acetonitrile (10 mL) was added with vigorous stirring to a solution of **7** (150 mg, 0.4 mmol) in dry acetonitrile (60 mL), and the mixture was maintained at room temperature for 6 h. After evaporation of solvent and excess thionyl chloride in vacuo, the residue was basified with NH_4OH and extracted with several portions of $CHCl_3$. The combined organic phases were dried ($MgSO_4$) and evaporated to give a syrup, which was subjected to dry column chromatography on silica gel with EtOAc–NH_4OH (100:1). Treatment of the base in EtOAc with ethanolic HCl gave the dihydrochloride salt, which was recrystallized from EtOAc to afford 91 mg (54%) of **3**·2HCl: mp 203–204 °C; EIMS, *m/e* 408, 410, 412 (15, 10, and 3 M^+), 345 (6, $M^+ - CH_2CH_2Cl$), 268 [20, $M^+ - N(CH_2CH_2Cl)_2$]; NMR (HCl salt in pyridine-d_5) δ 5.68–5.64 (m, 2 H, H-6 and H-7), 4.87 (d, 1 H, H-5), 3.02 (m, 1 H, H-8), 2.63 (m, 1 H, H-14). Anal. ($C_{21}H_{26}O_2N_2Cl_2 \cdot 2HCl \cdot H_2O$) C, H, N, Cl.

8β-Azido-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methylmorphinan (8). Compound **8** was synthesized by a method similar to that of Bognar et al.[17] Treatment of morphine with *p*-tosyl chloride afforded 7,8-didehydro-3,6-ditosyl-4,5α-epoxy-17-methylmorphinan, which was reacted with sodium azide to give 8β-azido-6,7-didehydro-4,5α-epoxy-17-methyl-3-tosylmorphinan. Detosylation with KOH–EtOH afforded compound **8**. The free base was recrystallized from EtOAc: mp 185–187 °C; EIMS, *m/e* 310 (M^+); IR 2100 (azide band) cm^{-1}; NMR (base in $CDCl_3$) δ 5.87–5.87 (m, 2 H, H-6 and H-7), 2.58 (m, 1 H, H-8), 2.45 (m, 1 H, H-14). Anal. ($C_{17}H_{18}N_4O_2$) C, H, N.

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Registry No. **3**, 90246-16-5; **3**·2HCl, 90246-15-4; **4**, 76-57-3; **5**, 467-08-3; **6**, 90246-17-6; **7**·2HCl, 90246-18-7; **8**, 55781-29-8; **8** (tosylate), 90246-19-8; 7,8-didehydro-3,6-ditosyl-4,5α-epoxy-17-methylmorphinan, 90246-20-1; morphine, 57-27-2; diethanolamine, 111-42-2.

The Preferred Solution Conformation of Warfarin at the Active Site of Cytochrome P-450 Based on the CD Spectra in Octanol/Water Model System

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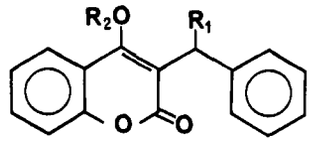
An octanol/water model system and circular dichroism (CD) spectroscopy have been used to study the solution conformation of warfarin in aqueous and lipid environments. Upon partitioning of (*S*)-warfarin from buffer pH 7.4 into octanol, the position of the absorption band due to the α,β-unsaturated carbonyl chromophore shifts from 210 nm in the aqueous phase to 220 nm in the octanol phase. The shift is coupled to an increase in the molecular ellipticity of the band, suggesting the formation of a dissymmetric chromophore. Comparison of CD spectra of conformationally fixed analogues of warfarin to that of warfarin in solution suggests that the compound shifts from the open side chain keto form in the aqueous phase at pH 7.4 to the cyclic hemiketal form after partitioning into the lipid octanol phase. On the basis of these results, the hemiketal form is proposed as the preferred solution conformation of warfarin in the lipid environment of the active site of cytochrome P-450 and the relationship between solution conformation and stereoselectivity of warfarin metabolism by β-naphthoflavone inducible cytochrome P-450 is discussed.

Warfarin (**1**) and phenprocoumon (**2**), two structurally related coumarin anticoagulants, are hydroxylated in the



1a, $R_1 = -CH_2COCH_3$; $R_2 = -H$

1b, $R_1R_2 = -CH_2C(OH)(CH_3)-$

2, $R_1 = -C_2H_5$; $R_2 = -H$

3, $R_1 = -CH_2COCH_3$; $R_2 = -CH_3$

4, $R_1R_2 = -CH_2C(OCH_3)(CH_3)-$

4′-, 6-, 7-, and 8-positions by rat liver microsomal preparations. Previous studies have shown the rate and regio- and stereoselectivity of aromatic hydroxylation are a function of induction state.[1] The highest rates of metabolism are observed with microsomes obtained from either 3-methylcholanthrene or β-naphthoflavone (BNF) pretreated rats. Induction with either compound leads to a high degree of regioselectivity for the 6- and 8-positions coupled to pronounced stereoselectivity but for opposite enantiomers of the two drugs. Thus, although a given absolute configuration for both compounds is spatially related about their asymmetric center, i.e., (*R*)-warfarin corresponds to (*R*)-phenprocoumon, the same enzymic preparation while selective for (*R*)-(+)-warfarin is also selective for (*S*)-(−)-phenprocoumon.

One possible explanation for the opposite stereoselectivity is provided by previous studies on the solution conformation of warfarin. Both nuclear magnetic resonance (NMR) and circular dichroism (CD) spectra of warfarin, phenprocoumon, and some conformationally restricted warfarin analogues indicate warfarin exists in solution in a tautomeric equilibrium of an open side chain keto form, **1a**, and a diastereomeric pair of ring closed hemiketals, **1b**.[2-4] In organic solvents warfarin exists principally as its hemiketal. It was the CD spectra of warfarin and phenprocoumon in organic solvent that suggested a structural relationship between opposite enantiomers of phenprocoumon and warfarin hemiketal. The CD spectra of like enantiomers of the two compounds are

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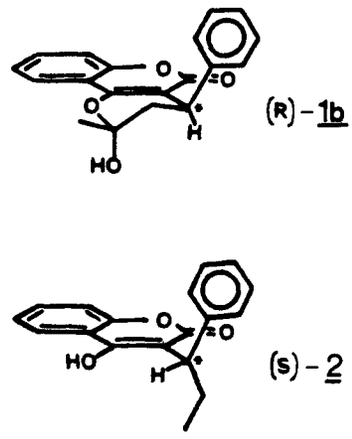

Figure 1. Comparison of the preferred conformations of (*R*)-warfarin hemiketal [(*R*)-1b] and (*S*)-phenprocoumon [(*S*)-2].

virtually mirror images. This peculiar relationship is due to the fact that a common spatial relationship of the phenyl group relative to the coumarin nucleus exists for opposite enantiomers of phenprocoumon and warfarin hemiketal[4] (Figure 1). If warfarin was metabolized in its hemiketal form, it would therefore be possible for opposite enantiomers of warfarin and phenprocoumon to spatially mimic one another at the active site of the BNF-induced enzyme(s) responsible for 6-and 8-hydroxylation.

In apparent conflict to the hypothesis that warfarin is metabolized in its hemiketal form, the CD spectrum of the drug indicates that the open side chain form is the predominant tautomer[5] in aqueous solution at physiological pH. However, cytochrome P-450 resides in a hydrophobic enviroment[6] and it is possible that warfarin may convert from its open side chain form to its hemiketal form upon partitioning from the aqueous to the lipid phase of the microsomal membrane. In this study circular dichroism is used to monitor the tautomeric equilibrium of warfarin and evidence is presented that suggests that the preferred conformation at the active site of P-450 is the hemiketal form.

Results and Discussion

Aqueous buffer pH 7.4 and octanol were used as a model system to simulate the respective aqueous phase and the lipid environment of the microsomal membrane found in an in vitro biological system. Warfarin was partitioned between the two phases and the resultant CD spectrum obtained from each phase recorded.

The position of the tautomeric equilibrium can be monitored by observing the wavelength and intensity of the CD active band due to the α,β-unsaturated carbonyl chromophore. As stated earlier, the preferred solution conformation of warfarin in organic solvent is known to be the hemiketal. The CD spectra of warfarin and its cyclic analogue, cyclocoumarol (**4**), in organic solvent, has an absorption band at 220 nm attributable to the α,β-unsaturated carbonyl chromophore.[4] The same band in the CD spectra of phenprocoumon and the open side chain analogue, warfarin 4-methyl ether (**3**), shifts to 210 nm. If warfarin is dissolved in aqueous buffer at pH 7.4, the band is again found at 210 nm. At this pH warfarin is known to exist in the open side chain tautomeric form.[5] Below pH 5 the hemiketal is the predominant tautomer and the wavelength of the band shifts to 220 nm. Thus, monitoring the band in the 210–220-nm region provides

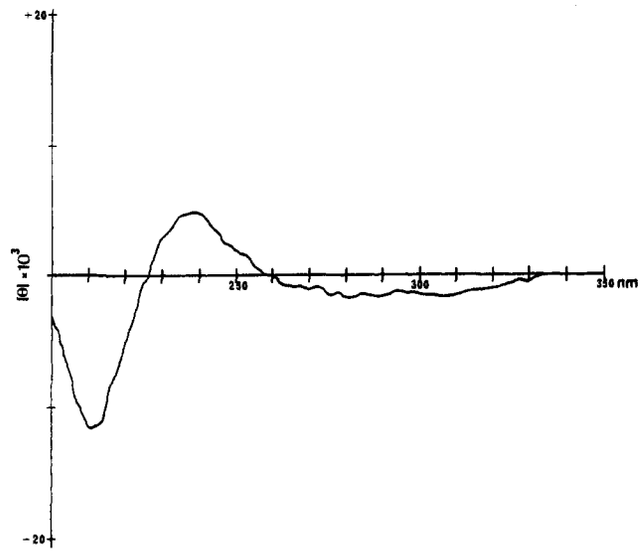

Figure 2. The CD spectrum of (*S*)-warfarin in 50 mM phosphate buffer, pH 7.38.

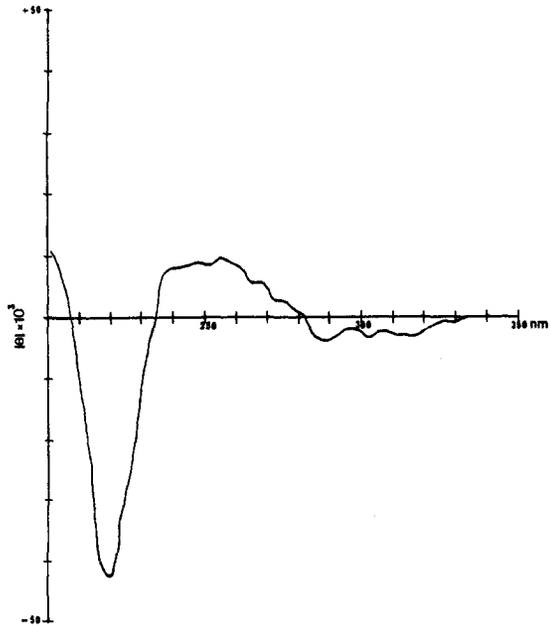

Figure 3. The CD spectrum of (*S*)-warfarin in octanol.

direct experimental evidence as to the tautomeric form of the drug in a specific solvent.

Also characteristic of hemiketal formation is an increased intensity of the 210–220-nm band. Upon cyclization a slight twist is imparted to the lactone ring bearing the α,β-unsaturated carbonyl, thus creating a dissymmetric chromophore that results in a more intense CD absorbance.[4]

In Figures 2 and 3 the respective CD spectra of (*S*)-warfarin in buffer and octanol after partitioning between the two phases are presented. As a control (*S*)-phenprocoumon was subjected to the same treatment (Figures 4 and 5). The CD spectra (in octanol) of the warfarin conformational analogues **3** and **4** were also recorded (Figure 6).

Upon partitioning from the aqueous to the octanol phase, the wavelength of the α,β-unsaturated carbonyl chromophore of warfarin shifts from 210 to 220 nm, indicating the hemiketal is now the predominant tautomer. In contrast, phenprocoumon, which cannot cyclize, exhibits no shift in the wavelength of the chromophore after it has partitioned into octanol (Figure 5). Likewise the α,β-un-

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(6) J. R. Gillette, *Prog. Drug. Res.*, 6, 22 (1963).

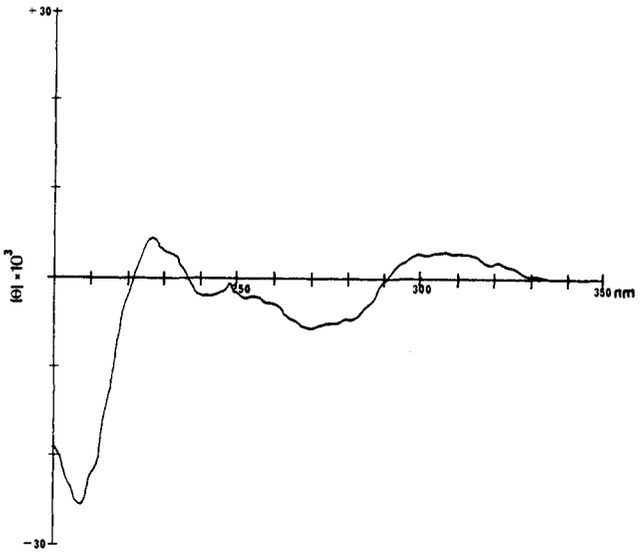

Figure 4. The CD spectrum of (*S*)-phenprocoumon in 50 mM phoshate buffer, pH 7.35.

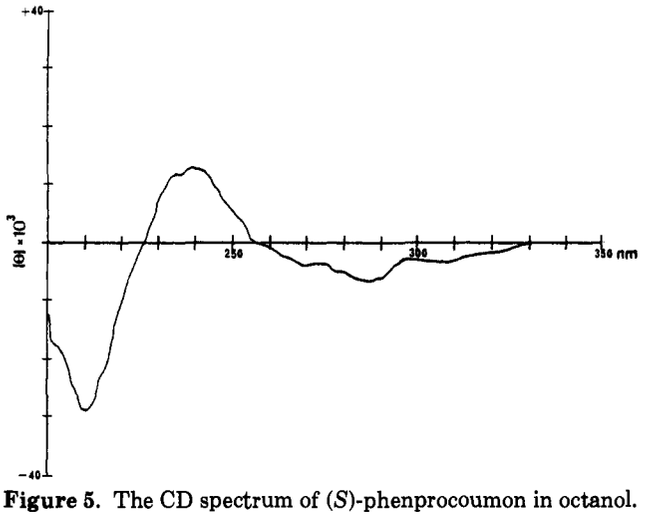

Figure 5. The CD spectrum of (*S*)-phenprocoumon in octanol.

saturated carbonyl chromophore of the open side chain analogue **3** occurs at 210 nm while the same band occurs at 220 nm in the spectrum of the cyclic methyl ketal **4** (Figure 6).

The increased intensity of the 210–220-nm band that should accompany the shift in wavelength upon hemiketal formation is also observed in the CD spectrum of (*S*)-warfarin partitioned into octanol. In Table I are listed the molecular ellipticity values of the major CD-active bands for (*S*)-(−)-**1** and (*S*)-(−)-**2** in buffer pH 7.4 and octanol and (*S*)-(−)-**3** and (*S,S*)-(+)-**4** in octanol. Upon partitioning from buffer to octanol, the magnitude of the cotton effect at 210–220 nm observed for (*S*)-(−)-**1** increased from θ = −11 800 in buffer to θ = −42 400 in octanol. However, with (*S*)-(−)-**2** the same band shows a decrease from θ = −29 100 in buffer to θ = −25 600 in octanol. Both the wavelength and intensity of the α,β-unsaturated carbonyl chromophore of warfarin in octanol are therefore strongly indicative of a preferred hemiketal conformation for warfarin in a hydrophobic environment.

Since P-450 is embedded in a lipid membrane and has a hydrophobic binding site, the environment a substrate would encounter upon binding to the enzyme would be lipoidal in nature.[6-8] Although a crude approximation,

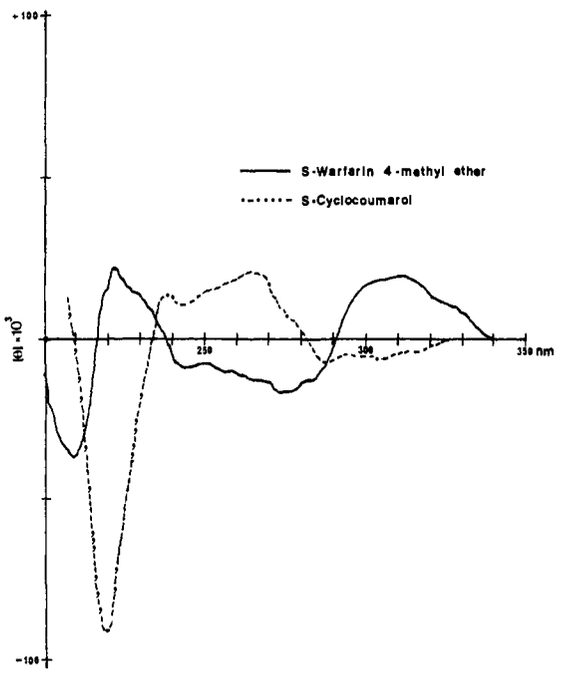

Figure 6. The CD spectra of (*S*)-warfarin 4-methyl ether (**3**) and (*S,S*)-cyclocoumarol (**4**) in octanol.

Table I. Circular Dichroism Spectral Data of 4-Hydroxycoumarin Derivatives in Phosphate Buffer and Octanol

comp, solvent	λ, nm	θ
(*S*)-(−)-**1**, 50 mM phosphate buffer, pH 7.38	290	−4 000
	240	+10 700
	210	−11 800
(*S*)-(−)-**2**, 50 mM phosphate buffer, pH 7.35	310	+4 600
	270	−7 200
	227	+5 800
	210	−29 100
(*S*)-(−)-**1**, octanol	305	−3 600
	262	+9 000
	220	−42 400
(*S*)-(−)-**2**, octanol	290	−6 000
	240	+11 500
	210	−25 600
(*S,S*)-(+)-**4**, octanol	305	−7 100
	265	+21 400
	220	−91 600
(*S*)-(−)-**3**, octanol	310	+18 900
	275	−15 900
	222	+21 900
	210	−35 800

octanol, nevertheless, is more representative of the kind of environment a substrate would encounter at the active site of the P-450 enzyme than is an aqueous system. The evidence presented here demonstrates that, although warfarin exists primarily in the open side chain tautomer in aqueous buffer at physiological pH after partitioning into a lipid environment, it converts to its hemiketal form. Therefore it is quite probable that warfarin assumes the hemiketal form at the active site of the enzyme and it is this preferred conformation that by spatially mimicking the opposite enantiomer of phenprocoumon is responsible for the opposite stereoselectivity observed in the 6- and 8-hydroxylation process of the two structurally similar drugs.

Experimental Section

Optical rotations were measured on a Jasco DIP-4 digital polarimeter using a 10 mm × 100 mm cell. The pH of the buffer

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solutions were determined with an Orion Research Model 701A digital pH meter. The CD spectra were recorded on a Jobin Yvon Dichrograph II using 0.1- and 1.0-cm cells, a scan rate of 12 nm/min, and a 10-s time constant.

Racemic warfarin and 1-octanol were purchased from Sigma Biochemicals. All other chemicals were of reagent grade.

(*S*)-(−)-Warfarin [3-(1-Phenyl-3-oxobutyl)-4-hydroxycoumarin (1)]. Racemic warfarin was resolved by the method of West:[9] $[\alpha]^{25}_D = -148.8$ (0.4)° (c 1.2, 0.5 N NaOH); CD (×10³) (0.0967 mg/mL of 50 mM phosphate buffer, pH 7.38) $[\theta]_{332}$ 0, $[\theta]_{290}$ −4.0, $[\theta]_{260}$ 0, $[\theta]_{240}$ +10.7, $[\theta]_{250}$ 0, $[\theta]_{210}$ −11.8; CD (×10³) (0.1127 mg/mL of octanol) $[\theta]_{340}$ 0, $[\theta]_{305}$ −3.6, $[\theta]_{286}$ 0, $[\theta]_{260}$ + 9.0, $[\theta]_{235}$ 0, $[\theta]_{225}$ −42.4.

(*S*)-(−)-Phenprocoumon [3-(1-Phenylpropyl)-4-hydroxycoumarin (2)]. Phenprocoumon was prepared by the method of Link[10] and resolved by the method of West:[11] $[\alpha]^{25}_D$ = −120.0(1.0)° (c 1.5, 95% EtOH); CD (×10³) (0.1084 mg/mL of 50 mM phosphate buffer, pH 7.35) $[\theta]_{330}$ 0, $[\theta]_{310}$ +4.6, $[\theta]_{290}$ 0, $[\theta]_{270}$ −7.2, $[\theta]_{240}$ 0, $[\theta]_{227}$ +5.6, $[\theta]_{220}$ 0, $[\theta]_{210}$ −29.1; (0.0995 mg/mL of octanol) $[\theta]_{340}$ 0, $[\theta]_{290}$ −6.0, $[\theta]_{255}$ 0, $[\theta]_{240}$ +11.5, $[\theta]_{225}$ 0, $[\theta]_{210}$ −25.6.

(*S*)-(−)-Warfarin 4-Methyl Ether [3-(1-Phenyl-3-oxobutyl)-4-methoxycoumarin (3)]. This compound was prepared from (−)-1 as previously described:[12] $[\alpha]^{25}_D = -9.1$ (2.0)° (c 1.8 MeOH); CD (×10³ (0.1070 mg/mL of octanol) $[\theta]_{340}$ 0, $[\theta]_{310}$ +18.9, $[\theta]_{290}$ 0, $[\theta]_{275}$ −15.9, $[\theta]_{240}$ 0, $[\theta]_{222}$ +21.8, $[\theta]_{215}$ 0, $[\theta]_{210}$ −35.8.

(*S*,*S*)-(+)-Cyclocoumarol [2(3*H*)-2-Methyl-2-methoxy-4-phenyl-5-oxobenzopyrano[3,4-*b*]dihydropyran (4)]. This compound was prepared from (−)-1 as previously described:[12] $[\alpha]^{25}_D$ = +33.1 (0.6)° (c 1.9, $CHCl_3$); CD (×10³) (0.1044 mg/mL of octanol) $[\theta]_{325}$ 0, $[\theta]_{305}$ −7.1, $[\theta]_{260}$ 0, $[\theta]_{265}$ +21.4, $[\theta]_{235}$ 0, $[\theta]_{220}$ −91.6.

Partition Experiments. Saturated solutions of (*S*)-(−)-1 and (*S*)-(−)-2 in 50 mM phosphate buffer, pH 7.4, were agitated with equal volumes of 1-octanol on a shaker bath at 25 °C for 16 h. Aliquots were removed from each phase and the CD spectra promptly recorded.

Acknowledgment. This investigation was supported by a Grant GM 25136 from the National Institutes of Health.

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2,5-Anhydro-1-deoxy-1-phosphono-D-altritol, an Isosteric Analogue of α-D-Ribofuranose 1-Phosphate[1]

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Condensation of tetramethyl methylenebisphosphonate with 2,3-*O*-isopropylidene-5-*O*-trityl-D-ribose gave a mixture of 2,5-anhydro-1-deoxy-1-(diethoxyphosphinyl)-2,3-*O*-isopropylidene-5-*O*-trityl-D-altritol and -allitol. Separation of the isomers and deprotection gave 2,5-anhydro-1-deoxy-1-phosphono-D-altritol and -allitol. The former is the stable isosteric methylenephosphonate analogue of α-D-ribose 1-phosphate, the ribose donor in nucleoside phosphorylase catalyzed nucleoside biosynthetic reactions. It did not, however, inhibit purine nucleoside phosphorylase at concentrations of 6 mM.

Investigations into the mechanism of inborn immunodeficiency diseases have pointed out the role of the purine salvage pathways in the viability of T and B lymphocytes. A deficiency in purine nucleoside phosphorylase has been identified as the cause of loss of immunocompetence in T lymphocytes.[3] The loss of this enzymic activity is responsible for the accumulation of 2′-deoxyguanosine, which apparently is selectively toxic to T lymphocytes.[4] It seems reasonable, therefore, that a compound that inhibits purine nucleoside phosphorylase might be a highly selective antineoplastic agent for leukemias and lymphomas derived from T lymphocytes.

Purine nucleoside phosphorylase catalyzes the reversible phosphorolysis of inosine (1) or guanosine (2) to give the corresponding purine, hypoxanthine (3) or guanine (4), and α-D-ribofuranose 1-phosphate (R-1-P, 5). Our initial approach to the design of inhibitors of this enzyme was to prepare an unreactive isosteric analogue of R-1-P. The substitution of the phosphate ester oxygen with a methylene has given a number of biologically active compounds in the past,[5] and we undertook the preparation of 2,5-anhydro-1-deoxy-1-phosphono-D-altritol[6] (6) to see if this strategy would yield an inhibitor of purine nucleoside phosphorylase.

The synthesis of this class of phosphonate analogue of glycosyl phosphates has very few literature precedents. Very recently, Russo and co-workers have prepared the methylenephosphonate analogues of α- and β-D-glucosyl phosphate via the Michaelis–Arbuzov reaction from blocked 1-bromo-1-deoxy-2,6-anhydroheptitols.[7] We have found a method, however, that gives blocked ribosyl methanephosphonates in one step from readily prepared blocked ribose derivatives.

The synthetic plan was based on the recently successful preparation of derivatives of 3,6-anhydro-D-*altro*- and -*allo*-heptonic acids 8 and 9 from 2,3-*O*-isopropylidene-5-

(1) A preliminary account of this work has appeared: Jesthi, P. K.; Stone, T. E.; Meyer, R. B., Jr. "Abstracts of Papers", 185th National Meeting of the American Chemical Society, Seattle, WA, March 1983; American Chemical Society: MEDI 28.
(2) Present address: Millipore Corp., Bedford, MA.
(3) Giblett, E. L., Amman, A.J.; Wara, D. W.; Sandman, R.; Diamond, L. K. *Lancet* 1975, 1010.
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(5) Engel, R. *Chem. Rev.* 1977, *77*, 349.
(6) The nomenclature of 6 is the preferred name by both IUPAC and current Chemcial Abstracts practice. Another descriptive, if trivial, name might be α-D-ribofuranosylmethanephosphonic acid. A similar name was used in ref 7 for the glucosyl analogue. McClard (see ref 17) named compound 6 as D-*altro*-2,5-anhydrodeoxyhexanophosphonate.
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