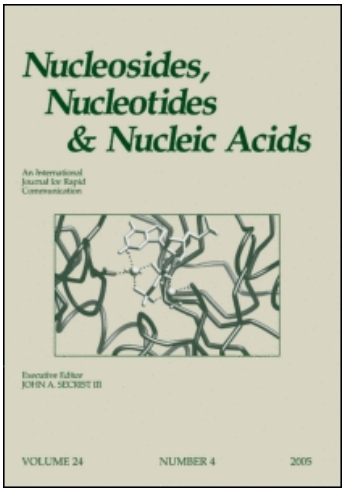

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$O^{5',6}$-Methanocytidine — Synthesis, Conformational Properties and Deamination by Cytidine Deaminase

S. Megati[a]; S. E. Ealick[b]; F. N. M. Naguib[c]; M. H. el Kouni[c]; R. S. Klein[a]; B. A. Otter[a]
[a] Department of Oncology, Montefiore Medical Center, Albert Einstein College of Medicine Cancer Center and Medicinal Chemistry Laboratory, Bronx, NY [b] Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY [c] Department of Pharmacology and Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham

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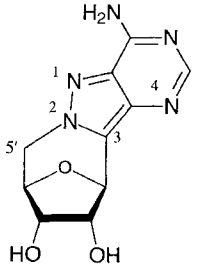
$O^{5',6}$-METHANOCYTIDINE — SYNTHESIS, CONFORMATIONAL PROPERTIES AND DEAMINATION BY CYTIDINE DEAMINASE

Sreenivasulu Megati[†], Steven E. Ealick[‡], Fardos N. M. Naguib[¶], Mahmoud H. el Kouni[¶], Robert S. Klein[†] and Brian A. Otter[†]

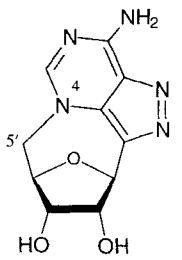
[†]Albert Einstein College of Medicine Cancer Center and Medicinal Chemistry Laboratory, Department of Oncology, Montefiore Medical Center, Bronx, NY 10467,
[‡]Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853,
[¶]Department of Pharmacology and Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294

Abstract: The synthesis of $O^{5',6}$-methanocytidine (**4**), a pyrimidine nucleoside restricted to the *anti* conformation, is described. Molecular modeling studies suggest that **4** is more flexible than conventional cyclonucleosides because of its larger-than-usual bridging system and that it can exist in a number of low energy conformations where the glycosyl rotation angles (χ) cover an ~80° segment of the *anti* range. However, while both N-type (C2'-*exo*) and S-type (C3'-*exo*) sugar puckerings are possible, none of the low energy conformers adopt the C3'-endo or C2'-endo puckering modes generally seen for unconstrained nucleosides. The lowest energy conformer predicted for **4** (χ = -152°, γ = 73°, P = 206°) is similar to the X-ray structure of a related compound, namely 5-hydroxy-$O^{5',6}$-methanouridine (**12** , χ = -138°, γ = 63°, P = 200°). In solution, NMR evidence suggests an equilibrium between C2'-*exo* and C3'-*exo* puckerings for **4**, and CD evidence suggests an average glycosyl rotation angle (χ) of around -160°. $O^{5',6}$-Methanocytidine (**4**) is slowly deaminated by crude cytidine deaminase from mouse liver to give $O^{5',6}$-methanouridine (**3**). Assuming that **4** interacts with the normal active site, it is concluded that cytidine deaminase from that particular source requires its ordinary substrates to adopt the *anti* conformation.

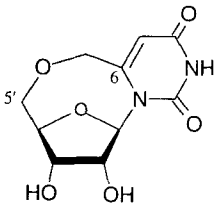
Introduction. In addition to their traditional roles as synthetic intermediates, cyclonucleosides and cyclonucleotides are useful for probing the conformational specificities of the enzymes of nucleic acid metabolism. For example, the finding[1a,b] that the *anti* nucleoside 2,5'-anhydroformycin (**1**) is a substrate for adenosine deaminase under conditions where its *syn* counterpart 4,5'-anhydroformycin (**2**) is completely resistant suggested that the *anti* conformation[2] is a normal requirement



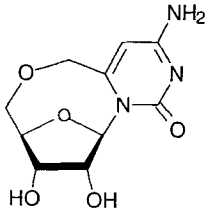
2,5'-Anhydroformycin (1)



4,5'-Anhydroformycin (2)



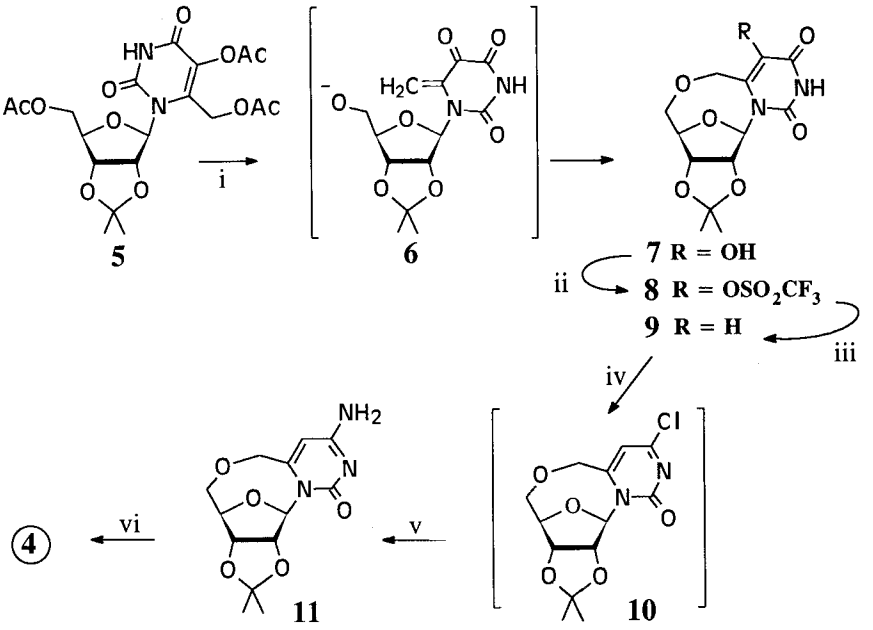
O 5',6-Methanouridine (3)



O 5',6-Methanocytidine (4)

for the enzymatic deamination of adenosine and its analogues. This conclusion was subsequently confirmed by X-ray crystallographic studies of enzyme-inhibitor complexes.[3] Nevertheless, the majority of cyclonucleosides suffer from electronic and/or conformational shortcomings that make them less than ideal probes. Nucleosides **1** and **2**, for instance, feature tautomeric differences from each other and from the parent compound formycin A, and their pronounced rigidity freezes the sugar moiety in envelope conformations of the O4′-*exo* type ($_{O}$E) that at best are only very minor contributors to the conformational populations of unconstrained nucleosides. We have been interested in preparing new types of conformationally restricted nucleosides that more closely simulate the electronic and conformational properties of normal nucleosides. An example[4] from the pyrimidine series is *O* 5′,6-methanouridine (**3**), an *anti* nucleoside in which the pyrimidine ring retains a full complement of normal hydrogen bonding groups, and in which the bridging system is sufficiently large to restore some flexibility to the furanose sugar ring. In continuation of this theme, we report in the present paper the synthesis of the corresponding *O* 5′,6-methanocytidine (**4**), we examine the range of conformations available to this molecule, and we show that the compound is in fact deaminated by cytidine deaminase from mouse liver.

Chemistry. In the approach that we developed[4] for the synthesis of *O* 5′,6-methanouridine (**3**) from the 6-acetoxymethyl nucleoside **5** (Scheme I), cyclization of the intermediate enone **6** leads to the 5-hydroxy cyclonucleoside **7** as the initial product.



Reagents: **i)** N NaOH, rt; **ii)** K_2CO_3, $(CF_3SO_2)_2NPh$, dioxane-water, rt; **iii)** $NaBH_4$, Et_3N, DMF, 65 °C; **iv)** Ph_3P, CCl_4, MeCN, reflux; **v)** NH_3-dioxane, rt; **vi)** 5% HCl-MeOH, rt.

Scheme 1

Removal of the 5-hydroxyl group from **7** is therefore a key step in the process, and this was originally achieved by treating the derived triflate **8** with tetrakis(triphenylphosphine)palladium(0) in the presence of lithium chloride and tributyltin hydride in refluxing THF. We have since examined some alternative methods. One possibility, namely the reported[5] reduction of aryl triflates with zinc powder in the presence of a nickel(0) complex, was not effective when applied to **8**. Another possibility, which was based on the demonstration by Ciattini *et al*[6] that vinyl triflates undergo Pd-catalyzed cross-coupling with tetraarylborates ($NaBAr_4$), was more successful. Thus, the C5-O bond of **8** can be cleaved efficiently by treatment with sodium borohydride in the presence of triethylamine and $[Ph_3P]_4Pd$. Moreover, further study has shown, at least in our application, that the costly palladium reagent can be dispensed with, and that **9** can be obtained in 80% yield simply by treating **8** with sodium borohydride and triethylamine in DMF at 65 °C. The scope and mechanism of this useful reaction, which is possibly of the addition-elimination type, remain to be determined.

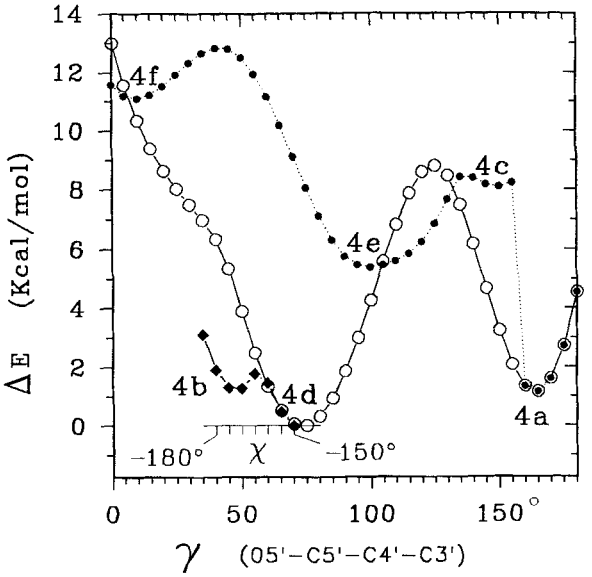
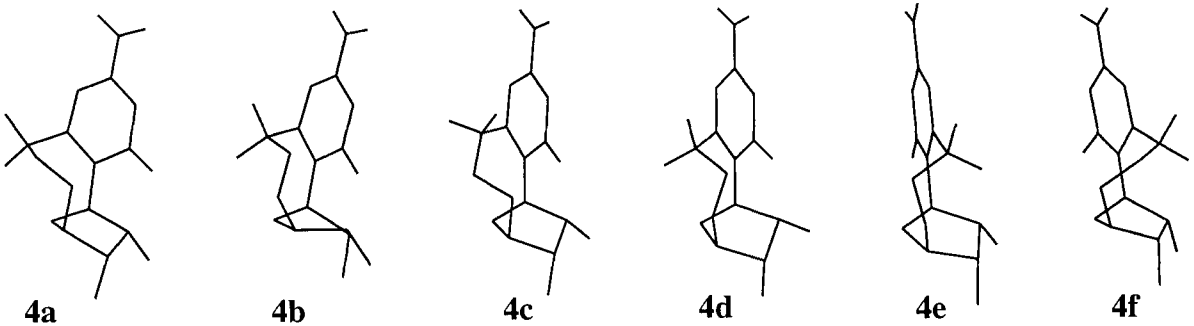


FIGURE 1. Energy profiles for rotation about the O5′-C5′-C4′-C3′ bond of **4** (open circles and filled circles), and about the glycosidic bond (O4′-C1′-N1-C2, filled diamonds), as determined by molecular mechanics calculations (MM+). See Experimental Section for details.

For the conversion of **9** into the corresponding cytosine nucleoside **11**, we have used the method that De Napoli and co-workers[7] reported recently for the synthesis of 4-substituted pyrimidine 2′,3′-dideoxynucleosides. In our case, treatment of **9** with triphenylphosphine and carbon tetrachloride in acetonitrile generated the 4-chloro nucleoside **10**, which, without rigorous purification, was converted into the 4-amino compound **11** on exposure to aqueous ammonia in dioxane. Simple removal of the isopropylidene blocking group from **11** then afforded the title nucleoside **4**.

Conformational Studies. Apart from the sugar puckering, the conformations of ordinary pyrimidine nucleosides depend largely on the values of two torsional angles, namely γ (C3′-C4′-C5′-O5′) and χ (O4′-C1′-N1-C2)[2]. The same is true for $O^{5',6}$-

TABLE I. **Conformational parameters for structures corresponding to the global (4d) and local energy minima of $O^{5',6}$-methanocytidine as determined by molecular mechanics (MM+). The structures are arranged from left to right with decreasing values of the glycosyl rotation angle χ**



4a **4b** **4c** **4d** **4e** **4f**

Structure	Glycosidic torsion angle χ (C2-N1-C1′-O4′)°	Steric energy (kcal)	ΔE (kcal)	Phase angle P (deg)	Puckering type	Puckering amplitude ν_m (deg)	Torsion angle γ (O5′-C5′-C4′-C3′)°
4a	-175	18.5	1.1	238	^4E	43	163
4b	-170	18.6	1.2	336	$_2E$	38	58
4c	-157	25.5	8.1	227	$^4T_3$	39	149
4d	-152	17.4	-	206	$_3E$	35	73
4e	-121	22.9	5.5	229	$^4T_3$	21	99
4f	-95	28.6	11.2	269	$_0E$	36	9
12*	-138			200	$_3E$	36	63

* X-ray structure

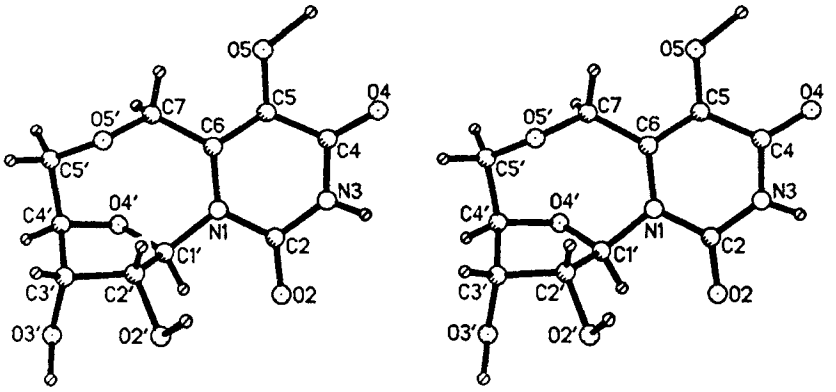


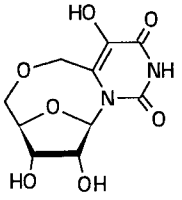
FIGURE 2. Stereoscopic View of the X-ray structure of 5-Hydroxy-$O^{5',6}$-methanouridine dihydrate (**12**)

methanocytidine (**4**) except that the values of γ and χ become mutually dependent because of the presence of the methano bridge. In order to identify combinations of γ and χ that might result in stable conformations we have carried out molecular modeling studies using the Hyperchem™ implementation of the MM2 force field. Conformational searching was conducted by restraining either γ or χ to a particular value while allowing the rest of the molecule to relax. As shown by the examples in Figure 1, this process revealed six energy minima. Further geometry optimizations performed after removal of the angle constraints then led to six low energy conformers, **4a** - **4f**, which are shown along with their associated parameters in Table I.

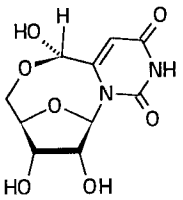
It will be seen that the glycosyl rotation angles (χ) of the six conformers vary by almost 80° within the *anti* range, and the highest energy conformer **4f** approaches the *high-anti* region. The lowest energy conformers (**4a**,**b** and **d**), in which the methano bridge projects towards the furanose ring oxygen atom, fall within a much narrower segment (ca. 23°) of the *anti* range. Regarding the sugar puckering, both N-type (**4b**, C2′-*exo*) and S-type (**4d**, C3′-*exo*) conformers are apparently favored, but it will be noted that the pseudorotation phase angles (*P*) fall outside the ranges generally seen for unconstrained nucleosides.[2,8] In other words, none of the conformers predicted by molecular mechanics for **4** adopt the familiar C3′-*endo* or C2′-*endo* puckering modes. The presence of the methano bridge *does* extend the range of allowed sugar conformations relative to those of more rigid cyclonucleosides such as **1** and **2**, but it

apparently cannot accommodate the full range of furanose ring puckering. Excluding **4e**, which features a flattened furanose ring, the average puckering amplitude (ν_m) of the remaining conformers is 38°, which is the same as the average value seen in the X-ray structures of normal nucleosides.[8]

The prediction from molecular mechanics that **4d** represents a favored conformation receives support from the X-ray structure of a related compound, 5-hydroxy-$O^{5',6}$-methanouridine dihydrate (**12**). As seen in the stereo view shown in Figure 2 and the parameters listed in Table I, the solid state conformation of **12** is very similar to **4d**. Compound **12** also features C3′-*exo* sugar puckering (P = 200°), and it differs from **4d** mainly in that the glycosyl rotation angle χ is some 14° smaller. Interestingly, the X-ray structure of **12** closely resembles the solid state structure reported recently by Groziak *et al* for the isomeric compound **13** (P = 197°, ν_m = 38°, χ = 137°).[9]

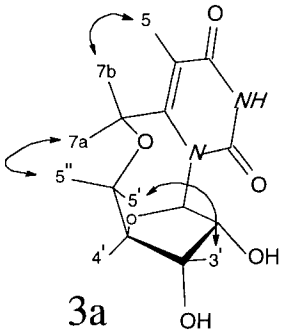


12



13

The basic orientation of the methano bridge of conformer **4d,** and of the crystal structures **12** and **13,** is apparently conserved in the predominant *solution* structures of $O^{5',6}$-methano-pyrimidine nucleosides. For example, in the case of $O^{5',6}$-methanouridine (**3**) it was possible to deduce from the NOE contacts shown in structure **3a** that the methano group is still directed towards the furanose ring oxygen in the solution conformation. Unfortunately, similar NOE experiments cannot be done with **4** because of the equivalence of the C5′ protons in all of the NMR solvents examined. However, a strong indication that the general orientation of the methano bridge of **4** must be essentially the same as that of **3** follows from the similarity of their CD spectra. For **4** the molar ellipticity [θ], which equals 20,300 at the wavelength corresponding to the main UV absorption band, is almost identical to that observed for **3**. The sign and magnitude of [θ] in pyrimidine nucleosides are known[10] to depend on the glycosyl rotation angle χ, and we



3a

have concluded previously[4] that the value of [θ] observed for **3** translates to a χ value of about -160°, which presumably reflects a weighted average of several contributing conformations.

In other respects, the solution conformations of *O* $^{5',6}$-methano-pyrimidine nucleosides diverge from the crystal structures of **12** and **13**. For example, the coupling constants $J_{4',5'}$ and $J_{4',5''}$, which are 0 Hz and 3.2 Hz, respectively, for compounds **3**, **4**[11] and **12**, indicate that the almost perfect *gauche*$^+$ orientation of O5′ (γ = 64°) seen in the solid state for **12** is not preserved in solution. The zero coupling constant means that the torsional angle H4′-C4′-C5′-H5′ must average about 90°, which is closer to the 80° expected for **4d** than to the 65° seen for **12**. The zero value of $J_{4',5'}$ also indicates that conformer **4a** is not a major contributor *in solution*. The *trans* relationship between H4′ and H5′ in **4a** would lead to a much larger coupling constant, perhaps as large as 12 Hz, so that even small populations might be expected to measurably affect $J_{4',5'}$.

Turning now to the sugar puckering, the furanose ring coupling constants observed for **4** ($J_{1',2'}$ = 3.2 and $J_{3',4'}$ = 3.7 Hz) suggest the presence of a mixture of conformations in solution rather than the single $_3$E conformation seen in the solid state for **12** and **13**, and predicted by conformer **4d**. If the $_3$E conformation were the only one present in solution, $J_{1',2'}$ would be much larger and $J_{3',4'}$ would be much smaller than the observed values. The opposite would be true if the $_2$E puckering predominated. Given the small energy differences involved in the $_3$E (**4d**) $\rightleftharpoons$ $_2$E (**4b**) interconversion indicated in Figure 1, it is highly likely that these two forms are in equilibrium in solution.[12]

Of the conformational types shown in Table I, it therefore appears from the NMR evidence that **4b** and **4d** are the major types present in solution, and that **4a** is present to a smaller extent than might be expected from the molecular modeling results. The conformational parameters of **4b** and **4d** are not unreasonable for cytidine itself; in fact computer models of cytidine restrained to these conformations can be superimposed quite satisfactorily on those of **4b** and **4d**. However, significant differences are apparent when **4b** or **4d** are superimposed on cytidine with the 3′-*endo* puckering found in the solid state[13], and which certainly contributes to the low energy conformer populations of cytidine in solution.

Interaction of $O^{5',6}$-methanocytidine (4) with Cytidine Deaminase. In spite of the inability of $O^{5',6}$-methanocytidine to adopt the low-energy conformations of cytidine, the compound is still deaminated by cytidine deaminase from mouse liver, although at a slow rate. Using a crude enzyme preparation, we found that **4** was deaminated to the extent of 60 - 70% in 32 hours whereas cytidine was deaminated to the extent of 90% during 3.5 hours. The deamination of **4** was monitored by changes in the UV spectrum and by HPLC analysis, which served to positively identify the product as $O^{5',6}$-methanouridine (**3**). In inhibition studies, **4** binds to cytidine deaminase from mouse liver with an apparent K_i value of 2.62 ± 0.86 mM. Since **4** is a substrate for cytidine deaminase, its apparent K_i value represents its apparent K_m,[14] which should be compared with the apparent K_m of cytidine, namely 0.127 ± 0.029 mM.[15] Assuming that **4** interacts with the normal active site of cytidine deaminase, we can conclude from the above results that this particular enzyme binds and deaminates cytosine nucleosides in the *anti* conformation. This conclusion is strongly supported by the recent work of Betts *et al* [16] on the X-ray structure of cytidine deaminase from *E. coli*, which appeared after the completion of the present study. Thus the glycosyl rotation angle of the transition-state inhibitor 1-(β-D-ribofuranosyl)-5-fluoropyrimidin-2-one bound covalently to the enzyme is clearly in the *anti* range, with a value of about -160°. As shown above, this is essentially the same as the glycosyl rotation angle found for **4** in solution. Further studies of the interaction of **4** and its 4-unsubstituted analogue with purified cytidine deaminases from a variety of sources would appear to be warranted.

It should be noted that besides any conformational considerations, the substitution pattern of $O^{5',6}$-methanocytidine (**4**) *per se* would be expected to reduce its affinity for cytidine deaminase. For example, it is known that while the 5′-hydroxy group is not essential for substrate activity with cytidine deaminases from various sources, 5′-O-substitution results in reduced binding.[17] Also, 6-substitution, as in 6-methylcytidine, either precludes binding or reduces it substantially.[17,18] In those cases where 6-methylcytidine and 6-methyl-Ara-C are deaminated slowly,[17a] the compounds probably still bind in the *anti* conformation even though they exist predominantly as *syn* conformers in solution. The same interpretation applies to 2-methylformycin,[19] a predominantly *syn* nucleoside that is nevertheless deaminated efficiently by the *anti*-requiring adenosine deaminase. An actual example[20] of a nucleoside that is *syn* in solution and in the solid state, yet is bound to an enzyme in the *anti* conformation is seen in the X-ray structure of the complex between 8-bromoadenosine-5′-

diphosphoribose and horse liver alcohol dehydrogenase. These examples serve to emphasize the point that even minor solution conformers can be bound by enzymes, and that analogues that are not adequately constrained can therefore give misleading results. On the other hand, it appears that a cyclonucleoside such as **4**, which is covalently restricted to a part of the normal conformational range, can give more reliable information.

EXPERIMENTAL SECTION.

General Chemical Procedures: ^{1}H-NMR spectra were obtained relative to internal TMS on Varian XL-200 and VXR-500 spectrometers. ^{13}C-NMR spectra were recorded on a Varian XL-200 instrument operating at 50.3 MHz, and the solvent resonance was used as a reference. Selective irradiation experiments were used whenever possible to confirm peak assignments. UV and CD spectra were recorded on Gilford Response II and JASCO J-720 instruments, respectively. Preparative TLC separations were performed on 1000 μm 20 X 20 cm silica gel plates (Uniplates from Analtech, Inc.). All evaporations were carried out under reduced pressure in a rotary evaporator. Microanalyses were performed by M. H. W. Laboratories, Phoenix, Arizona.

2′,3′-*O*-Isopropylidene-*O*$^{5',6}$-methanouridine (9). Triethylamine (68 mg, 0.67 mmol) was added to a stirred solution of triflate[4] **8** (300 mg, 0.67 mmol) and sodium borohydride (13.2 mg, 0.35 mmol) in DMF (5 mL), and the mixture was heated to 65°C for 4 h. The solvent was then removed and the products were isolated using preparative TLC, with double development in ethyl acetate-hexane-acetic acid (100:100:1, v/v). The appropriate zones were extracted with dichloromethane-methanol (85:15, v/v) to afford a small amount of unchanged triflate **8** (15 mg) and the reduced product **9** (150 mg, 80%, based on amount of starting material that reacted), identical (NMR, TLC) with material prepared by the original method.[4] Nucleoside **9** was obtained in 85% yield from a similar reaction (80 °C, 3h) conducted in the presence of 50 mg of $[(Ph)_3P]_4Pd$.

2′,3′-*O*-Isopropylidene-*O*$^{5',6}$-methanocytidine (11). Carbon tetrachloride (1.5 g, 10 mmol) was added to a suspension of 2′,3′-*O*-isopropylidene-*O*$^{5',6}$-methanouridine (170 mg, 0.57 mmol) and triphenylphosphine (600 mg, 2.2 mmol) in acetonitrile (6 mL) at room temperature, and the mixture was refluxed for 3 h. The clear reaction mixture was evaporated to dryness and the residue was rapidly fractionated on a column of neutral alumina[21] with ethyl acetate-hexane (2:3, v/v) as the eluting solvent. Fractions

containing the 4-chloro nucleoside **10** were pooled and solvents were removed. Since this intermediate readily undergoes hydrolysis to regenerate the starting material, the residue was treated without delay with a mixture of 28% ammonium hydroxide-dioxane (1:3, v/v, 9 mL), and the solution was stored at room temperature for 16 h. The reaction mixture was then evaporated to dryness and the residue was purified on a column of silica gel using chloroform-methanol (9:1, v/v) as the eluting solvent. Evaporation of the appropriate fractions and crystallization of the combined residues from ethanol afforded **11** (85 mg, 50%) as colorless needles, mp 254-258 °C (dec., darkens above 170 °C); UV (pH 7) λ_{max} 278 nm, UV (pH 3) λ_{max} 288 nm; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.14 and 7.08 (two bs, 2H, NH_2), 6.32 (d, 1H, H-1′), 5.54 (s, 1H, H-5), 4.70 and 4.68 (two overlapping d, 2H, H-3′ and H-7a), 4.51 and 4.52 (m, 2H, H4′ d overlapping H2′ dd), 4.49 (d, 1H, H-7b), 3.87 (d, 1H, H-5′), 3.66 (dd, 1H, H-5″), 1.44 and 1.25 (two 3H s, *Me*C*Me*), $J_{1',2'}$ = 1.7, $J_{2',3'}$ = 6.1, $J_{3',4'}$ = $J_{4',5'}$ ≃ 0, $J_{4',5''}$ = 3.2, $J_{5',5''}$ = 12.8 Hz, $J_{7a,7b}$ = 14.3 Hz; ^{13}C NMR (DMSO-d_6) δ 164.81 (C-4), 156.00 (C-2), 151.11 (C-6), 111.23 (Me*C*Me), 95.31 (C-1′), 94.52 (C-5), 87.96 and 87.56 (C-4′ and C-2′), 82.67 (C-3′), 73.79 (C-7), 72.19 (C-5′), 26.55 and 24.69 (*Me*C*Me*). *Anal.* Calcd. for $C_{13}H_{17}N_3O_5$: C, 52.87; H, 5.80; N, 14.23. Found: C, 52.78, H, 5.82; N, 14.06.

O $^{5',6}$**-Methanocytidine (4).** The blocked nucleoside **11** (0.1 g, 0.34 mmol) was dissolved in 10 mL of 5% (w/w) HCl (gas) in methanol and the solution was stirred at room temperature for 4 h. The reaction mixture was evaporated to dryness and an aqueous solution of the residue was passed through a small column of Dowex 50 (H^+). The column was washed thoroughly with water before elution of the product with 1N ammonium hydroxide solution. Evaporation of the appropriate fractions and crystallization from absolute methanol then afforded crystalline **4** (56 mg, 65%), mp 232 - 235 °C (dec); UV (pH 7-11) λ_{max} 278 nm (ε 8,700), UV (pH 3) λ_{max} 215 nm (ε 8,500), λ_{max} 287 nm (ε 12,130), λ_{min} 244 nm (ε 1,330); CD (water) $[\theta]_{max}$ (nm) 20,300 (277); ^{1}H NMR (200 MHz, D_2O) δ 6.31 (d, 1H, H-1′), 5.89 (s broadened by allylic couplings, 1H, H-5), 4.97 (dd, 1H, H-7a), 4.63 (d, 1H, H-7b), 4.49 (dd, 1H, H-2′), 4.44 (5-line m, 1H, H-4′), 4.34(dd, 1H, H-3′), 3.93 (pseudo d, 2H, H-5′ and H-5″), $J_{1',2'}$ = 3.2, $J_{2',3'}$ = 4.9, $J_{3',4'}$ = 3.7, $J_{7a,7b}$ = 14.3, $J_{5,7a}$ = 0.8 Hz;[11] ^{13}C NMR (DMSO-d_6) δ 164.92 (C-4), 156.49 (C-2), 151.62 (C-6), 96.41 (C-5), 93.13 (C-1′), 85.69 (C-4′), 76.99 (C-2′), 73.54 (C-7), 71.17 (C-3′), 70.21 (C-5′). *Anal.* Calcd. for $C_{10}H_{13}N_3O_5$: C, 47.06; H, 5.13; N, 16.46. Found: C, 47.21, H, 5.15; N, 16.47.

X-ray Crystallographic Methods: A sample of 5-hydroxy-$O^{5',6}$-methanouridine[4] (**12**, $C_{10}H_{12}N_2O_7 \cdot 2H_2O$ MW = 290.23) was recrystallized from water. The resulting

orthorhombic crystals belong to the $P2_12_12_1$ space group, with a = 5.410(3) Å, b = 13.064(8) Å, c = 17.239(14) Å, V = 1218(2) Å^3, $\alpha = 90°$, $\beta = 90°$, $\gamma = 90°$, Z = 4 and $D_{calc} = 1.680$ g/cm^3. Intensities were measured using ω-2θ scans with an Enraf-Nonius CAD-4 diffractometer using Cu Kα radiation and a graphite monochromator (λ = 1.5418Å). Corrections were made for Lorentz and polarization factors and for X-ray absorption (μ = 12.5 cm^{-1}). A total of 999 independent intensities were measured with $2\theta < 114$; 11 reflections had net intensities of < 0 and were omitted from all further calculation. The structure was determined by direct methods using the program MULTAN80. [22] All hydrogen atom positions and two disordered water molecules of solvation were located using difference Fourier maps. The structure was refined using full-matrix least-squares techniques in which non-hydrogen atoms were given anisotropic temperature factors, and hydrogen atoms were assigned a fixed isotropic temperature factor equal to 1.5 times the equivalent isotropic temperature factor of the atom to which it was attached. The final R factor for 988 observations and 245 variables was 0.040, and the goodness-of-fit was 2.804. All crystallographic calculations were carried out using the Enraf-Nonius Structure Determination Package.[23] Positional parameters for **12** are given in Table 2.

Molecular modeling. Energy minimizations were performed using Hyperchem™ Release 3 for Windows™ (Autodesk Inc.). All calculations were done with the MM+ force field and default values were used for all parameters. For the conformational searches shown in Figure 1, the pyrimidine ring was maintained in a planar state by applying restraints to keep the torsional angles C7-C6-N1-C1′ and O2-C2-N1-C1′ at 0°, and the torsional angles C5-C6-N1-C1′ and N3-C2-N1-C1′ at 180°. In addition, the plane defined by C7-N1-C1′-C2 was assigned an improper torsional angle of 180°. A force constant of 1600 kcal/mol^{-1}degree^{-2} was used for all restraints. The curve represented by the filled circles in Figure 1 was generated by starting with an arbitrary structure with $\chi = -96.6°$ and $P = 269°$ ($_OE$, $\nu_0 = 39$, $\nu_1 = -22$, $\nu_2 = -0.5$, $\nu_3 = 23$, $\nu_4 = -39$) and optimizing the geometry for fixed values of γ over the range 0° to 180° in 5° increments using the Polak-Ribière conjugate gradient method. Optimization at each step was continued until the root-mean-square gradient was less than 0.1 kcal/(Å mol). In the reverse direction, starting from the $\gamma = 180°$ structure (where $\chi = -173°$ and $P = 242°$ [^4T_O, $\nu_0 = 32$, $\nu_1 = -4$, $\nu_2 = -22$, $\nu_3 = 43$, $\nu_4 = -50$]), optimization at 5° intervals of γ produced the curve represented by the open circles, and ended with a structure where $\chi = -97°$, $\gamma = 0°$, and $P = 240°$ ($_4E$, $\nu_0 = 29$, $\nu_1 = -4$, $\nu_2 = -21$, $\nu_3 = 38$, $\nu_4 = -41$). The open circle curve can be generated in either direction. The curve represented by

TABLE 2. Positional parameters and their estimated standard deviations

Atom	x	y	z	B(A2)
N1	0.2686(5)	0.8322(2)	0.1691(2)	2.21(5)
C2	0.4387(7)	0.9096(2)	0.1759(2)	2.25(6)
O2	0.5790(5)	0.9194(2)	0.2318(1)	2.98(5)
N3	0.4457(6)	0.9801(2)	0.1167(2)	2.73(5)
C4	0.2883(7)	0.9846(3)	0.0547(2)	2.90(7)
O4	0.3069(6)	1.0528(2)	0.0054(1)	4.45(6)
C5	0.1065(7)	0.9039(3)	0.0518(2)	2.84(7)
O5	-0.0512(6)	0.9019(2)	-0.0090(1)	4.39(6)
C6	0.1029(6)	0.8279(2)	0.1060(2)	2.43(7)
C7	-0.0659(8)	0.7388(3)	0.0932(2)	3.17(7)
C1'	0.2665(6)	0.7584(2)	0.2348(2)	2.26(6)
C2'	0.0599(7)	0.7784(2)	0.2930(2)	2.46(6)
O2'	0.1478(5)	0.8400(2)	0.3548(1)	3.75(6)
C3'	-0.0127(7)	0.6690(2)	0.3177(2)	2.78(7)
O3'	0.1511(6)	0.6322(2)	0.3756(2)	3.96(6)
C4'	0.0298(7)	0.6086(2)	0.2442(2)	2.71(7)
O4'	0.2434(4)	0.6564(2)	0.2079(1)	2.52(4)
C5'	-0.1799(7)	0.6106(3)	0.1857(2)	2.93(7)
O5'	-0.2177(5)	0.7126(2)	0.1580(1)	2.73(5)
OW1A	0.3245(8)	0.4370(3)	0.1057(2)	3.99(8)
OW1B	0.393(2)	0.412(1)	0.1059(6)	6.8(3)
OW2A	0.473(2)	0.2603(4)	0.0210(3)	9.4(2)
OW2B	0.184(4)	0.265(1)	0.0113(9)	11.5(5)
HN3	0.55(1)	1.031(3)	0.115(3)	3.9*
HO5	-0.09(1)	0.979(4)	-0.056(3)	6.4*
H7A	0.01(1)	0.679(3)	0.071(2)	4.6*
H7B	-0.18(1)	0.758(3)	0.051(3)	4.6*
H1'	0.426(9)	0.773(3)	0.260(3)	3.2*
H2'	-0.06(1)	0.809(3)	0.272(2)	3.7*
HO2'	-0.00(1)	0.920(3)	0.349(3)	5.6*
H3'	-0.19(1)	0.669(4)	0.339(3)	5.7*
HO3'	0.13(1)	0.669(4)	0.435(3)	5.7*
H4'	0.08(1)	0.532(3)	0.264(2)	4.0*
H5'A	-0.17(1)	0.554(3)	0.145(3)	4.5*
H5'B	-0.33(1)	0.585(4)	0.215(3)	4.5*

Anisotropically refined atoms are given in the form of the isotropic equivalent displacement parameter defined as: (4/3) * [a2*B(1,1) + b2*B(2,2) + c2*B(3,3) + ab(cos gamma)*B(1,2) + ac(cos beta)*B(1,3) + bc(cos alpha)*B(2,3)]. Isotropic B factors for starred atoms were not refined. Tables of torsion angles, bond angles and bond lengths, and other crystallographic parameters are available on request.

filled diamonds was generated by releasing the restraints on γ and optimizing the geometry while varying χ in 5° steps over the range -150° to ±180°, and then back to -150°. Since in this case the same energy minima are found in each direction, the lower energy at each point was plotted. Structures in Figure 1 that correspond to an energy minimum were further optimized (RMS gradient < 0.02) after removal of *all* restraints, the only exceptions being conformers **4e** and **4f** where it was necessary to keep the planar pyrimidine restraints in order to avoid pronounced distortion. The resulting structures (**4a-4f**) are those shown in Table I.

Cytidine deaminase preparation. Livers were obtained from female Swiss Albino (CD1) mice (Charles River Laboratories, Boston, MA). Mice were killed by cervical dislocation and the livers were removed, weighed, minced and homogenized in three volumes of 50 mM Tris-Cl (pH 8) containing 1 mM dithiothreitol and 1 mM EDTA using a Polytron homogenizer (Brinkman Instruments, Westbury, NJ). The homogenate was centrifuged at 105,000 x g for 1 h at 4 °C in a Beckman L1-M ultracentrifuge and the supernatant fluid (cytosol) was used as the enzyme source.

Cytidine deaminase assay. The deamination of cytidine to uridine was determined isotopically. The assay mixture contained 50 mM Tris-Cl buffer (pH 8), 1 mM dithiothreitol, 1 mM EDTA, 125 µM [^{14}C]cytidine (1.8 Ci/mol, Research Products International Corp.), and 30 µL of the enzyme preparation in a final volume of 60 µL in the presence or absence of 0.1 - 1.0 mM $O^{5',6}$-methanocytidine. Reactions were started by the addition of extract, incubated at 37 °C for 30 min, and terminated by heating for 2 min in a boiling water bath followed by freezing. The precipitated proteins were removed by centrifugation. Cytidine was separated from uridine in the supernatant by TLC on silica gel plates (G/UV$_{254}$, Brinkmann Instruments) developed with chloroform:methanol:acetic acid (90:5:5, v/v/v). The R_f values for cytidine and uridine were 0.1 and 0.38, respectively. The radioactivity in the spots was determined on a percentage basis using a Berthold LB-2821 Automatic TLC Linear Analyzer. Under these conditions activity was linear with time and enzyme concentration. The apparent K_i value for $O^{5',6}$-methanocytidine (2.62 ± 0.86 mM) was estimated from Dixon's plots ($1/v$ vs. [I])[24] of the data by a computer program with least squares fitting. The program was written by Dr Sungman Cha and modified for IBM BASIC by Dr. Fardos N. M. Naguib.

Deamination of $O^{5',6}$-methanocytidine. The deamination of $O^{5',6}$-methanocytidine by cytidine deaminase was monitored by changes in the UV spectrum, and by HPLC

using a reversed phase (C-18) silica gel column eluted isocratically with 8% methanol containing 0.1% acetic acid. In acidic solution (pH about 3) $O^{5',6}$-methanocytidine shows λ_{max} at 287nm, whereas the product ($O^{5',6}$-methanouridine) shows a λ_{max} at 271 nm. The reaction mixture contained in a final volume of 0.2 mL, 20mM of potassium phosphate buffer (pH 8), 1mM of EDTA, 1mM of dithiothreitol, 0.1 mL of the enzyme preparation, and with and without 1mM of $O^{5',6}$-methanocytidine. The reaction mixtures were incubated at 37 °C. Aliquots (15 µL) were periodically withdrawn, diluted with water (380 µL) and acidified with 2 drops of 1N $HClO_4$. The mixture was centrifuged (10 min at 14000 rpm), and the clear supernatant fluid was used to determine the UV spectrum (0.4 mL capacity self-masking cuvettes; wavelength scan from 350 to 200 nm) relative to the appropriate reference solution. From zero time to 25 h, the UV absorption shifted from 287 nm to 274 nm, indicating that deamination was considerable but not complete. In a second run — when the enzyme preparation was not as active because of several freeze/thaw cycles — over 90% of cytidine (1 mM) was converted into uridine in 3.5 h at 37 °C (UV λ_{max} shifts from 280 nm to 261.5 nm). In this same run, $O^{5',6}$-methanocytidine was deaminated to the extent of 60 - 70% in 32 h as seen by HPLC, which also serves to positively identify the product. No further change was seen at 48 h.

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REFERENCES AND NOTES.

(1) a) Makabe, O.; Nakamura, M; Umezawa, S. Cyclonucleoside Derivatives of Formycin, *J. Antibiot., Tokyo*, **1975**, *28A*, 492-495. b) Žemlička, J. Formycin Anhydronucleosides. Conformation of Formycin and Conformational Specificity of Adenosine Deaminase. *J. Amer. Chem. Soc.*, **1975**, *97*, 5896-5903.

(2) a) For conformational nomenclature and conventions used for nucleosides see Saenger, W. *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, 1984, chapter 2. See also: IUPAC-IUB Joint Commission on Biochemical Nomenclature, Abbreviations and Symbols for the Description of Polynucleotide Chains, *Eur. J. Biochem.*, **1983**, *131*, 9 - 15. b) Davies, D. B. Conformations of Nucleosides and Nucleotides. *Progress in NMR Spectroscopy*; Emsley, J. W.; Feeney, J.; Sutcliffe, L. H., Eds.; Pergamon: Oxford, **1978**, *12*, pp 135 - 225.

(3) Scharff, A. J.; Wilson, D. K.; Chang, Z.; Quiocho, F. A. Refined 2.5 Å Structure of Murine Adenosine Deaminase at pH 6.0. *J. Mol. Biol.*, **1992**, *226*, 917-21.

(4) Otter, B. A.; Patil, S. A.; Spada, M. R.; Jelicks, L. A.; Yoshimura, Y.; Matsuda, A.; Klein, R. S. Synthesis and Conformational Studies of $O^{5',6}$-Methanouridine — A New Type of Pyrimidine Cyclonucleoside. *Nucleosides & Nucleotides* **1992**, *11*, 615-635.

(5) Sasaki, K.; Sakai, M.; Sakakibara, Y.; Takagi, K. Nickel(0)-Catalyzed Reduction of Aryl Triflates with Zinc Powder and Methanol. *Chem. lett.*, **1991**, 2017-2018.

(6) Ciattini, P. G.; Morera, A.; Ortar, G. Palladium-Catalyzed Cross-Coupling Reactions of Vinyl and Aryl Triflates with Tetraarylborates. *Tetrahedron lett.*, **1992**, *33*, 4815-4818.

(7) De Napoli, L.; Messere, A.; Montesarchio, D.; Picialli, G.; Santacroce, C. Synthesis of 4-Substituted Pyrimidine-2′,3′-Dideoxynucleosides. *Nucleosides & Nucleotides*, **1991** *10*, 1719-1728.

(8) Altona, C.; Sundaralingham, M. Conformational Analysis of the Sugar Ring of Nucleosides and Nucleotides. A New Description using the Concept of Pseudorotation. *J. Amer. Chem. Soc.*, **1972,** *94*, 8205-8212.

(9) Groziak, M. P.; Koohang, A.; Stevens, W. C.; Robinson, P. D. A New Class of Nucleosides Possessing Unusual Physical Properties: Syntheses, Hydration, and Structural Equilibria of 1-(β-D-Glycofuranosyl)uracil-6-carboxaldehydes. *J. Org. Chem.*, **1993**, *58*, 4054-4060 ; and personal communication from Dr. M. P. Groziak.

(10) Yoshimura, Y.; Otter, B. A.; Ueda, T.; Matsuda, A. Nucleosides and Nucleotides. 108. Synthesis and Optical Properties of *Syn*-Fixed Carbon-Bridged Pyrimidine Cyclonucleosides. *Chem. Pharm. Bull.*, **1992**, *40*, 1761-1769, and references therein.

(11) For **4**, where the equivalence of the C5′ protons precludes the direct measurement of $J_{4',5'}$ and $J_{4',5''}$, values were obtained by NMR simulation. Thus, the frequencies observed for the 5-line H-4′ resonance can be reproduced satisfactorily when the coupling constants are set to 0 and 3.2 Hz, respectively, which are also the values obtained from the non-equivalent C5′ proton resonances of the cyclouridine **3**. If $J_{4',5'}$ in **4** were as large as 1 Hz, 6 lines would be resolvable for the H-4′ resonance.

(12) As with ordinary nucleosides, the idea of a predominantly two-state equilibrium in solution is supported by the fact that the sum of $J_{1',2'}$ and $J_{3',4'}$ is approximately constant.[2b] This sum is 6.9 Hz for **4** (3.2 and 3.7 Hz, respectively), 6.9 for **3** (4.2 and 2.7 Hz)[4] and 6.6 for **12** (4.6 and 2.0 Hz).[4] However, these sums are about 3 Hz less than those of unconstrained *anti*-β-D-pyrimidine nucleosides, whereas the average value of 4.8 Hz for $J_{2',3'}$ in **3**, **4**, and **12** is slightly smaller than usual (5.1 - 5.3 Hz). Because of these differences, the use without appropriate modification of equations[2b] that employ

$J_{1',2'}$ or $J_{3',4'}$ to estimate the mole fractions of N-type and S-type conformers in *ribo*furanose rings (see also, for example, van den Hoogen, Y. Th.; Treurniet, S. J.; Roelen, H. C. P. F.; de Vroom, E.; van der Marel, G.A.; Van Boom, J. H.; Altona. C. *Eur J. Biochem.*, **1988**, *171*, 155) is not appropriate for our compounds.

(13) Furberg, S.; Petersen, C. S.; Rømming, C.; A Refinement of the Crystal Structure of Cytidine. *Acta Crystallogr.*, **1965**, *18*, 313-320.

(14) Cha, S. *Mol. Pharmacol.*, **1968**, *4*, 621-629.

(15) Naguib, F. N. M.; el Kouni, M. H. Unpublished results.

(16) Betts, L.; Xiang, S.; Short, S. A.; Wolfenden, R.; Carter, C. W., Jr. Cytidine Deaminase. The 2.3Å crystal structure of an enzyme:transition-state analog complex. *J. Mol. Biol.* **1994**, *235*, 635-656.

(17) a) Kreis, W.; Watanabe, K. A.; Fox, J. J. Structural Requirements for the Enzymatic Deamination of Cytosine Nucleosides. *Helv. Chim. Acta.*, **1978**, *61*, 1011-1016. b) Holý, A.; Ludziša, A.; Votruba, I.; Šedivá, K.; Pischel. H. Preparation of Analogues of Cytosine and 2-Pyrimidone Nucleosides and their Effect on Bacterial (*Escherichia coli* A19) Cytidine Aminohydrolase. *Coll. Czech. Chem. Commun.*, **1985**, *50*, 393-417.

(18) DeYoung, J. L,; Notari, R. E.; Feller, D. R. Comparative Reactivity of Cytidine and Arabinosylcytosine to their 6-Methyl Derivatives in the Presence of Cytidine Deaminase. *Res. Comm. Chem. Pathol. Pharm.*, **1974**, *9*, 163-172.

(19) Crabtree, G. W.; Agarwal, R. P.; Parks, R. E., Jr,; Lewis, A. F.; Wotring, L. L.; Townsend, L. B. *N*-Methylformycins. Reactivity with Adenosine Deaminase, Incorporation into Intracellular Nucleotides of Human Erythrocytes and L1210 Cells and Cytotoxicity to L1210 Cells. *Biochem. Pharmacol.*, **1979**, *28*, 1491-1500.

(20) Abdallah, M. A.; Biellmann, J. F.; Nordström, B.; Brändén, C-I. The Conformation of Adenosine Diphosphoribose and 8-Bromoadenosine diphosphoribose when Bound to Liver Alcohol Dehydrogenase. *Eur. J. Biochem.*, **1975**, *50*, 475-481.

(21) The 4-chloro intermediate (λ_{max} [MeOH] = 311 nm) was extensively hydrolyzed to give the 4-oxo compound when silica gel was used instead of neutral alumina. Omission of the alumina column step resulted in much lower yields of **11**.

(22) Main, P.; Fiske, S. J.; Lessinger, L.; Germain, De Clercq, J.P.; Woolfson, M. M. *MULTAN80, A System of computer programs for the automatic solution of crystal structures from X-ray diffraction data*; Universities of York, England, and Louvain, Belgium, 1980.

(23) Frenz, B. S.; Okaya, Y. *Enraf-Nonius Structure Determination Package*; Enraf -Nonius, Delft, Holland, 1980.

(24) Dixon, M.; Webb, E. C. *Enzymes*, Academic Press, New York, 1979.

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