 polarimeter. Positive-ion fast-atom bombardment mass spectra (FAB MS) were obtained on a VG 7070E mass spectrometer at an accelerating voltage of 6 kV and a resolution of 2000. Glycerol was used as the sample matrix, and ionization was effected by a beam of xenon atoms. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA.

(1*R*,2*S*,4*S*,5*S*)-1-[(Benzyloxy)methyl]-2-(*tert*-butyloxy)-4-(5-methyl-2,4(1*H*,3*H*)-dioxopyrimidin-1-yl)bicyclo[3.1.0]-hexane (9). A solution of triphenylphosphine (4.18 g, 15.93 mmol) in anhydrous THF (40 mL) was treated with diethyl azodicarboxylate (DEAD; 2.75 g, 15.8 mmol) and stirred under argon at 0 °C for 30 min. After cooling to −45 °C, a suspension of N^3-benzoylthymine (3.0 g, 13.0 mmol) in THF (50 mL) was added followed by a solution of carbocyclic alcohol **6** (1.89 g, 6.50 mmol) in THF (30 mL). The reaction mixture was stirred at −45 °C for 2 h and allowed to reach room temperature overnight. The solvent was evaporated at reduced pressure and the residue was purified first by flash chromatography (silica gel, 0−40% EtOAc in hexane) to give a mixture of *N*- and *O*-alkylated products, which was rechromatographed (silica gel, 0−5% ether in CH_2Cl_2) to give 0.87 g (27%) of the desired *N*-alkylated product **7** as a semisolid and 1.30 g (40%) of the *O*-alkylated product as an oil. The *N*-alkylated product **7** was dissolved in MeOH (200 mL), treated with concentrated NH_4OH (15 mL), and stirred at room temperature overnight. The solvent was evaporated under reduced pressure, and the crude product was purified by flash column chromatography (silica gel, 0−50% EtOAc in hexane) to give **9** (0.25 g, 78%) as a white solid: mp 158−160 °C; ^{1}H NMR ($CDCl_3$) δ 8.20 (br s, 1 H, NH), 7.88 (d, J = 1.1 Hz, 1 H, H-6), 7.60−7.35 (m, 5 H, ArH), 4.93 (d, J = 6.8 Hz, 1 H, H-4′), 4.62−4.42 (m, 3 H, H-2′, $PhCH_2O$), 4.13 (AB d, J = 9.9 Hz, 1 H, $PhCH_2OCH$ H), 3.09 (AB d, J = 9.9 Hz, 1 H, $PhCH_2OCH$*H*), 1.90−1.60 (m, 2 H, H-3′$_{a,b}$), 1.48 (s, 3 H, CH_3), 1.25 (m, 1 H, H-5′), 1.15 (s, 9 H, $C(CH_3)_3$), 0.92 (m, 1 H, H-6′$_{endo}$), 0.65 (dd, J = 8.6, 6.3 Hz, H-6′$_{exo}$). Anal. ($C_{23}H_{30}N_2O_4$·0.33H_2O) C, H, N.

(1*R*,2*S*,4*S*,5*S*)-1-(Hydroxymethyl)-2-hydroxy-4-(5-methyl-2,4(1*H*,3*H*)-dioxopyrimidin-1-yl)bicyclo[3.1.0]hexane (2). A solution of **9** (0.60 g, 1.50 mmol) in dry CH_2Cl_2 (100 mL) was cooled to −78 °C under argon and treated with boron trichloride (1 M in CH_2Cl_2, 13 mL). The reaction mixture was stirred at −78 °C for 4 h, and the solvent was removed under reduced pressure. The residual material was treated with several portions of MeOH (4 × 20 mL) and evaporated to dryness under reduced pressure. The crude product was purified by flash chromatography (silica gel, 0−25% MeOH in CH_2Cl_2) to give 0.291 g (76%) of **2** as a solid. This material was further purified by reverse phase chromatography (Baker octadecyl C-18) using a gradient of 0−5% MeOH in water to give 0.231 g of a white crystalline solid: mp 239−241 °C; $[\alpha]^{25}_D$ = +47° (*c* 0.28, MeOH); ^{1}H NMR (Me_2SO-d_6) δ 11.20 (s, 1 H, NH), 7.91 (s, 1 H, H-6), 5.01 (t, J = 4.8 Hz, 1 H, OH), 4.70 (d, J = 6.8 Hz, 1 H, H-4′), 4.65−4.50 (m, 2 H, OH, H-2′), 4.07 (dd, J = 11.2, 4.9 Hz, 1 H, C*H*HOH), 3.05 (dd, J = 11.2, 4.5 Hz, 1 H, CH*H*OH), 1.80−1.45 (m, 2 H, H-3′$_{a,b}$), 1.22 (dd, J = 8.4, 3.5 Hz, 1 H, H-5′), 0.78 (irregular t, 1 H, H-6′$_{endo}$), 0.54 (dd, J = 8.4, 5.3 Hz, 1 H, H-6′$_{exo}$); ^{13}C NMR (CH_3OH-d_4) δ 10.58, 12.39, 26.09, 37.80, 39.19, 57.56, 63.52, 71.27, 111.22, 139.82, 152.93, 167.23; FAB MS (*m*/*z*, rel intensity) 253 (MH^+, 100), 127 (b + 2 H, 40). Anal. ($C_{12}H_{16}N_2O_4$·0.33H_2O) C, H, N.

(1*R*,2*S*,4*S*,5*S*)-1-[(Benzyloxy)methyl]-2-(*tert*-butyloxy)-4-(2,4(1*H*,3*H*)-dioxopyrimidin-1-yl)bicyclo[3.1.0]hexane (10). Following a similar procedure for the synthesis of **9**, the desired *N*-alkylated product **8** (0.360 g, 44%) was hydrolyzed to the title compound **10** (0.27 g, 98%), which was obtained as a foam: ^{1}H NMR ($CDCl_3$) δ 8.25 (br s, 1 H, NH), 8.15 (d, J = 8.0 Hz, 1 H, H-6), 7.60−7.25 (m, 5 H, ArH), 5.32 (dd, J = 8.0, 2.4 Hz, H-5), 4.95 (d, J = 6.6 Hz, 1 H, H-4′), 4.60−4.45 (m, 3 H, H-2′, $PhCH_2O$), 4.15 (AB d, J = 9.8 Hz, 1 H, $PhCH_2OCH$H), 3.10 (AB d, J = 9.8 Hz, 1 H, $PhCH_2OCH$*H*), 1.88−1.60 (m, 2 H, H-3′$_{a,b}$), 1.23 (dd, J = 8.5, 3.7 Hz, 1 H, H-5′), 1.15 (s, 9 H, $C(CH_3)_3$), 0.95 (dd, J = 5.8, 3.9 Hz, 1 H, H-6′$_{endo}$), 0.70 (dd, J = 8.3, 6.1 Hz, H-6′$_{exo}$). Anal. ($C_{22}H_{26}N_2O_4$) C, H, N.

(1*R*,2*S*,4*S*,5*S*)-1-(Hydroxymethyl)-2-hydroxy-4-(2,4-(1*H*,3*H*)-dioxopyrimidin-1-yl)bicyclo[3.1.0]hexane (3). After a similar deblocking procedure used for the preparation of **2**, the crude material was purified by column chromatography (silica gel, 25% 2-propanol in CH_2Cl_2), and following recrystallization from MeOH/ether, 0.069 g (93%) of **3** was obtained as a white solid: mp 157−159 °C; $[\alpha]^{25}_D$ = +51° (*c* 1, MeOH); ^{1}H NMR (Me_2SO-d_6) δ 11.20 (s, 1 H, NH), 7.91 (d, J = 8.0 Hz, 1 H, H-6), 5.51 (dd, J = 8.0, 2.2 Hz, 1 H, H-5), 4.90 (br s, 1 H, OH), 4.52 (d, J = 6.9 Hz, 1 H, H-4′), 4.51 (t, J = 8.5 Hz, 1 H, H-2′), 4.05 (d, J = 11.3 Hz, 1 H, C*H*HOH), 3.35 (br s, 2 H, OH), 3.10 (d, J = 11.3 Hz, 1 H, CH*H*OH), 1.75 (dd, J = 14.7,

8.0 Hz, 1 H, H-3′$_a$), 1.58 (m, 1 H, H-3′$_b$), 1.24 (dd, J = 8.4, 3.4 Hz, 1 H, H-5′), 0.75 (irregular t, 1 H, H-6′$_{endo}$), 0.55 (dd, J = 8.4, 5.3 Hz, 1 H, H-6′$_{exo}$); ^{13}C NMR (CH_3OH-d_4) δ 9.28, 24.05, 36.51, 37.78, 55.43, 61.31, 69.08, 100.93, 141.80, 150.89, 163.18; FAB MS (m/z, rel intensity) 239 (MH^+, 85), 113 (b + 2 H, 100). Anal. ($C_{11}H_{14}N_2O_4 \cdot 0.25H_2O$) C, H, N.

(1*R*,2*S*,4*S*,5*S*)-1-(Hydroxymethyl)-2-hydroxy-4-(4-amino-2(1*H*)-oxopyrimidin-1-yl)bicyclo[3.1.0]hexane (4). Uracil nucleoside **3** (0.527 g, 2.21 mmol) was stirred at room temperature for 2 h in the presence of acetic anhydride (20 mL) and pyridine (30 mL). The reaction mixture was concentrated under reduced pressure, and excess pyridine was removed by azeotropic distillation first with toluene and then with diethyl ether. The residue was recrystallized from 2-propanol/ether to give 0.544 g (first crop) and 0.086 g (second crop) of the diacetate of **3** (88% yield) as a white solid: mp 126−127 °C; ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1 H, NH), 7.60 (d, J = 8.0 Hz, 1 H, H-6), 5.72 (dd, J = 8.0, 2.2 Hz, 1 H, H-5), 5.55 (t, J = 8.4 Hz, 1 H, H-2′), 5.05 (d, J = 7.5 Hz, 1 H, H-4′), 4.51 (d, J = 12.1Hz, 1 H, C*H*OH), 3.81 (d, J = 12.1 Hz, 1 H, CH*H*OH), 2.25 (dd, J = 15.7, 8.4 Hz, 1 H, H-3′$_a$), 1.80 (m, 1 H, H-3′$_b$), 1.45 (dd, J = 8.7, 3.9 Hz, 1 H, H-5′), 1.08−0.88 (m, 2 H, H-6′). Anal. ($C_{15}H_{28}N_2O_6$) C, H, N.

An ice-cold solution of 1,2,4-triazole (0.54 g, 7.76 mmol) in CH_3CN (30 mL), which was maintained under argon, was treated with $POCl_3$ (0.71 mL, 7.76 mmol) and stirred cold for 15 min. The diacetate (0.25 g, 0.776 mmol) and triethylamine (1.1 mL, 0.76 mmol) were added, and the reaction mixture was stirred at room temperature overnight. After removing the solvent under reduced pressure, the orange residue was dissolved in CH_2Cl_2 (100 mL), extracted with water (3 × 75 mL) and brine (75 mL), dried ($MgSO_4$), and filtered. The filtrate was reduced to dryness under vacuum, and the residue was purified by flash column chromatography (silica gel, 10% MeOH in EtOAc). The collected fractions were evaporated, and the solid material was triturated with a mixture of EtOAc and petroleum ether to give the solid triazole intermediate (0.138 g, 46%). This material was dissolved in a mixture of dioxane (16 mL) and concentrated NH_4OH (4 mL) and stirred overnight at room temperature. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (silica gel, 5% MeOH in CH_2Cl_2) to give the semisolid diacetate of **4** (0.105 g, 92%), which was immediately dissolved in saturated methanolic ammonia (35 mL) and stirred overnight at room temperature. The solvent was removed under reduced pressure, and the residue was redissolved and reconcentrated from $CHCl_3$ (3 × 50 mL). The residue was warmed with $CHCl_3$ and allowed to stand at room temperature. The solid formed was collected by filtration to give 0.038 g (65%) of **4**. An analytical sample of **4** was obtained by recrystallization from MeOH to give white crystals: mp 283−290 °C; $[\alpha]^{25}_D$ = +74° (c 1, MeOH); ^{1}H NMR (Me_2SO-d_6) δ 7.90 (d, J = 7.3 Hz, 1 H, H-6), 7.00 (br d, 2 H, NH_2), 5.65 (d, J = 7.3 Hz, 1 H, H-5), 4.88 (t, J = 5.0 Hz, 1 H, OH), 4.75 (d, J = 6.5 Hz, 1 H, H-4′), 4.60−4.40 (m, 2 H, OH, H-2′), 4.05 (dd, J = 11.4, 5.2 Hz, 1 H, C*H*OH), 3.12 (dd, J = 11.4, 5.0 Hz, 1 H, CH*H*OH), 1.72−1.43 (m, 2 H, H-3′$_{a,b}$), 1.20 (dd, J = 8.4, 3.5 Hz, 1 H, H-5′), 0.75 (irregular t, 1 H, H-6′$_{endo}$), 0.55 (dd, J = 8.2, 5.2 Hz, 1 H, H-6′$_{exo}$); ^{13}C NMR (Me_2SO-d_6) δ 9.33, 24.41, 36.44, 38.16, 55.75, 61.50, 69.18, 93.18, 142.34, 155.48, 165.14; FAB MS (m/z, rel intensity) 238 (MH^+, 100), 112 (b + 2 H, 60). Anal. ($C_{11}H_{15}N_3O_3$) C, H, N.

(1*R*,2*S*,4*S*,5*S*)-1-[(Benzyloxy)methyl]-2-(*tert*-butyloxy)-4-[2-amino-6-(benzyloxy)-9-purinyl]bicyclo[3.1.0]-hexane (11). A stirred solution of triphenylphosphine (1.37 g, 5.22 mmol) in anhydrous THF (30 mL) was treated with DEAD (0.91 g, 1.64 mmol) at room temperature. After 30 min, a suspension of 2-amino-6-chloropurine (0.248 g, 1.60 mmol) in anhydrous THF (15 mL) was added, and 30 min later a solution of carbocyclic alcohol **6** (0.50 g, 1.72 mmol) in THF (30 mL) was added. The resulting mixture was stirred at room temperature for 20 h. The suspension was filtered, and the filtrate was concentrated under reduced pressure. The crude product was purified by flash column chromatography (silica gel, 0−50% EtOAc in hexane) to give the 6-chloro intermediate (0.26 g, 34%) as a foam. This compound was reacted with a freshly made solution of $PhCH_2ONa$ (prepared from 10 mL of benzyl alcohol and 0.30 g of Na) and stirred at room temperature for 30 min. After the reaction was quenched with water (50 mL), the mixture was extracted with CH_2Cl_2 (2 × 75 mL). The combined organic extract was washed with water until the pH of the washings was neutral, dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, 0−50% EtOAc in hexane) to give 0.215 g (72%) of **11** as a solid: mp 180−181 °C; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1 H, H-8), 7.20−7.50 (m, 10 H, ArH), 5.60 (AB q, J = 12.1 Hz, 2 H, $PhCH_2O$), 5.20 (br s, 2 H, NH_2), 4.95 (d, J = 6.0 Hz, 1 H, H-4′), 4.63 (t, J = 8.3 H, 1 Hz, H-2′), 4.55 (AB q, J = 12.1 Hz, 2 H, $PhCH_2O$), 4.06 (AB d, J = 9.9 Hz, 1 H, $PhCH_2OC$*H*HO), 3.03 (AB d, J = 9.9 Hz, 1 H, $PhCH_2OCH$*H*O), 1.92−1.67 (m, 2 H, H-3′$_{a,b}$), 1.49 (dd, J = 8.4, 3.7 Hz, 1 H, H-5′), 1.07 (s, 9 H, $C(CH_3)_3$), 1.00 (dd, J = 5.7, 4.0 Hz, 1 H, H-6′$_{endo}$), 0.68 (dd, J = 8.0, 6.2 Hz, 1 H, H-6′$_{exo}$). Anal. ($C_{30}H_{35}N_5O_3$) C, H, N.

(1*R*,2*S*,4*S*,5*S*)-1-(Hydroxymethyl)-2-hydroxy-4-(2-amino-1,9-dihydro-6*H*-6-oxopurin-9-yl)bicyclo[3.1.0]hexane (5). A stirred solution of **11** (0.130 g, 0.253 mmol) in CH_2Cl_2 (10 mL) was maintained under argon, cooled to −78 °C, and treated with boron trichloride (1.0 M in hexane, 2 mL). The solution was stirred at that temperature for 4 h, the reaction quenched with MeOH (2 mL), and the mixture allowed to reach room temperature. The solvent was removed, and additional amounts of MeOH (4 × 5 mL) were added and evaporated successively. The crude product was purified by reverse phase chromatography (Baker octadecyl C-18) using a gradient of 0−10% MeOH in water to give 0.054 g (77%) of **5** as a foam: $[\alpha]^{25}_D$ = +18° (c 0.4, DMF); ^{1}H NMR (Me_2SO-d_6) δ 10.50 (br s, 1 H, NH), 8.00 (s, 1 H, H-8), 6.45 (s, 2 H, NH_2), 4.92 (t, J = 4.9 Hz, 1 H, OH), 4.68 (m, 1 H, H-2′), 4.61 (d, J = 6.0 Hz, 1 H, H-4′), 4.05 (dd, J = 11.3, 4.7 Hz, 2 H, C*H*HOH), 3.15 (dd, J = 11.3, 4.2 Hz, CH*H*OH), 1.78 (dd, J = 14.3, 7.5 Hz, 1 H, H-3′$_a$), 1.70−1.50 (m, 1 H, H-3′$_b$), 1.45 (dd, J = 8.1, 3.3 Hz, 1 H, H-5′), 0.84 (t, J = 4.3 Hz, 1 H, H-6′$_{endo}$), 0.59 (dd, J = 8.0, 5.4 Hz, 1 H, H-6′$_{exo}$); ^{13}C NMR (Me_2SO-d_6) δ 9.29, 24.96, 35.87, 37.86, 53.45, 61.49, 69.30, 116.56, 135.19, 150.61, 153.44, 156.89; FAB MS (m/z, rel intensity) 278 (MH^+, 100), 152 (b + 2 H, 57); high-resolution FAB MS MH^+ calcd 278.1253, found 278.1251. Since the value for nitrogen was off in the conventional combustion analysis, the purity of the sample (99.6%) was assessed by HPLC (column, Altex ODS, 250 × 4.6 mm; mobile phase, 75% CH_3CN, 10 mM phosphate buffer; λ = 255 nm).

Synthesis of Phosphorothioate 5′-CTTCATTTTTTCT-TC-3′. The (N)-methanocarba-T was incorporated in a phosphorothioate ODN using a hydrogen phosphonate protocol.[31] The synthon suitable for automated synthesis was prepared by protection of the primary 1-hydroxymethyl alcohol (equivalent to the 5′-hydroxyl in a nucleoside) with the dimethoxytrityl (DMT) group (1 equiv of DMT-Cl in pyridine) and purification by silica gel flash chromatography followed by phosphitylation[32] (1.5 equiv of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in pyridine), triethylammonium bicarbonate/CH_2Cl_2 extraction, and flash chromatography. The automated synthesizer method used pivaloyl chloride as the H-phosphonate activator and terminated in a S_8/pyridine/CS_2 oxidation.[31] Standard ammonia deprotection and gel purification protocols yielded the final ODN.

Cytopathic Effect Inhibition Assay.[16] Low-passage (3−10) human foreskin fibroblast (HFF) cells were trypsinized, counted, and seeded into 96-well tissue culture plates at a cell concentration of 2.5 × 10^4 cells in 0.1 mL of minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS). The cells were then incubated for 24 h at 37 °C in a 5% CO_2−95% air, 90% humidified atmosphere. The media were then removed, and 100 μL of MEM containing 2% FBS was added to all but the first row. In the first row, 125 μL of media containing the experimental compound was added in triplicate wells. Media alone were added to both cell and virus control wells. The compound in the first row of wells was then diluted serially 1:5 throughout the remaining wells by transferring 25 μL using the Cetus Liquid Handling Machine. The plates were then incubated for 1 h, and 100 μL of the appropriate virus concentration was added to each well,

excluding cell control wells which received 100 μL of MEM. For HSV-1 and HSV-2 assays, the virus concentration utilized was 1000 PFU/well. For HCMV the virus concentration added was 2500 PFU/well. The plates were then incubated at 37 °C in a CO_2 incubator for 3 days for HSV-1 and HSV-2 and 14 days for HCMV. After the incubation period, media were aspirated and the cells stained with a 0.1% crystal violet solution for 30 min. The stain was then removed, and the plates were rinsed with tap water until all excess stain was removed. The plates were allowed to dry for 24 h and then read on a BioTek Multiplate Autoreader.

Virus Plaque Reduction Assay (Using Semisolid Overlay).[16] On the date of the assay, the drug was made up at 2 times the desired concentration in 2× MEM and then serially diluted 1:5 in 2× MEM to give six concentrations of drug. The drug concentrations utilized were usually 200 down to 0.06 μg/mL. The virus to be used was diluted in MEM containing 10% FBS to a desired concentration which would give 20−30 plaques/well. The media were then aspirated from the wells, and 0.2 mL of virus was added to each well in duplicate with 0.2 mL of media being added to drug toxicity wells. The plates were then incubated for 1 h with shaking every 15 min. After the incubation period, an equal amount of 1% agarose was added to an equal volume of each drug dilution. This gave a final drug concentration beginning with 100 and ending with 0.03 μg/mL and a final agarose overlay concentration of 0.5%. The drug agarose mixture was applied to each well in 2 mL volume, and the plates were incubated for 3 days, after which the cells were stained with a 1.5% solution of neutral red. At the end of the 4−6 h incubation period, the stain was aspirated, and plaques were counted using a stereomicroscope at 10× magnification.

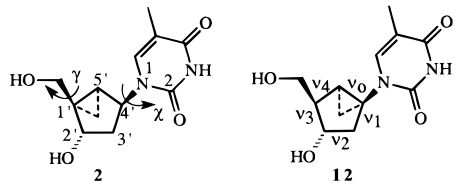
The procedure for HCMV is nearly the same. The agarose used for both the initial overlay and the two subsequent overlays was 0.8% rather than 1%. The assay was incubated for 14 days with the additional 1 mL overlay being applied on days 4 and 8.

RNase H Susceptibility. This analysis was performed as previously reported[22] with the modification that the complementary RNA strand (5′-GAAGAAAAAAUGAAG-3′) was synthesized chemically and 5′-radiolabeled using [γ-^{32}P]ATP and T_4 kinase.

Conformational Analysis. The pseudorotational angle P was calculated according to eq 1.[27] The endocyclic torsion

$$\tan P = (\nu_4 + \nu_1) - (\nu_3 + \nu_0)/2 \sin \nu_2(\sin 36° + \sin 72°) \quad (1)$$

angles ν_0−ν_4 were measured directly from the X-ray structures. For the cyclopentane ring, these angles correspond to equivalent torsion angles as defined for a sugar moiety in ref 27. The torsion angles χ (pseudoglycosyl bond) and γ correspond, respectively, to C_2−N_1−$C_{4'}$−$C_{5'}$ and HO−CH_2−$C_{1'}$−$C_{2'}$. These torsion angles are equivalent to C_2−N_1−$C_{1'}$−$O_{4'}$ and $O_{5'}$−$C_{5'}$−$C_{4'}$−$C_{3'}$ in pyrimidine nucleosides.[27]



2 **12**

Molecular mechanics calculations were performed with the CaChe Scientific, Oxford Molecular, program version 4.0 using Allinger's standard MM2 force field parameters. The optimization method employed was the Block Diagonal Newton Raphson with a convergence criterion equal to to 0.001 kcal/mol. Exhaustive potential energy maps were generated by defining the torsion angles χ and γ as indicated above and allowing them to rotate 360° in 15° increments.

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References

(1) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984; pp 51−104.
(2) The concept of pseudorotation was introduced and applied for the first time to substituted furanoses by Altona, C.; Sundaralingam, M. Conformational Analysis of the Sugar Ring in Nucleosides and Nucleotides. A New Description of Using the Concept of Pseudorotation. *J. Am. Chem. Soc.* **1972**, *94*, 8205−8212.
(3) Plavec, J.; Tong, W.; Chattopadhyaya, J. How do the Gauche and Anomeric Effects Drive the Pseudorotational Equilibrium of the Pentofuranose Moiety of Nucleosides? *J. Am. Chem. Soc.* **1993**, *115*, 9734−9746.
(4) DeLeeuw, H. P. M.; Haasnoot, C. A. G.; Altona, C. Empirical Correlations Between Conformational Parameters in β-D-Furanoside Fragments Derived from a Statistical Survery of Crystal Structures of Nucleic Acid Constituents. *Israel J. Chem.* **1980**, *20*, 108−126.
(5) Appelt, K.; Bacquet, R. J.; Bartlett, C. A.; Booth, C. L. J.; Freer, S. T.; Fuhry, M. A. M.; Gehring, M. R.; Herrmann, S. M.; Howland, E. F.; Janson, C. A.; Jones, T. R.; Kan, C.-C.; Kathardekar, V.; Lewis, K. K.; Marzoni, G. P.; Matthews, D. A.; Mohr, C.; Moomaw, E. W.; Morse, C. A.; Oatley, S. J.; Ogden, R. C.; Reddy, M. R.; Reich, S. H.; Schoettlin, W. S.; Smith, W. W.; Varney, M. D.; Villafranca, J. E.; Ward, R. W.; Webber, S.; Webber, S. E.; Welsh, K. M.; White, J. Design of Enzyme Inhibitors Using Iterative Protein Crystallographic Analysis. *J. Med. Chem.* **1991**, *34*, 1925−1934.
(6) Sharma, R.; Lee, J.; Wang, S.; Milne, G. W. A.; Lewin, N. E.; Blumberg, P. M.; Marquez, V. E. Conformationally Constrained Analogues of Diacylglycerol. 10. Ultrapotent Protein Kinase C Ligands Based on a Racemic 5-Disubstituted Tetrahydro-2-furanone Template. *J. Med. Chem.* **1996**, *39*, 19−28.
(7) Rodriguez, J. B.; Marquez, V. E.; Nicklaus, M. C.; Barchi, J. J., Jr. Synthesis of Cyclopropane-fused Dideoxycarbocyclic Nucleosides Structurally Related to Neplanocin C. *Tetrahedron Lett.* **1993**, *34*, 6233−6236.
(8) Altmann, K.-H.; Kesselring, R.; Francotte, E.; Rihs, G. 4′,6′-Methano Carbocyclic Thymidine: A Conformationally Constrained Building Block for Oligonucleotides. *Tetrahedron Lett.* **1994**, *35*, 2331−2334.
(9) Rodriguez, J. B.; Marquez, V. E.; Nicklaus, M. C.; Mitsuya, H.; Barchi, J. J., Jr. Conformationally Locked Nucleoside Analogues. Synthesis of Dideoxycarbocyclic Nucleoside Analogues Structurally Related to Neplanocin C. *J. Med. Chem.* **1994**, *37*, 3389−3399.
(10) Altmann, K.-H.; Imwinkelried, R.; Kesselring, R.; Rihs, G. 1′,6′-Methano Carbocyclic Thymidine: Synthesis and X-ray Crystal Structure, and Effect on Nucleic Acid Duplex Stability. *Tetrahedron Lett.* **1994**, *35*, 7625−7628.
(11) Ezzitouni, A.; Barchi, J. J., Jr.; Marquez, V. E. A Simple Approach to 1,1′a-Methano Carbocyclic Thymidine. *J. Chem. Soc., Chem. Commun.* **1995**, 1345−1346. A full account of this work has been recently submitted to *J. Chem. Soc., Perkin Trans. 1*.
(12) Siddiqui, M. A.; Ford, H., Jr.; George, C.; Marquez, V. E. Synthesis, Conformational Analysis, and Biological Activity of a Rigid Carbocyclic Analogue of 2′-Deoxyaristeromycin Built on a Bicyclo[3.1.0]hexane Template. *Nucleosides Nucleotides* **1996**, *15*, 235−250.
(13) Mitsunobu, O. The Use of Diethyl Azodicarboxylate and Triphenylphosphine in Synthesis and Transformation of Natural Products. *Synthesis* **1981**, 1−28.
(14) Cruickshank, K. A.; Jiricny, J.; Reese, C. B. The Benzoylation of Uracil and Thymine. *Tetrahedron Lett.* **1984**, *25*, 681−684.
(15) Divakar, K. J.; Reese, C. B. 4-(1,2,4-Triazol-1-yl)- and 4-(3-Nitro-1,2,4-triazol-1-yl)-1-(β-D-2,3,5-tri-*O*-acetylarabinofuranosyl)pyrimidin-2(1*H*)-ones. Valuable Intermediates in the Synthesis of Derivatives of 1-(β-D-Arabinofuranosyl)cyosine (Ara-C). *J. Chem. Soc., Perkin Trans. I* **1982**, 1171−1176.
(16) Antiviral testing was performed by Dr. Earl Kern of the University of Alabama at Birmingham and supported by USPHS Contract N01-AI-35177 from the National Institute of Allergy and Infectious Diseases, NIH.
(17) Shealy, Y. F.; O'Dell, C. A.; Shannon, W. M.; Arnett, G. Carbocyclic Analogues of 5-Substituted Uracil Nucleosides: Synthesis and Antiviral Activity. *J. Med. Chem.* **1983**, *26*, 156−161.
(18) Shealy, Y. F.; O'Dell, C. A.; Arnett, G.; Shannon, W. M. Synthesis and Antiviral Activity of the Carbocyclic Analogues of 5-Ethyl-2′-deoxyuridine and 5-Ethynyl-2′-deoxyuridine. *J. Med. Chem.* **1986**, *29*, 79−84.

(19) Beres, J.; Sagi, G.; Tomoskozi, I.; Gruber, L.; Baitz-Gacs, E.; Otvos, L.; De Clercq, E. Stereospecific Synthesis and Antiviral Properties of Different Enantiomerically Pure Carbocyclic 2′-Deoxyribonucleoside Analogues Derived from Common Chiral Pools: (+)-(1R,5S)- and (-)-(1S,5R)-2-Oxabicyclo[3.3.0]oct-6-en-3-one. *J. Med. Chem.* **1990**, *33*, 1353−1360.
(20) See ref 1, pp 220−241.
(21) Ivanov, V. I.; Minchenkova, L. E. The A-Form of DNA: in Search of Biological Role (a Review). *Mol. Biol.* **1995**, *28*, 780−788 (translated from *Mol. Biol.* **1994**, *28*, 1258−1271).
(22) Wagner, R. W.; Matteucci, M. D.; Lewis, J. G.; Gutierrez, A. J.; Moulds, C.; Froehler, B. C. Antisense Gene Inhibition by Oligonucleotides Containing C-5 Propyne Pyrimidines. *Science* **1993**, *260*, 1510−1513.
(23) Monia, B. P.; Lesnik, E. A.; Gonzalez, C.; Lima, W. F.; McGee, D.; Guinosso, C. J.; Kawasaki, A. M.; Cook, P. D.; Freier, S. M. Evaluation of 2′-Modified Oligonucleotides Containing 2′-Deoxy Gaps as Antisense Inhibitors of Gene Expression. *J. Biol. Chem.* **1993**, *268*, 14514−14522.
(24) Moulds, C.; Lewis, J. G.; Froehler, B. C.; Grant, D.; Huang, T.; Milligan, J. F.; Matteucci, M. D.; Wagner, R. W. Site and Mechanism of Antisense Inhibition by C-5 Propyne Oligonucleotides. *Biochemistry* **1995**, *34*, 5044−5053.
(25) Kawasaki, A. M.; Casper, M. D.; Freier, S. M.; Lesnik, E. A.; Zounes, M. C.; Cummins, L. L.; Gonzalez, C.; Cook, P. D. Uniformly Modified 2′-Deoxy-2′-fluoro Phosphorothioate Oligonucleotides as Nuclease-Resistant Antisense Compounds with High Affinity and Specificity for RNA Targets. *J. Med. Chem.* **1993**, *36*, 831−841.
(26) McKay, R. A.; Cummins, L. L.; Graham, M. J.; Lesnik, E. A.; Owens, S. R.; Winniman, M.; Dean, N. M. Enhanced activity of an antisense oligonucleotide targeting murine protein kinase C-alpha by the incorporation of 2′-O-propyl modifications. *Nucleic Acids Res.* **1996**, *24*, 411−417.
(27) See ref 1, pp 9−28.
(28) Green, E. A.; Rosenstein, R. D.; Shiono, R.; Abraham, D. J.; Trus, B. L.; Marsh, R. E. The Crystal Structure of Uridine. *Acta Crystallogr.* **1975**, *B31*, 102−107.
(29) Young, D. W.; Tollin, P.; Wilson, H. R. The Crystal and Molecular Structure of Thymidine. *Acta Crystallogr.* **1969**, *B25*, 1423−1432.
(30) Kalman, A.; Koritsanszky, T.; Beres, J.; Sagi, G. Crystal and Molecular Structure of (+)-Carba-Thymidine C11H16N2O6. *Nucleosides Nucleotides* **1990**, *9*, 235−243.
(31) Froehler, B. C. In *Protocols for Oligonucleotides and Analogs: Synthesis and Properties*; Agrawal, S., Ed.; Humana: Totowa, NJ, 1993; pp 63−80.
(32) Marugg, J. E.; Tromp, M.; Kuyl-Yehskiely, E.; van der Marel, G.; van Boom, J. H. *Tetrahedron Lett.* **1986**, *27*, 2661−2664.

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