BIOORGANIC CHEMISTRY 18, 165–178 (1990)

Stable and Nonmetabolizable C-Glycosylphosphonyl Analogs of 5-Phosphorylribose 1-α-Diphosphate That Act as Inhibitors of Orotate Phosphoribosyltransferase

RONALD W. MCCLARD[1] AND JOHN F. WITTE

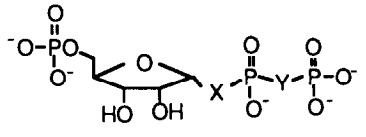
Arthur F. Scott Laboratory of Chemistry, Reed College, Portland, Oregon 97202

Received January 2, 1990

Phosphonate analogs of 5-phosphorylribose 1-α-diphosphate (PRPP), in which the anomeric oxygen has been replaced by carbon (C-glycoside analogs), have been prepared in multistep syntheses. The exact "ribosyl" analog could not be elaborated from the 3,4-isopropylidene precursor, **9**, owing to attack of the 3-OH onto the phosphonyl phosphorus under conditions for removal of the blocking group. Nevertheless, this isopropylidene derivative is a competitive inhibitor ($K_i/K_{m(PRPP)}$ = 16.4), with respect to PRPP, of yeast orotate phosphoribosyltransferase. This result indicates that the enzyme can accommodate a good deal of bulk at the corresponding 2- and 3-hydroxyl groups of PRPP. The "α-arabinosyl" analog, **15**, was successfully synthesized with ultimate stereospecificity that results from the preferential hydrolysis of the "β-anomer" in the same fashion as for the hydrolysis of **9**. This α-arabinosyl analog is also a competent competitive inhibitor of yeast orotate phosphoribosyltransferase ($K_i/K_{m(PRPP)}$ = 54). Both compounds are considerably more stable than PRPP both chemically and biochemically.

INTRODUCTION

5-Phosphorylribose 1-α-diphosphate, PRPP (**1**), is the sole donor metabolite of the sugar moiety of nucleotides, deoxynucleotides, and thus for all (deoxy)nucleic acids and other essential metabolites such as histidine and NAD(P)(H). PRPP fulfills this role through enzymes called phosphoribosyltransferases (PRTases), which catalyze the condensation of a wide variety of nitrogenous bases (e.g., adenine, ammonia, nicotinic acid, orotate) to form their respective nucleotides. Analogs of PRPP thus hold potential as important antimetabolites as well as tools for probing the active sites of the enzymes in this class. Thus a chemically and metabolically stable analog would be of particular utility.



1 X = Y = -O-
2 X = -O-, Y = $-CH_2-$
3 X = $-CH_2-$, Y = -O-

[1] To whom correspondence should be addressed.

0045-2068/90 $3.00

The first analogs of PRPP were reported by Murray *et al.* (*1*), who described a 5-phosphorothioate analog and PRPCP (**2**), the methylenebisphosphonate analog that exhibits competitive inhibition of mammalian and yeast orotate PRTase (*2*). Since then, Smithers and O'Sullivan reported other thioate analogs of PRPP (*3, 4*). All previously reported analogs of PRPP have two common features—(i) they are generally readily metabolized by the enzymes that act upon PRPP and (ii) they have excellent leaving groups at the anomeric carbon of ribose and are thereby particularly susceptible to hydrolysis under even weakly acidic or basic conditions. Thus they are probably not good candidates as potential pharmaceutical agents or as stable active site probes. We reasoned that since PRPCP, **2,** which has one methylene in place of an oxygen, is still a good inhibitor of orotate PRTase (indeed it has a $K_i/K_m < 1$ for the Ehrlich ascites enzyme (*2*)), a repositioning of the methylene to the anomeric position of the ribose should endow metabolic stability to analogs like **3** above while losing little, if any, strength of binding interaction.

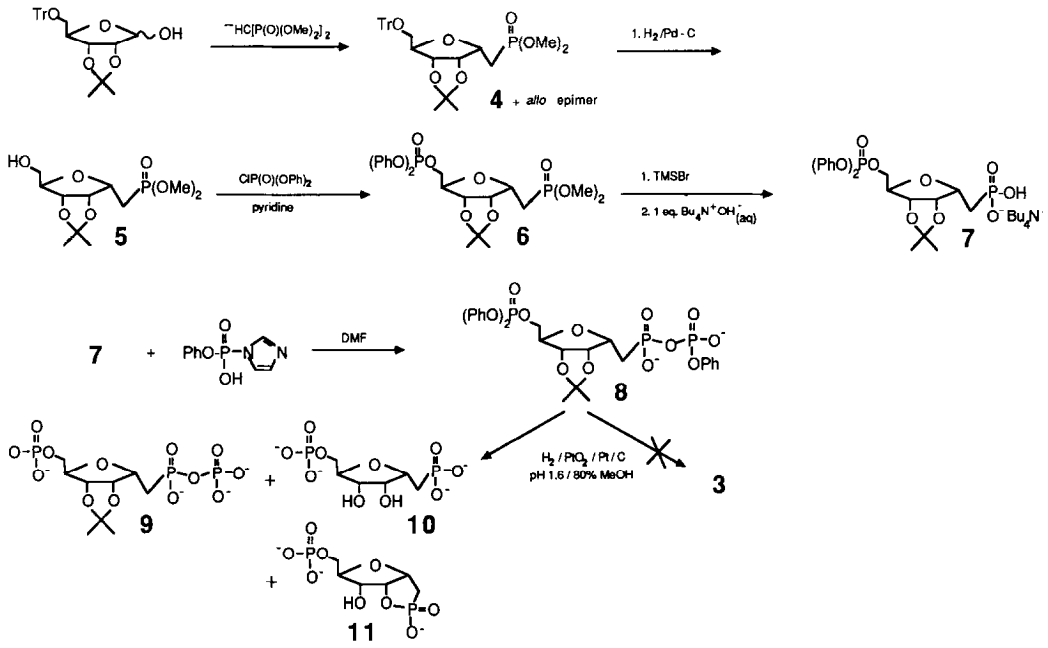
An analysis of possible synthetic strategies leading to compounds such as **3** revealed three potential major difficulties. The first involved the introduction of the requisite methylenephosphonate moiety, with the "α" configuration at the "anomeric carbon," onto the sugar fragment. From previous work in our laboratory and elsewhere (*5–7*) it was apparent that a suitably protected ribose or arabinose could serve as starting material since the methylphosphonate moiety could be introduced by a simple Wittig or Horner–Emmons reaction.

Second, our synthetic scheme required activation at C6 to allow phosphorylation with a reagent that would then permit selective activation of the phosphonyl group at C1. We expected that diphenyl chlorophosphate would fulfill this requirement since the *O*-phenyl groups as well as the isobutyl-like phosphate ester at C6 should be relatively inert to $(CH_3)_3SiBr$, which we had chosen to remove the phosphonyl ester groups prior to the formation of the crucial phosphonyl-phosphate anhydride bond. We were confident that this anhydride could be formed from the imidazolide of monophenylphosphate and the monoanion of the phosphonate at C1. In addition, this method would leave a negative charge on the anhydride at each of the two P atoms, thus endowing the most sensitive bond with resistance to hydrolysis (*8*) during workup and subsequent deprotection steps.

Third, our ultimate concern with this entire scheme rested on the final set of deprotection steps wherein the sugar hydroxyls, phosphate, and phosphonyl-phosphate groups would be unmasked. Under such conditions, the carefully preserved negative charges at the C1 phosphorus atoms could be neutralized, rendering the anhydride susceptible to nucleophilic attack. In fact, compound **3** itself falls victim to this final problem. Nevertheless, we report here the successful syntheses of two *C*-glycosyl PRPP analogs that act as competent competitive inhibitors of yeast orotate phosphoribosyltransferase and are resistent to chemical hydrolysis as well as metabolism by orotate PRTase.

RESULTS AND DISCUSSION

Scheme 1 outlines our synthetic route used to attempt the synthesis of the simplest and most exact *C*-glycoside analog, **3,** of PRPP. 2,3-*O*-Isopropylidene-5-



SCHEME 1

O-trityl-D-ribofuranose was subjected to a Horner–Wadsworth–Emmons reaction with tetramethyl methylenebisphophonate (*6*, *7*) to yield the *C*-glycosyl phosphonate **4** as a mixture of epimers which were separated on column chromatography. The *altro* epimer (α "anomer") was detritylated to give **5.** This compound was phosphorylated with diphenyl chlorophosphate to yield **6,** which was de-esterified with TMSBr to yield **7.** The methyl groups were selectively removed in this step and the 6-phosphoryl group, which is effectively a hindered "isobutyl" ester, remains intact as predicted. Compound **7** was converted to its monotetrabutylammonium salt and allowed to condense with phenyl imidazolylphosphate to yield **8.** Attempts to form the fully esterified counterparts of **8** were prone to rapid hydrolysis upon workup. A bare oxygen anion left on each of the two anhydride phosphorus atoms predictably provided resistance to nucleophilic attack by water or other nucleophiles. Compound **8** was then treated with Pt on C under H_2 at pH 1.6 in an attempt to simultaneously effect removal of the isopropylidene and hydrogenolysis of the phenyl esters. Higher values of pH were unsuitable for removal of this rather stable acetonide. We were, however, only able to recover **9** (the 3,4-isopropylidene derivative of **3**) and a mixture of **10,** a compound which clearly results from attack of the newly released 3-OH group (via removal of the isopropylidene moiety therefrom) onto the phosphonyl phosphorus, and its phostone[2] precursor **11** (see Fig. 1).

[2] The identity of a phostone was indicated by its sizable downfield shift (ca. 20 ppm) from the normal phosphonate resonance. This large shift is expected upon restriction of phosphorus to a 5-membered ring and indeed observed in the analogous formations of 5-phosphorylribose 1-α-2-cyclic-phosphate from PRPP and of ribose 1-α-2-cyclic phosphate from ribose 1-α-phosphate ($\Delta\delta \sim +17$ ppm) (*9*).

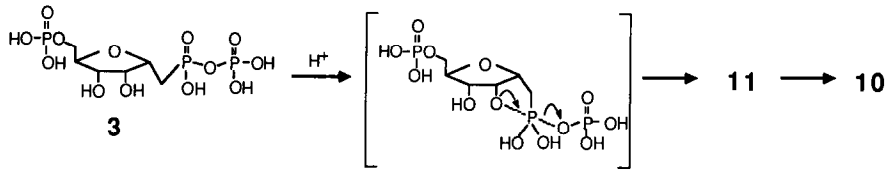
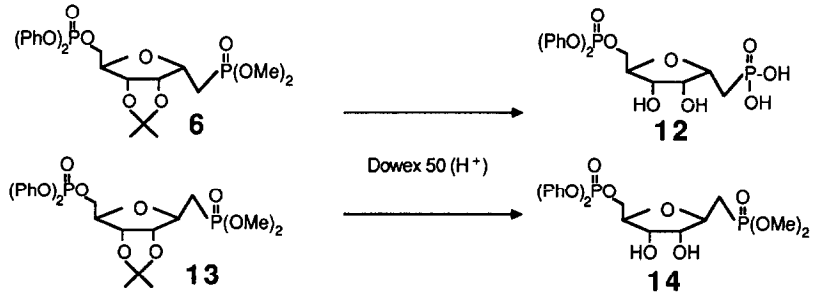


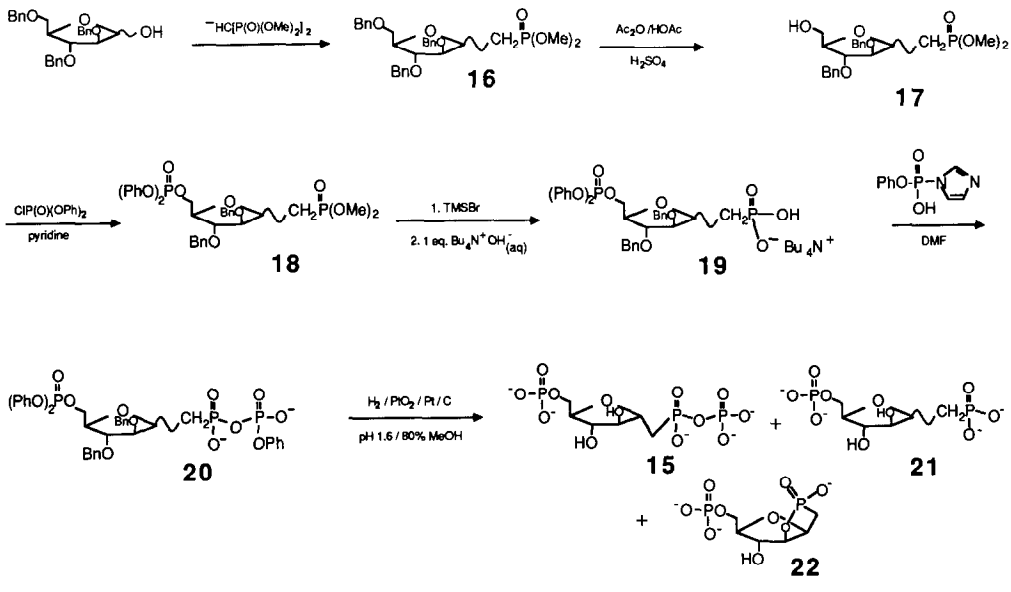
FIG. 1. Likely mechanism for the acid-catalyzed destruction of PRPP analog **3** (not isolated).

This conclusion was confirmed by the following two results. When compound **6** was treated with an aqueous methanolic suspension of Dowex 50 (H^+) in order to remove the acetonide group, we found that the methyl esters were also removed to yield **12** (Scheme 2) plus some of its corresponding phostone. Accordingly, when the *allo* epimer of **6,** compound **13,** was utilized we obtained only the desired deisopropylidenated methyl diester **14.** All of these results support the mechanism in Fig. 1 and indirectly confirm the assigned structure of **9.** Further corroboration of this conclusion is offered by the following case.

The *C*-glycoside PRPP analog **15** was synthesized from tribenzylarabinose by an analogous route, which is outlined in Scheme 3. The phosphonate **16** cannot be resolved into its epimers (*7*) and was thus carried through the synthesis as the mixture. The 6-*O*-benzyl ether was specifically removed by the acetolysis procedure of Eby *et al.* (*10*) to give the alcohol **17.** The syntheses of **18–20** were then carried out in close analogy to the methods for **6–8,** respectively. ^1H and ^{31}P NMR revealed that compounds **17–20** existed as mixtures of the D-*manno* and D-*gluco* epimers in the approximate ratio 3:2, respectively. The phosphonylphosphate anhydride intermediate, **20,** was treated first with Pd/C and H_2 to remove the benzyl ether groups followed by the treatment with Pt/C, pH 1.6, to remove the phenyl esters. This deprotection afforded the desired D-*manno* isomer of **15** plus the cleavage product **21,** mostly the D-*gluco*-1-phosphonate-6-phosphate, plus the intermediate phostone **22,** which was isolated and fully characterized by NMR; for example, this compound shows a three-bond coupling of P to C4 which can only be true for the proposed structure. It comes as no surprise that the D-*gluco* epimer (β anomer) of **20** readily and solely undergoes 3-OH-assisted hydrolysis to



SCHEME 2



SCHEME 3

22 in the same manner as does **8** → **10** and **11** (refer to Fig. 1). It is entirely consistent that one infer the structure of **15** to be correct using the same argument as described for the formation of **9.**

The analogs **9** and **15** are considerably more stable than PRPP. At pH 7 and 10 both analogs are virtually indefinitely stable, whereas PRPP has a half-life at 37°C of about 30 h (*2*). At pH 4 (50 mM formate buffer) and 37°C compounds **9** and **15** were found to have half-lives of 18 and 180 days, respectively (corresponding pseudo-first-order rate constants given in Table 1). These results indicate that at pH 4 compound **9** is approximately 70 times more stable than PRPP and that compound **15** is about 700 times more stable than PRPP. During the course of hydrolysis of **9** we observed the formation of the phosphate-phosphonate (**10**), the

TABLE 1

Kinetic Parameters for the Competitive Inhibition of Yeast Orotate PRTase by PRPP Analogs **9** and **15**

Compound	K_i (μM)	K_i/K_m[a]	$\Delta\Delta G$ (kcal/mol)[b]	k_{hydrol}[c] (h^{-1})
9	76 ± 15	16.4 ± 2.2	1.7	0.0016
15	207 ± 40	54 ± 8	2.3	0.00016

[a] The K_m for PRPP was 4.6 μM under the conditions of the assay.

[b] Calculated using the relationship $\Delta\Delta G = -RT \ln(K_i/K_m)$. See also footnote 3.

[c] Pseudo-first-order rate constants for the hydrolysis of the compound at pH 4.0 and 37°C. The value for PRPP is 0.107 under similar conditions (*2*).

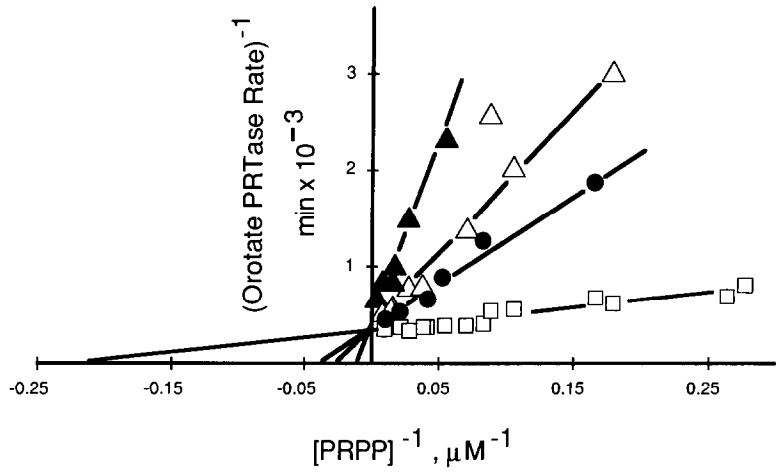


FIG. 2. Inhibition of orotate PRTase by *C*-glycoside phosphonate analogs of PRPP. The enzyme was assayed with (□) no analog present, (●) plus 1.0 mM **9**, (▲) plus 2.0 mM **9**, or (△) 1.0 mM **15**. Appropriate sets of data were fitted to the competitive inhibition model by nonlinear regression analysis, which gave rise to the double-reciprocal lines shown here.

expected phostone intermediate, and a very small amount of another anhydride species which we interpret to be **3**, which quickly collapses to **10** via anchimeric attack of the 3-OH and formation of the phostone. Accordingly, the hydrolysis of **15** gives rise only to the expected phosphate-phosphonate species (the D-*manno* epimer of **21**) as a result of simple cleavage of the anhydride. It was not unexpected that **9** should hydrolyze some 10 times faster than **15** in view of the fact that the former is allowed the hydrolysis pathway afforded by the *cis* hydroxyl and subsequent formation of the phostone.

Both analogs of PRPP, **9** and **15**, act as competitive inhibitors of orotate PRTase from yeast (see Fig. 2). The values of K_i and K_i/K_m and corresponding $\Delta\Delta G_{binding}$ values relative to PRPP[3] for these analogs are summarized in Table 1. It is somewhat surprising that the enzyme permits the bulk of an acetonide group (the case of **9**) at the corresponding 2- and 3-OH groups of ribose with a loss of binding energy of only about 1.7 kcal, some or all of which could be attributable to the substitution of the anomeric oxygen with the less electronegative -CH_2-group. Inversion of configuration of the 2-OH of PRPP (the case of **15**) results in the loss of about 2.3 kcal, which might reflect (i) the loss of a favorable hydrogen bonding interaction enjoyed by PRPP, (ii) steric congestion imposed by the "wrong" stereochemistry at the C2 of ribose, or (iii) the phosphoryl to phosphonyl conversion discussed above. Such tolerance by PRTases of the disruption of structure about the 2- and 3-OH groups of PRPP has been indirectly observed previously (*12–14*). For example, orotate PRTase from *Escherichia coli* (*12*) and hypoxan-

[3] It has been shown (*11*) that the kinetic K_m for PRPP and its K_i as a competitive inhibitor in the reverse reaction are identical within experimental uncertainty. Thus it is reasonable to calculate loss in binding interaction from values of $K_i/K_{m(PRPP)}$ for these analogs.

thine-guanine PRTase from mammalian sources (*13*) can be highly purified using affinity resins derived from periodate cleavage of the *cis* diols of OMP and GMP, respectively. Also, the latter enzyme is inhibited by acyclovir (*14*). Both **9** and **15** bind significantly more tightly to yeast orotate PRTase than does PRPCP, **2**, whose K_i is 550 μM under similar conditions (*2*).

Both compounds were unaffected by the addition of orotate PRTase. Thus, the chemical stability of these new compounds and their recognition by (but inability to be cleaved by) PRTases make them excellent choices as "inert" PRPP analogs.

EXPERIMENTAL

General Methods

All solvents were dried by conventional means except where noted. ^{1}H NMR spectra were obtained using either a Bruker AC-300 (300.13 MHz) or, when noted, a Varian EM-360 (60 MHz) instrument. ^{31}P NMR spectra were performed on either a JEOL FX90Q spectrometer (36.2 MHz) or a Bruker AC-300 (121.49 MHz). ^{13}C NMR spectra were obtained on the Bruker AC-300 (75.47 MHz). Assignments of ^{13}C resonances were made from ^{1}H–^{13}C shift correlation NMR experiments. The assignments of configuration at C2 could be unambiguously assigned from the values of $\Delta\delta$ for the two acetonide methyl groups (*5, 15, 16*). Phosphorus was determined in some samples as phosphate according to the method of Ames (*17*); generally the procedure was modified to monitor the chromatography of **9** and **15** in the following way: 40 μl of a fraction was incubated with 0.6 ml of Ames reagent at 45°C. Elemental analyses were performed by Galbraith Laboratories. Chlorine was determined in one sample by neutron activation analysis. All reactions that required anhydrous conditions were performed under a positive pressure of dry N_2. Orotate PRTase (mixed with orotidine 5′-phosphate decarboxylase in the approximate ratio 1 : 10, respectively) from baker's yeast was obtained from Sigma Chemical Co.

*2,5-Anhydro-1-deoxy-1-(dimethoxyphosphinyl)-2,3-O-isopropylidine-6-O-trityl-*D*-altro-hexitol* (**4**). To a suspension of sodium hydride (0.555 g, 23.1 mmol) in dry glyme (30 ml) was added with stirring a solution of tetramethyl methylenebisphosphonate (5.35 g, 23.1 mmol) in dry glyme (30 ml) dropwise for 15 min at room temperature. After the addition was complete and the evolution of hydrogen had ceased, the resulting solution was allowed to stir for 10 min, whereupon a solution of 2,3-*O*-isopropylidene-5-*O*-trityl-D-ribofuranose (9.53 g, 22.0 mmol) in glyme (20 ml) was added all at once. The reaction was allowed to proceed for 60 min. At this time, TLC (EtOAc/$CHCl_3$, 3/2) showed that the starting material had disappeared. The reaction was quenched with saturated NaH_2PO_4 (200 ml) and the glyme removed under reduced pressure. The aqueous residue was extracted with CH_2Cl_2 (3 × 50 ml). The combined extracts were dried (Na_2SO_4) and the solvent removed under reduced pressure to give 11.5 g of a pale yellow viscous oil. Chromatography of this material over silica gel (EtOAc) yielded three fractions. The fastest moving fraction (4.69 g) corresponded to the desired D-*altro* isomer **4**, the slowest moving fraction (1.2 g) to the D-*allo* isomer (β anomer), and the middle fraction

(3.50 g) to a mixture of these. The middle fraction was rechromatographed as before to yield an additional 2.59 g of **4** (total yield 73% of theory) and 0.95 g of the D-*allo* compound (total yield 19%). This compound has also been described by Meyer *et al.* (*6*).

2,5-Anhydro-1-deoxy-1-dimethoxyphosphinyl-2,3-O-isopropylidine-D-altro-hexitol (**5**). To a solution of **4** (9.01 g, 16.7 mmol) in MeOH (70 ml) was added 1.0 g Pd/C. The mixture was stirred under 1 atm H_2 until uptake ceased (4.5 h) at 100% of theory. The reaction mixture was filtered and the catalyst washed with EtOH (50 ml). Solvent was removed under diminished pressure to yield 9.0 g of residue that contained crystalline Ph_3CH. This material was used without purification in the next step. ^1H NMR: δ 1.28 (3H, s, CH_3-), 1.49 (3H, s, CH_3-), 2.15 (2H, dd, P-CH_2-), 3.68 ("dd," 6H, -OCH_3), 3.8-4.5 (6H, sugar envelope), 7.2 (Ph_3CH).

2,5-Anhydro-1-deoxy-1-dimethoxyphosphinyl-6-diphenoxyphosphinyl-2,3-O-isopropylidine-D-altro-hexitol (**6**). The semicrystalline residue from the previous step was dissolved in anhyd pyridine (20 ml) and the solvent removed *in vacuo*. This procedure was repeated with anhyd pyridine (2 × 10 ml). Dry ether (75 ml) was added followed by anhyd pyridine (5.30 g, 65 mmol) and the resulting solution was stirred while a solution of diphenyl chlorophosphate (8.82 g, 32.8 mmol) in dry ether (30 ml) was added dropwise for 30 min. The reaction mixture was allowed to stand at room temperature for 24 h. The precipitated pyridinium chloride was filtered off and washed with dry ether. The ether was removed under reduced pressure and the residue stirred with H_2O (10 ml). All volatiles were removed under high vacuum. Chromatography (EtOAc/IPA, 10/1) of the residue on silica gel yielded 7.8 g (87%) of a clear oil. ^1H NMR (60 MHz, CCl_4): δ 1.20 and 1.39 (3 H each s, -CH_3), 2.04 (2 H, m, CH_2P), 3.48 and 3.64 (3 H each, s, -OCH_3), 3.87-4.64 (6 H, m, H-2,3,4,5,6,6′), 7.14 (10 H, s, Ph). *Anal.* Calcd for $C_{23}H_{30}O_{10}P_2$: C, 52.27; H, 5.68; P, 11.74. Found: C, 51.46; H, 5.85; P, 11.92.

The D-*allo* epimer, **13**, was prepared in a like manner from the D-*allo* epimer of **4** (see above). ^1H NMR (60 MHz, CCl_4): δ 1.22 and 1.45 (3 H each, s, -CH_3), 2.00 (2 H, m, C-1), 3.48 and 3.65 (s, 3 H each, -OCH_3), 3.77-4.50 (6 H, m, H-2,3,4,5,6,6′), 7.05 (10 H, s, Ph). *Anal.* Calcd for $C_{23}H_{30}O_{10}P_2$: C, 52.27; H, 5.68; P, 11.74. Found: C, 51.49; H, 5.97; P, 11.90.

2,5-Anhydro-1-deoxy-6-diphenoxyphosphinyl-1-((hydroxyphenoxyphosphinyl)oxy)hydroxyphosphinyl-2,3-O-isopropylidine-D-altro-hexitol (**8**). To a stirred solution of **6** (4.65 g, 9.7 mmol) in dry CCl_4 (40 ml) was added dropwise via syringe $(CH_3)_3SiBr$ (3.93 g, 25.7 mmol). The resulting solution was allowed to stand for 16 h. All volatiles were removed under high vacuum. The residue was dissolved in methanol (10 ml) and the solvent removed *in vacuo* to give 4.28 g of the desired *altro* phosphonic acid in quantitative yield. Aqueous tetrabutylammonium hydroxide (27.4 ml, 0.391 M, 9.7 mmol) was then added and the solution was evaporated to dryness. The resulting monotetrabutylammonium salt, **7**, was dried by repeated evaporation with dry (distilled from CaH_2 and passed over activated alumina) CH_3CN (4 × 10 ml). The material from this reaction was used in the next step.

Disodium phenyl phosphate dihydrate (3.36 g, 13.20 mmol in water, 10 ml) was converted to the free acid by passage over a column of Dowex 50 (H^+ form).

Aqueous effluent was collected until the pH reached 5.5. The acidic solution was evaporated *in vacuo* and the crystalline residue (phenyl dihydrogen phosphate) was dried by evaporation with dry CH_3CN (3 × 15 ml). The solid phosphoric acid ester was dissolved in dry (passed over activated alumina) DMF (7.0 ml) and carbonyl diimidazole (2.15 g, 13.2 mmol) was added. The addition was accompanied by vigorous gas (CO_2) evolution. The resulting solution was allowed to stir at 45°C for 45 min whereupon a solution of the monotetrabutylammonium salt of **7** (4.28 g, 9.7 mmol) in dry DMF (5 ml) was added with stirring. The reaction was allowed to proceed at 45°C for 18 h whereupon the solvent was removed *in vacuo* and the resulting pale yellow gum was dissolved in water (100 ml) and passed over a column containing Dowex 50 (NH_4^+ form). The material was eluted with water until the pH of the efflucent was neutral. The eluate was lyophilized to give 7.52 g of a pale brown solid. The solid was dissolved in $CH_3CN/H_2O/NH_3$, 100/2/1 (15 ml), and chromatographed over silica gel, eluting with a gradient of $CH_3CN/H_2O/NH_3$, 100/0/1–80/20/1 (2.5 liter), to give, after evaporation of the CH_3CN and lyophilization, 3.89 g (61%) of **8** as an amorphous solid that was pure by ^{1}H-decoupled ^{31}P NMR (50% D_2O/50% MeOH): δ +12.36 (d, $J_{P,P'}$ = 26.49 Hz, phosphinyl P), −12.27 (s, phosphate ester), −16.66 (d, $J_{P,P'}$ = 26.49 Hz, phosphonyl P).

2,5-Anhydro-1-deoxy-6-dihydroxyphosphinyl-1-((dihydroxyphosphinyl)oxy)hydroxyphosphinyl-2,3-O-isopropylidine-D-altro-hexitol (**9**). A solution of **8** (1.70 g, 2.43 mmol) in 50% aqueous methanol (15 ml) was adjusted to pH 1.6 with 60% $HClO_4$ and PtO_2 (0.50 g) and 10% Pt/C (0.50 g) were added. The mixture was stirred at 22°C under 1 atm H_2 until uptake ceased (2.0 h). The catalyst was removed by filtration and washed with water. The filtrate was adjusted to pH 7.2 with concd NH_3 and loaded onto a column of Dowex 1-X8 (205 ml, HCO_3^- form, 200–400 mesh); the column was washed with H_2O (1 liter) and then eluted with a linear gradient (0–0.4 M, 1 liter) of aqueous NH_4HCO_3 and 25-ml fractions were collected. Phosphorus (*17*) appeared in fractions 24–31 (orthophosphate), 39–41 (**10** + **11**[2]), and 42–48 (**9**). The fractions containing **9** were evaporated *in vacuo* and then evaporated several times with methanol (4 × 25 ml) to give **9** (652 mg) as a white powder. This residue was dissolved in methanol and precipitated as the sodium salt from $NaClO_4$/acetone as described by Hoard and Ott (*18*). The resulting hygroscopic powder was absolutely pure by ^{13}P NMR and gave the expected resonances (D_2O, ^{1}H-decoupled): δ + 11.24 (d, $J_{P,P'}$ = 25.64 Hz, phosphinyl P), +2.38 (s, phosphate ester), −8.52 (d, $J_{P,P'}$ = 25.64 Hz, phosphonyl P). ^{1}H NMR (D_2O, relative to TMS-propionate): δ 1.40 (3H, s, CH_3-), 1.53 (3H, s, H_3C-), 2.00 (1H, ddd, -CH**H**-P), 2.19 (1H, ddd, -C**H**H-P), 3.92 (2H, br m, PO-CH_2-), 4.23 (1H, br m, C(5)H), 4.47 (br m, 1H, C(2)H), 4.9 (2H, C(3)H and C(4)H); assignments were determined by the COSY experiment. ^{1}H-decoupled ^{13}C NMR (vs TMS-propionate): δ 15.49 and 16.84 (2 acetonide -CH_3), 19.72 (d, C1, J_{CP} = 138.6 Hz), 55.85 (C6), 68.95 (d, C2, J_{CCP} = 27.4 Hz), 73.24 (d, C3, J_{CCCP} = 7.8 Hz), 73.74 (C4), 74.13 (d, C5, J_{CCOP} = 7.4 Hz), 104.37 (ketal).

An analytical sample was obtained by trituration in methanol and recovery of ~50% of the finely powdered residue, which gave the following analytical data: *Anal.* Calcd for $C_9H_{14}O_{13}P_3Na_{3.5}H_{1.5} \cdot 9H_2O$ (this amount of H_2O was confirmed

approximately by ^{1}H NMR of the solid dissolved in D_2O): C, 16.20; H, 5.06; P, 13.93; Na, 12.06. Found: C, 15.90; H, 4.59; P, 14.22; Na, 12.08.

*2,5-Anhydro-1-deoxy-2,3-di-O-benzyl-1-dimethoxyphosphinyl-*D*-manno- and -*D*-gluco-hexitol* (**17**). A mixture of epimers **16** was prepared essentially as previously described (*7*). A solution of **16** (10.99 g, 20.9 mmol) in 50 ml of Ac_2O under nitrogen was treated with 30 drops of concd H_2SO_4. An aliquot of this solution was transferred to an NMR tube and spectra were recorded at 2-h intervals. The reaction was judged to be complete (4.5 h at room temperature) when the integrated areas of the benzylic protons (of the forming benzyl acetate) and the aromatic protons in the NMR spectrum were approximately in the theoretical ratio 2:15. Excess Ac_2O was decomposed by pouring the reaction onto 300 g of ice with stirring overnight. Acetic acid was removed by evaporation of the reaction mixture with water (2 × 300 ml) under reduced pressure. The aqueous residue was extracted with CH_2Cl_2 (4 × 20 ml) and dried (Na_2SO_4), and all volatiles were removed, first under aspirator and then under high vacuum at 40–50°C. Crude yield of the 6-acetoxy intermediate was 10.48 g. A small amount was chromatographed over silica gel (EtOAc) to yield an analytical sample (^{1}H NMR ($DCCl_3$): δ 2.05-2.07 (3H, two s, $CH_3C(O)$), 2.25 (2H, m, $-CH_2P$), 3.72 (6H, m, $-POCH_3$), 4.20 (10H, br m, sugar envelope and $-CH_2Ph$), 7.35 (10H Ph). ^{1}H-decoupled ^{31}P NMR ($CDCl_3$): δ +31.52 (s) and +30.28 (s)).

The crude material was dissolved in absolute methanol (75 ml), 15 drops of concd H_2SO_4 were added, and the solution was heated under reflux for 3.5 h at which time TLC indicated that the starting acetate(s) was no longer present. The reaction was quenched with 5% Na_2HPO_4 (200 ml), the methanol removed under reduced pressure, and the residue extracted with CH_2Cl_2 (3 × 20 ml). The combined extracts were dried (Na_2SO_4), and the solvent was removed under reduced pressure to give 9.08 g (99%) of a mixture of the D-*gluco* and D-*manno* epimers of **17,** obtained as a viscous colorless oil. A sample chromatographed over silica gel (EtOAc: IPA, 9:1) gave ^{1}H NMR ($CDCl_3$): δ 2.03 (2H, m, $-CH_2P$), 3.72 (6H, m, $-POCH_3$), 4.71 (10H, br m, sugar envelope and $-CH_2Ph$), 7.33 (10H, Ph). ^{1}H-decoupled ^{31}P NMR ($CDCl_3$): δ +30.61 (s) and +32.11 (s).

*2,5-Anydro-1-deoxy-2,3-di-O-benzyl-1-(dimethoxyphosphinyl)-6-diphenoxyphosphinyl-*D*-manno- and -*D*-gluco-hexitol* (**18**). A solution of **17** (3.84 g, 8.81 mmol) in dry toluene (20 ml) was dried by evaporation of the solvent under vacuum. The process was repeated with 2 × 12-ml portions of anhyd pyridine (passed over basic-activated alumina). The residue was dissolved in a mixture of dry THF (50 ml) and dry pyridine (0.92 ml, 11.4 mmol). Diphenyl chlorophosphate (3.08 g, 11.4 mmol) in dry THF (15 ml) was added dropwise with stirring. After the addition was complete, the reaction was allowed to stand under N_2 for 24 h. Water (10 ml) was added and the reaction mixture stirred for 1 h. THF was removed under reduced pressure and the aqueous residue extracted with ether (3 × 50 ml). The combined ether extracts were dried (Na_2SO_4) and all volatiles were removed at reduced pressure. Chromatography of the residue on silica gel (EtOAc/iPrOH, 6/1) yielded 5.25 g (98%) of a mixture of the D-*manno* and D-*gluco* epimers. ^{1}H NMR ($CDCl_3$): δ 2.03 (2H, m, $-CH_2P$), 3.72 (6H, m, $-OCH_3$) 4.71 (10H, br m, sugar envelope and $-CH_2Ph$), 7.34 (20H, br m, Ph). ^{1}H-decoupled ^{31}P NMR ($CDCl_3$): δ +30.22 and +31.53 (two s, phosphonyl P), −11.63 (phosphoryl P).

2,5-Anhydro-1-deoxy-2,3-di-O-benzyl-6-diphenoxyphosphinyl-1-((hydroxyphenoxyphosphinyl)oxy)hydroxyphosphinyl-D-manno- and D-gluco-hexitol (**20**). To a stirred solution of **18** (4.07 g, 5.3 mmol) in dry CCl_4 (30 ml) was added dropwise via syringe $(CH_3)_3SiBr$ (2.28 g, 14.9 mmol). The resulting solution was allowed to stand under N_2 for 16 h. All volatiles were removed under high vacuum. The residue was dissolved in methanol (10 ml) and the solvent removed *in vacuo* to give a mixture of the desired D-*manno* and D-*gluco* phosphonic acids. This mixture was converted to the monotetrabutylammonium salt, **19,** by adding 1 eq of aqueous tetrabutylammonium hydroxide and drying as described for compound **7.**

Phenyl imidazolylphosphate (5.50 mmol) was prepared as described for the synthesis of **8.** A portion of compound **19** (3.07 g, 3.86 mmol) in dry DMF (5.0 ml) was added with stirring. The reaction was allowed to proceed at 45°C for 18 h. The solvent was removed *in vacuo* and the resulting pale yellow gum was dissolved in water (100 ml) and passed over a column containing Dowex 50 (NH_4^+ form). The material was eluted with water until the pH of the effluent was neutral. The eluate was lyophilized to give 4.10 g of a pale brown solid. The solid was dissolved in $CH_3CN/H_2O/NH_3$, 100/2/1 (15 ml), and chromatographed over silica gel, eluting with a gradient of $CH_3CN/H_2O/NH_3$, 100/0/1–80/20/1 (2.5 liter), to give, after lyophilization, 2.97 g (107%) of an amorphous solid that was shown by ^{31}P NMR (50% D_2O/50% methanol) to be a mixture of the α and β isomers: δ +13.76 (d, J = 27 Hz, phosphonyl anhydride P of minor isomer), +12.57 (d, J = 27 Hz, phosphonyl anhydride of major isomer), −12.34 (phosphate ester of minor isomer), −12.27 (phosphate ester of major isomer), −16.30 (d, J = 27 Hz, phosphoryl anhydride of minor isomer), −16.39 (d, J = 27 Hz, phosphoryl anhydride of major isomer). ^1H NMR ($DCCl_3$): δ 2.05 (2H, m, $-CH_2P$), 4.01 (10H, bm, sugar envelope and $-CH_2Ph$), 7.15 (25 H, Ph).

2,5-Anhydro-1-deoxy-6-dihydroxyphosphinyl-1-((dihydroxyphosphinyl)oxy)hydroxyphosphinyl-D-manno-hexitol (**15**) *and hydrolysis products.* To a solution of **20** (1.74 g, 2.16 mmol) in glacial AcOH (25 ml) was added 10% Pd/C (500 mg). The resulting suspension was stirred under H_2 (1 atm) until the uptake of H_2 ceased at 52 ml (30 min). The catalyst was filtered off and washed with AcOH (10 ml). Pt/C (10%, 0.50 g) and PtO_2 (10%, 0.50 g) were added to the filtrate and the reaction mixture was again stirred under H_2 (1 atm). Again uptake of H_2 was rapid and ceased at 300 ml. The reaction mixture was filtered and the catalyst washed with H_2O (20 ml). All volatiles were removed *in vacuo*, and then the residue was adjusted to pH 9.0 with aqueous ammonia and loaded onto a Dowex 1-X8 column (300 ml, HCO_3^- form, 200–400 mesh), which was then washed with water (1 liter) and eluted with a linear gradient of NH_4HCO_3 (1 liter, 0–0.4 M) followed by 0.4 M NH_4HCO_3 (0.5 liter) and 0.8 M NH_4HCO_3 (0.5 liter); 50-ml fractions were collected. Phosphorus (*17*) appeared in fractions 15–18 and 24–35 in varying intensities. Appropriate fractions were further characterized by ^{31}P NMR and, if appropriate, ^1H and ^{13}C NMR. Fractions 15–18 were found to consist of orthophosphate and 22–23 were the pure D-*gluco* phostone **22.** [^1H NMR (D_2O): δ 1.93 (1H, q, -CHHP), 2.08 (1H, dt, -CHHP), 3.94 (3H, m, $-CH_2OP$ and C5(H)), 4.23 (1H, bd, C4(H)), 4.53 (1H, t, C3(H)), 4.92 (1H, bm, C1(H)). ^1H-decoupled ^{31}P NMR (D_2O): δ +43.77 (s, phostone P) and +3.48 (s, phosphoryl P). ^{13}C NMR (D_2O): δ 87.36 (d,

C3, J_{POC} = 8.4 Hz), 87.11 (d, C5, J_{POCC} = 8.1 Hz), 82.23 (d, C2, J_{POC} = 2.7 Hz), 80.07 (d, C4, J_{POCC} = 7.7 Hz), 66.77 (d, C6, J_{POC} = 4.5 Hz), 29.90 (d, C1, J_{CP} = 140.4 Hz). Assignments of ^1H and ^{13}C resonances were confirmed by ^1H COSY and ^{31}P–^1H and ^{13}C–^1H shift correlation NMR experiments.] Fractions 24–26 consisted of a mixture of phostone **22** and its hydrolysis product(s) **21** (*7*). Fractions 27–28 showed the presence of phostone **22,** hydrolysis product(s) **21,** and the desired **15.** Finally fractions 29–35 were found to consist of pure compound **15.** Appropriate fractions were combined and concentrated under reduced pressure and NH_4HCO_3 was removed by repeated evaporation of the residue(s) with abs. EtOH. The material in fractions 29–35 was converted to the Na salt by the procedure of Hoard and Ott (*18*) and found to be pure by ^1H, ^{13}C, and ^{31}P NMR but determined by elemental analysis (27.0% Na, 5.2% P, 5.7% C, 38.0% Cl) to be about 30% compound **15** and the rest NaCl (due to incomplete removal of Cl^- from the Dowex resin during the regeneration cycle) which coprecipitated with the desired compound. Partial purification was accomplished by cation exchange (Dowex 50) to the corresponding lithium salt(s) followed by evaporation *in vacuo* and trituration with acetone, which selectively dissolved much of the LiCl. ^1H NMR (D_2O): δ 2.19 (2H, dd, -CH_2P, J_{HCP} = 18.0 Hz, J_{HCCH} = 6.4 Hz), 3.95 (2H, m, -CH_2OP), 4.00 – 4.22 (4H, m, C2(H), C3(H), C4(H), C5(H)). ^{13}C NMR (D_2O): δ 29.85 (C1, J_{CP} = 136.0 Hz), 61.70 (d, C6, J_{POC} = 5.1 Hz), 73.96 (s, probably C4), 76.07 (s, C2), 78.16 (d, C3 or C5, J = 7.2 Hz) 78.60 (d, C3 or C5, J = 7.9 Hz). ^{13}P NMR (D_2O): δ +11.80 (d, phosphonyl, J_{POP} = 25.64 Hz), +0.97 (s, phosphoryl at C6), −10.24 (d, phosphoryl anhydride, J_{POP} = 25.64 Hz). *Anal.* Calcd for $C_6H_{10}O_{13}P_3Li_{3.5}H_{1.5} \cdot 6H_2O \cdot 2.1$ LiCl: C, 11.89; H, 3.91; P, 15.33; Li, 6.41; Cl, 12.29. Found: C, 11.67; H, 3.44; P, 14.00; Li, 6.15; Cl, 12.28. Chloride was also present in substantial amounts in fractions 22–28. Again, partial purification was achieved by the ion-exchange method just described.

Deacetonidation of **6.** To a solution of the *altro* phosphonate-phosphate **6** (7.00 g, 13.2 mmol) in 60% aqueous MeOH (20 ml) was added 10 g of Dowex 50 (H^+ form). The reaction mixture was allowed to stand for 14 days at room temperature at which time TLC indicated that the starting material was no longer present. The resin was filtered off and the filtrate was evaporated to a thick syrup under reduced pressure to give 5.84 g (100%) of product. The ^1H NMR spectrum (60 MHz, $CDCl_3$) was in accord with the structure of **12** or its phostone (no isopropylidine methyl protons, no ester -OMe groups): δ 2.24 (2H, dd, -CH_2P), 4.42 (6H, m, H-2,3,4,5,6) 7.25 (15H, bs, -Ph).

Deacetonidation of **13.** To a solution of **13** (1.05 g, 2.0 mmol) in 60% aqueous MeOH (20 ml) was added 2 g of Dowex 50 (H^+ form). The reaction mixture was allowed to stand for 10 days at room temperature at which time TLC indicated that the starting material was no longer present. The resin was filtered off and the filtrate was evaporated to a thick syrup under reduced pressure to give 0.96 g (99%) of product. The NMR spectrum was in accord with the structure of compound **14** (no isopropylidine methyl protons and a 6H doublet for 2 -OMe groups). ^1H NMR (60 MHz, $CDCl_3$): δ 2.05 (2H, dd, -CH_2P), 3.62 and 3.75 (3H, s, -OCH_3), 4.12 (4H, m, H-2,3,4,5), 4.75 (2H, bs, -CH_2OP), 7.25 (15H, bs, -Ph).

Hydrolysis of compounds **9** *and* **15.** These experiments were performed at pH

4.0 in a 50 mM sodium formate buffer which contained 50% D_2O, 1 mM EDTA, and 0.01% NaN_3 essentially as described previously (*2*). At measured times the samples were subjected to inverse-gated ^1H-decoupled ^{31}P NMR, using a sufficient relaxation delay to allow integration. Under these conditions compound **9** ($\delta = +13.25$, $J = 25$ Hz; $\delta = +0.18$; $\delta = -10.62$, $J = 25$ Hz) gave rise to a phosphonate ($\delta = +20.41$), a phostone ($\delta = +45.11$), and another phosphonate anhydride ($\delta = +12.76$, $J = 25$ Hz), which was taken to indicate the formation of a small amount of ephemeral compound **3;** integration values for the phostone and phosphonate (relative to the total for both anhydride phosphonyl Ps) were used to quantitate the degree of P-O-P bond breakage. Under the same conditions compound **15** ($\delta = +12.96$, $J = 25.3$ Hz; $\delta = +0.48$; $\delta = -10.67$, $J = 25.3$ Hz) gave rise exclusively to a phosphonate ($\delta = 20.23$); integration values for the phosphonate (relative to the anhydride phosphonyl P) were used to quantitate the degree of P-O-P bond breakage.

Enzyme assays. Orotate PRTase was assayed spectrophotometrically according to the method of Umezu *et al.* (*19*) in the presence of a 10-fold excess of OMP decarboxylase activity. Reaction mixtures contained 40 mM tris(hydroxymethyl)aminomethane buffer, 5 mM MgCl, 200 μM orotate, varying amounts of PRPP, and **9** or **15** as indicated in Fig. 2. The actual concentrations of PRPP in assays were determined in runs which contained a large excess of orotate and the enzymes. Actual concentrations of either **9** or **15** were estimated by phosphorus determinations (*17*). Data were analyzed by fitting to the BMDP nonlinear regression analysis on a VAX 11/785 computer. Sets of data were tested for competitive and noncompetitive inhibition models. The K_i and K_m values reported were extracted from these statistical analyses using the competitive model, which gave the best fit (significantly lower sum of squares of residuals) for both **9** and **15.**

ACKNOWLEDGMENTS

This work was supported by grants from the American Cancer Society, National Institutes of Health (R 23 CA 33659 and 39530), and the Medical Research Foundation of Oregon and by funds from a NIH BRSG grant. We thank the Oregon Graduate Center for making their JEOL NMR spectrometer available to us.

REFERENCES

1. MURRAY, A. W., WONG, P. C. L., AND FRIEDRICHS, B. (1969) *Biochem J.* **112,** 741–746.
2. MCCLARD, R. W., FISCHER, A. C., MAULDIN, S. K., AND JONES, M. E. (1984) *Bioorg. Chem.* **12,** 339–348.
3. SMITHERS, G. W., AND O'SULLIVAN, W. J. (1984) *Biochemistry* **23,** 4767–4773.
4. SMITHERS, G. W., AND O'SULLIVAN, W. J. (1984) *Biochemistry* **23,** 4773–4778.
5. MCCLARD, R. W. (1983) *Tetrahedron Lett.* **24,** 2631–2634.
6. MEYER, R. L., STONE, T. E., AND JESHTI, P. K. (1984) *J. Med. Chem.* **27,** 1095–1098.
7. MCCLARD, R. W., TSIMIKAS, T., AND SCHRIVER, K. E. (1986) *Arch. Biochem. Biophys.* **245,** 282–286.
8. WESTHEIMER, F. H. (1987) *Science* **235,** 1173–1178.

9. FAHTI, R., AND JORDAN, F. (1988) *J. Org. Chem.* **53,** 1997–2001.
10. EBY, R., SONDHEIMER, S. J., AND SCHUERCH, C. (1979) *Carbohydr. Res.* **73,** 273–276.
11. VICTOR, J., GREENBERG, L. B., AND SLOAN, D. L. (1979) *J. Biol. Chem.* **254,** 2647–2655.
12. DODIN, G. (1981) *FEBS Lett.* **134,** 20–24.
13. GUTENSOHN, W. (1977) *Adv. Exp. Med. Biol.* **76A,** 586–590.
14. TUTTLE, J. V., KRENITSKY, T. A., AND ELION, G. B. (1983) *Biochem. Pharmacol.* **32,** 3011–3015.
15. OHRUI, H., JONES, G. H., MOFFATT, J. G., MADDOX, A. T., CHRISTENSEN, A. T., AND BYRAM, S. K. (1975) *J. Amer. Chem. Soc.* **97,** 4602–4613.
16. SECRIST, J. A. (1978) *J. Org. Chem.* **43,** 2925–2927.
17. AMES, B. N. (1966) *in* Methods in Enzymology (Neufeld, E. F., and Ginsburg, V., Eds.), Vol. 8, pp. 115–118, Academic Press, San Diego.
18. HOARD, D. E., AND OTT, D. G. (1965) *J. Amer. Chem. Soc.* **87,** 1785–1788.
19. UMEZU, K., AMAYA, T., YOSHIMOTO, A., AND TOMITA, K. (1971) *J. Biochem.* **70,** 249–267.