(Dpg)$_2$-O-*t*-Bu, 87113-51-7; Tfa-(Dpg)$_3$-O-*t*-Bu, 92844-91-2; Tfa-(Dpg)$_4$-O-*t*-Bu, 87112-71-8; Tfa-(Dpg)$_5$-O-*t*-Bu, 87112-73-0; H-L-Nva-OMe·HCl, 56558-30-6; H-(L-Nva)$_2$-OMe·HCl, 92844-92-3; H-(L-Nva)$_3$-OMe·HCl, 79780-94-2; H-(L-Nva)$_4$-OMe·HCl, 92844-93-4; H-(L-Nva)$_5$-OMe·HCl, 92900-57-7; Tfa-Aib-OH oxazolone, 705-20-4; H-(Aib)-O-*t*-Bu, 4512-32-7; H-(Aib)$_3$-O-*t*-Bu, 92844-94-5; H-(Aib)$_4$-O-*t*-Bu, 92844-95-6; trifluoroacetic anhydride, 407-25-0; chloroform, 67-66-3; *N*-methylmorpholine, 109-02-4; acetonitrile, 75-05-8; ethyl thiotrifluoroacetate, 383-64-2; trifluoroacetic acid, 76-05-1; acetic anhydride, 108-24-7.

^{18}O Isotope Effect in ^{13}C Nuclear Magnetic Resonance Spectroscopy. 9. Hydrolysis of Benzyl Phosphate by Phosphatase Enzymes and in Acidic Aqueous Solutions[1,2]

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Abstract: The ^{18}O isotope-induced shifts in ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectroscopy were used to establish the position of bond cleavage in the phosphatase-catalyzed and acid-catalyzed hydrolysis reactions of benzyl phosphate. The application of the ^{18}O-isotope effect in NMR spectroscopy affords a continuous, nondestructive assay method for following the kinetics and position of bond cleavage in the hydrolytic process. The technique provides advantages over most discontinuous methods in which the reaction components must be isolated and converted to volatile derivatives prior to analysis. In the present study, [α-^{13}C,*ester*-^{18}O]benzyl phosphate and [*ester*-^{18}O]benzyl phosphate were synthesized for use in enzymatic and nonenzymatic studies. Hydrolysis reactions catalyzed by the alkaline phosphatase from *E. coli* and by the acid phosphatases isolated from human prostate and human liver were all accompanied by cleavage of the substrate phosphorus–oxygen bond consistent with previously postulated mechanisms involving covalent phosphoenzyme intermediates. An extensive study of the acid-catalyzed hydrolysis of benzyl phosphate at 75 °C revealed that the site of bond cleavage is dependent on pH. At pH $\leq$1.3, the hydrolysis proceeds with C–O bond cleavage; at 1.3 < pH < 2.0, there is a mixture of C–O and P–O bond scission, the latter progressively predominating as the pH is raised; at pH $\geq$ 2.0, the hydrolysis proceeds with exclusive P–O bond scission. (*S*)-(+)-[α-^2H]Benzyl phosphate was also synthesized. Hydrolysis of this chiral benzyl derivative demonstrated that the acid-catalyzed C–O bond scission of benzyl phosphate proceeds by an A-1 (S_N1) mechanism with 70% racemization and 30% inversion at carbon.

The hydrolysis reactions of organic esters of phosphoric acid are of considerable chemical, biological, and technical importance in chemistry and enzymology.[3,4] The hydrolysis of a phosphate monoester (R–O–PO_3H_2) involves the scission of a bond to oxygen: either the P–O bond of the phosphate moiety or the C–O bond to the substituent (R) group. The position of bond cleavage, therefore, can be of key importance in studying the reaction mechanism.

The hydrolysis reactions of (primary) monoalkyl phosphate esters have been studied most extensively under three sets of experimental conditions: as the monoanion, as the neutral species in dilute acid, and in solutions of strong acid where further protonation of the neutral ester may occur. Most esters exhibit a similar reaction pattern under these conditions.[5-7] The pH-rate profile for hydrolysis of the monoanion generally shows a simple kinetic form with a rate maximum at pH 4–5; only P–O bond scission is observed for the monoanion. In solutions of acid to 1 M, the hydrolysis of the neutral species proceeds by C–O bond scission exclusively (at least within the often large limits of experimental error for isotopic analysis). In strong acid solutions ($\geq$1 M), hydrolysis of the conjugate acid of the neutral species can occur with the simultaneous operation of more than one mechanism such that both C–O and P–O bond cleavage patterns are found. Concise mechanistic explanations for many of these experimental observations are still lacking, and additional experimental evidence would be valuable.

One phosphomonoester where such data are of particular interest is benzyl phosphate. The acid-catalyzed hydrolysis of benzyl phosphate at 75 °C was initially studied by Kumamoto and Westheimer.[8] They found that the hydrolysis pH-rate profile differed markedly from that exhibited by simple alkyl monoesters.[5-7] In particular, whereas the rate of hydrolysis of simple alkyl monoesters, such as glycerol or ethyl phosphate,[9] generally displays a maximum at pH 4, the rate of hydrolysis of benzyl phosphate remains constant in the pH range 2–5. Tentative conclusions regarding the hydrolysis reaction of benzyl phosphate have been presented: (1) in strong acid, C–O bond cleavage is "probably" observed,[6] which would result in the formation of the moderately stable carbonium ion; (2) the neutral phosphate ester may (or must)[6,8] cleave at the C–O bond; and (3) the phosphate monoester monoanion was assumed[6] to display P–O bond cleavage by formation of a metaphosphate ion. These conclusions were based on the rate data, on the properties of the benzyl group,[10] and on extrapolations from the hydrolysis of other phosphate monoesters, but direct experimental evidence has not been presented to support or to refute these assertions. Moreover, conclusions about the involvement of metaphosphate ion in the hydrolysis of typical phosphate monoesters have been called into question as the result of a study of the stereochemistry of alcoholysis reactions.[11] A thorough understanding of the benzyl

(1) Part 8: Risley, J. M.; Van Etten, R. L.; Uncuta, C.; Balaban, A. T. *J. Am. Chem. Soc.* **1984**, *106*, 7836-7840.

(2) Taken in part from the M.S. thesis of J. E. Parente, Purdue University, 1982. Preliminary communications of portions of this work have been presented: (a) Parente, J. E.; Risley, J. M.; Van Etten, R. L. *Proc. Indiana Acad. Sci.* **1981**, *91*, 178. (b) Risley, J. M.; Parente, J. E.; Van Etten, R. L. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1982**, *41*, 906. (c) Van Etten, R. L. *Ann. N.Y. Acad. Sci.* **1982**, *390*, 27-51.

(3) Bruice, T. C.; Benkovic, S. J. "Bioorganic Mechanisms"; Benjamin: New York, 1966; Vol. 2, Chapter 5.

(4) Benkovic, S. J.; Schray, K. J. *Enzymes* **1970**, *8*, 201-238.

(5) Bunton, C. A. *Acc. Chem. Res.* **1970**, *3*, 257-265.

(6) Cox, J. R., Jr.; Ramsay, O. B. *Chem. Rev.* **1964**, *64*, 317-352.

(7) Barnard, P. W. C.; Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Silver, B. L.; Vernon, C. A. *Chem. Ind. (London)* **1955**, 760-763.

(8) Kumamoto, J.; Westheimer, F. H. *J. Am. Chem. Soc.* **1955**, *77*, 2515-2518.

(9) Bailly, M. C. *Bull. Soc. Chim.* **1942**, *9*, 314-339, 340-350. Desjobert, A. *Bull. Soc. Chim.* **1947**, *14*, 809-812.

(10) Hammett, L. P. "Physical Organic Chemistry"; McGraw-Hill: London, 1940; p 154. Lowry, T. H.; Richardson, K. C. "Mechanism and Theory in Organic Chemistry"; Harper & Row: New York, 1976; p 179.

0002-7863/84/1506-8156$01.50/0

phosphate hydrolysis problem remains elusive. Consequently, we sought to obtain relevant experimental evidence about the hydrolysis of benzyl phosphate by utilizing the ^{18}O isotope effect in ^{13}C NMR[12] and ^{31}P NMR.[13] [α-^{13}C,*ester*-^{18}O]Benzyl phosphate and [*ester*-^{18}O]benzyl phosphate were hydrolyzed at 75 °C in aqueous solutions containing perchloric acid. The products, benzyl alcohol and inorganic phosphate, were analyzed for their isotopic composition by using the ^{18}O-isotope-induced shift in ^{13}C and ^{31}P NMR spectroscopy. The reaction was further examined by utilizing a chiral [^2H]benzyl phosphate.

The experimental demonstration of the point of bond cleavage in an enzyme-catalyzed reaction is important in order to deduce the mechanism of the enzyme. Early studies with glucose 1-phosphate and phenyl phosphate demonstrated P–O bond fission in hydrolysis reactions catalyzed by alkaline phosphatase[14,15] and by human prostatic acid phosphatase.[14,16] The studies were done with preparations of crude enzymes and the products were analyzed indirectly by mass spectrometry. We decided to examine the scissile bond in reactions of benzyl phosphate catalyzed by acid phosphatase enzymes using homogeneous enzyme preparations from human prostate and from human liver; alkaline phosphatase from *Escherichia coli* was chosen as a standard because it is such a well-characterized enzyme.[17] The tracer was ^{18}O, and the reactions were analyzed by ^{13}C NMR and ^{31}P NMR using the ^{18}O-isotope-induced shift.[12,13] Either an ^{18}O-labeled substrate and normal water or ^{18}O-enriched water and unlabeled substrate may be employed in the design of such experiments. Both protocols were utilized in this study and the simultaneous evaluation of a number of properties of these reactions is demonstrated: course of the reaction, point of bond cleavage, and transphosphorylation.

Experimental Section

Crystalline phosphoric acid (MCB, Inc.), [^{18}O]water (98 atom % excess ^{18}O, normalized, Norsk Hydro, Oslo), [α-^{13}C]benzoic acid (90 atom % α-^{13}C, Merck), potassium [$^{18}O_4$]phosphate (monobasic),[18] deuterium oxide (99.75 atom % ^2H, Baker), deuteriochloroform (99.8 atom % ^2H containing 1% Me_4Si, Aldrich), tris[3-((heptafluoropropyl)-hydroxymethylene)-*d*-camphorato]europium(III) ($Eu(hfc)_3$, Aldrich), and doubly distilled, deionized water were used. All other reagents were analytical or spectrometric grade. Alkaline phosphatase (type III: bacterial (*Escherichia coli*), Sigma) (orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1) was used without further purification. The four esters of bis(cyclohexylammonium) benzyl phosphate (vide infra) were synthesized by a slight modification of a published procedure.[19] The ^{18}O isotopic enrichment was quantitated by ^{13}C or ^{31}P NMR.

Synthesis of Bis(cyclohexylammonium) Benzyl Phosphate. Benzyl alcohol (30 mmol), crystalline phosphoric acid (6 mmol), and triethylamine (12 mmol) were stirred until the acid dissolved. Trichloroacetonitrile (30 mmol) was added and the reaction was stirred at room temperature for 4 h. Excess trichloroacetonitrile was removed under vacuum, and the residue was diluted with water (1.5 mol) and extracted with ether. (The ether extract contains the excess benzyl alcohol, from which the alcohol may be recovered.) To the aqueous solution is added cyclohexylamine (60 mmol). The salt of the ester may be isolated by either of two methods: (1) lyophilization and recrystallization from water: acetone (1:10) or (2) acetone (approximately 1.2 mol) is added to the aqueous solution at 0 °C and the salt precipitates on standing at 4 °C overnight, after which it is collected, washed with acetone, and dried in a desiccator. Typical yields by isolation method 1 were 33 and 36%, and by method 2 were 52 and 53%: mp 227–229 °C (lit. 233 °C). Anal. Calcd for $C_{19}H_{35}N_2O_4P\cdot H_2O$: C, 56.39; H, 9.20. Found: C, 56.39; H, 9.19.

Synthesis of Bis(cyclohexylammonium) [*ester*-^{18}O]Benzyl Phosphate. Benzaldehyde diethyl acetal was prepared from benzaldehyde and triethylorthoformate[20,21] and hydrolyzed with [^{18}O]water. The [^{18}O]-benzaldehyde was reduced with sodium borohydride[22] to [^{18}O]benzyl alcohol. The bis(cyclohexylammonium) [*ester*-^{18}O]benzyl phosphate contained 30% ^{18}O.

Synthesis of Bis(cyclohexylammonium) [α-^{13}C,*ester*-^{18}O]Benzyl Phosphate. The [α-^{13}C,^{18}O]benzyl alcohol was synthesized in five steps from [α-^{13}C]benzoic acid in an overall yield of 30%. [α-^{13}C]Benzoyl chloride was distilled from an equimolar mixture of [α-^{13}C]benzoic acid and phosphorus pentachloride, and was reduced to [α-^{13}C]benzaldehyde with lithium tri-*tert*-butoxyaluminohydride.[23] [α-^{13}C]Benzaldehyde diethyl acetal[20,21] was hydrolyzed in [^{18}O]water and was reduced[22] to [α-^{13}C,^{18}O]benzyl alcohol (90 atom % α-^{13}C, 30 atom % ^{18}O). [^{18}O]-Benzyl alcohol was added to give [α-^{13}C,^{18}O]benzyl alcohol containing 17 atom % α-^{13}C, which was used to synthesize bis(cyclohexylammonium) [α-^{13}C,*ester*-^{18}O]benzyl phosphate (17 atom % α-^{13}C, 30 atom % *ester*-^{18}O).

Synthesis of Bis(cyclohexylammonium) (*S*)-(+)-[α-^2H]Benzyl Phosphate. (*S*)-(+)-[α-^2H]Benzyl alcohol (70% ee) was a gift from Prof. M. Mark Midland of the University of California, Riverside. It was synthesized by using a chiral trialkylborane reducing agent.[24] Bis(cyclohexylammonium) (*S*)-(+)-[α-^2H]benzyl phosphate (0.42 g) was synthesized in 52% yield from 1.0 mL of the alcohol as already described for the ^{18}O-labeled ester.

Enzyme Purification. Acid phosphatase enzymes (orthophosphoric-monoester phosphohydrolase (acid optimum); EC 3.1.3.2) were purified to homogeneity from human prostate[25] and from human liver.[26] Protein concentration was measured by the Lowry method[27] using bovine serum albumin as the standard. Enzyme activity was measured with *p*-nitrophenyl phosphate as the substrate at 25 °C in sodium acetate or Tris buffer. The specific activities of the enzymes were human prostatic acid phosphatase 286 units/mg, human liver acid phosphatase 76 units/mg, and *E. coli* alkaline phosphatase 38 units/mg.

Enzyme-Catalyzed Hydrolysis Reactions. A solution of bis(cyclohexylammonium) benzyl phosphate (0.25 mmol) in 40% [^{18}O]water, 20% deuterium oxide buffered with tris(hydroxymethyl)aminomethane (pH 8.0) (total volume 5.0 mL) was equilibrated at 18 °C; the reaction was initiated by the addition of *E. coli* alkaline phosphatase (0.05 mg) and was followed by ^{31}P NMR. To a solution of bis(cyclohexylammonium) [α-^{13}C,*ester*-^{18}O]benzyl phosphate (0.25 mmol) in 20% deuterium oxide buffered with sodum acetate (pH 5.0) (total volume 5.0 mL) and equilibrated at 18 °C was added a quantity of acid phosphatase enzyme and the reaction was followed by ^{13}C NMR. Bis(cyclohexylammonium) benzyl phosphate (0.25 mmol) was dissolved in a solution of sodium acetate (pH 5.0), 40% [^{18}O]water, 20% deuterium oxide, EDTA (0.1 mmol) (total volume 5.0 mL) and equilibrated at 18 °C; acid phosphatase enzyme was added and the reaction was followed by ^{31}P NMR. Addition of human prostatic acid phosphatase (0.05 mg) caused the complete hydrolysis of bis(cyclohexylammonium) (*S*)-(+)-[α-^2H]benzyl phosphate (0.125 mmol) in sodium acetate (pH 5.0), μ = 0.15 M with sodium chloride (total volume 5.0 mL) at 25 °C; the hydrolysis product [α-^2H]benzyl alcohol was isolated and analyzed by ^1H NMR as subsequently described. For all experiments, control reaction mixtures were also prepared with [α-^{13}C,^{18}O]benzyl alcohol, potassium [$^{18}O_4$]phosphate, and benzyl phosphate; in the absence of native enzymes no changes in the isotopic compositions of these compounds were detected.

Aqueous Acid-Catalyzed Hydrolysis Reactions. A solution of perchloric acid (1.04 M) was standardized with tris(hydroxymethyl)-

(11) Buchwald, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1982**, *104*, 1438–1440.
(12) Part 1: Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 252–253.
(13) Cohn, M.; Hu, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 200–203. Lutz, O.; Nolle, A.; Staschewski, D. *Z. Naturforsch., A* **1978**, *33*, 380–382.
(14) Cohn, M. *J. Biol. Chem.* **1949**, *180*, 771–781.
(15) Stein, S. S.; Koshland, D. E., Jr. *Arch. Biochem. Biophys.* **1952**, *39*, 229–230.
(16) Bunton, C. A.; Silver, B. L.; Vernon, C. A. *Proc. Chem. Soc.* (*London*) **1957**, 348–349.
(17) Coleman, J. E.; Chlebowski, J. F. *Adv. Inorg. Biochem.* **1979**, *1*, 1–66. Coleman, J. E.; Gettins, P. *Adv. Enzymol.* **1983**, *55*, 381–452. McComb, R. B.; Bowers, G. N., Jr.; Posen, S. "Alkaline Phosphatase"; Plenum Press: New York, 1979. Reid, T. W.; Wilson, I. B. *Enzymes* **1970**, *4*, 373–415. Wyckoff, H. W.; Handschumacher, M.; Murthy, H. W. K.; Sowadski, J. M. *Adv. Enzymol.* **1983**, *55*, 453–480.
(18) Risley, J. M.; Van Etten, R. L. *J. Labelled Compds. Radiopharm.* **1978**, *15*, 533–538.
(19) Cramer, F. D.; Weimann, G. *Chem. Ind.* (*London*) **1960**, *46*.
(20) Fife, T. H.; Jao, L. K. *J. Org. Chem.* **1965**, *30*, 1492–1495.
(21) Part 5: Risley, J. M.; DeFrees, S. A.; Van Etten, R. L. *Org. Magn. Reson.* **1983**, *21*, 28–35.
(22) Vogel, A. "Textbook of Practical Organic Chemistry"; Longman: London, 1978.
(23) Brown, H. C.; Subba Rao, B. C. *J. Am. Chem. Soc.* **1958**, *80*, 5377–5380.
(24) Midland, M. M.; Greer, S.; Tramontano, A.; Zderic, S. A. *J. Am. Chem. Soc.* **1979**, *101*, 2352–2355.
(25) Van Etten, R. L.; Saini, M. S. *Clin. Chem.* **1978**, *24*, 1525–1530.
(26) Saini, M. S.; Van Etten, R. L. *Arch. Biochem. Biophys.* **1978**, *191*, 613–624.
(27) Bailey, J. L. "Techniques in Protein Chemistry"; Elsevier: Amsterdam, 1967; pp 340–341.

aminomethane as a primary standard and this perchloric acid solution was used as a stock to prepare all subsequent solutions. Adjustments to pH were made with sodium hydroxide (2 M). All hydrolysis reactions were run at 75 °C. In 20% deuterium oxide was added bis(cyclohexylammonium) [*ester*-^{18}O]benzyl phosphate (0.125 mmol) (total volume 5.0 mL). Following hydrolysis, the solution was neutralized with sodium hydroxide (2 M) and EDTA (0.1 mmol) was added prior to ^{31}P NMR analysis. Bis(cyclohexylammonium) [α-^{13}C,*ester*-^{18}O]benzyl phosphate (0.125 mmol) was dissolved in 20% deuterium oxide at pH 1.9 (total volume 5.0 mL); prior to ^{13}C NMR analysis the solution was neutralized with sodium hydroxide (2 M). The product, [α-^{2}H]benzyl alcohol, from the hydrolysis of bis(cyclohexylammonium) (*S*)-(+)-[α-^{2}H]benzyl phosphate (0.125 mmol) (total volume 5.0 mL) was isolated as follows: the solution was cooled to room temperature, neutralized with sodium hydroxide (2M), saturated with sodium chloride, and extracted twice with ethyl ether. The ether extracts were dried over magnesium sulfate (anhydrous), the ether was removed under vacuum, and the residual alcohol was taken up into deuteriochloroform for ^{1}H NMR analysis.

NMR Measurements and Data Analysis. An NTC-200 spectrometer operating at 50.31 MHz was fitted with a 12-mm probe equilibrated at 18 °C for ^{13}C NMR analysis. Sweep widths of ±4000 and ±500 Hz (quadrature phase detection), a 90° pulse angle, data blocks of 32K and 16K, broad-band proton decoupling, and recycling times of 2.6 to 21.5 s were used. A line-broadening factor (0.1–1.0 Hz) was applied to the accumulated FID.

An NTC-200 spectrometer operating at 80.9 MHz was fitted with a 20-mm probe equilibrated at 18 °C for ^{31}P NMR analysis. A 12-mm NMR tube with a vortex plug was used. A sweep width of ±250 Hz (quadrature phase detection), a 90° pulse angle, an 8K data block, and recycling times of 11.2 to 23.2 s were used. Broad-band proton-decoupled and proton-coupled spectra were taken. A line-broadening factor was applied to the accumulated FID.

An NTC-470 spectrometer operating at 469.9 MHz was fitted with a 5-mm probe for ^{1}H NMR analysis. A ±5000 Hz sweep width (quadrature phase detection), a 90° pulse angle, a 32K data block, and varying acquisition times were used. A line broadening factor was applied to the accumulated FID. Spectra were taken of the [α-^{2}H]benzyl alcohol before and after the addition of the chiral NMR shift reagent, Eu(hfc)$_3$, using a 0.5–0.6 mol ratio of shift reagent to alcohol.

Quantitative spectral analysis data were obtained by deconvoluting the NMR spectra using a Nicolet curve resolving program. The relative concentration (as a percentage) of each species present was calculated from the measured area under each peak of interest; the error is ±3%. The error in the calculated enantiomeric excess (ee) is ±6%.

Results and Discussion

The synthesis of (mono)benzyl phosphate is surprisingly difficult, and only the reaction of crystalline phosphoric acid in the presence of trichloroacetonitrile and excess benzyl alcohol[19] gave satisfactory results. In order to facilitate the acquisition and evaluation of the data by ^{13}C NMR, α-^{13}C-enriched benzyl phosphate was synthesized. Upon substitution of ^{18}O for *ester*-^{16}O, the ^{13}C NMR signal of the benzyl carbon atom in benzyl phosphate is shifted upfield 0.024 ppm (±0.001 ppm) and in benzyl alcohol the upfield shift is 0.019 ppm (±0.001 ppm); the ^{31}P NMR signal is shifted upfield 0.016 ppm (±0.001 ppm) in the ester and 0.020 ppm (±0.001 ppm) in [^{18}O]phosphate ion.

Enzyme-Catalyzed Hydrolysis Reactions. The hydrolysis reaction of benzyl phosphate in [^{18}O]water buffered with 1.5 M Tris as catalyzed by *E. coli* alkaline phosphatase is illustrated in Figure 1. ^{31}P NMR spectra were recorded at regular intervals during the reaction and the stacked plot is generated from the spectra recorded at hourly intervals. The phosphate ester is hydrolyzed with scission of the P–O bond. Upon hydrolysis, the ^{18}O label from the solvent is incorporated into the product phosphate ion at the same isotopic enrichment as the solvent. Figure 1 also illustrates a concurrent reaction, transphosphorylation, catalyzed by the enzyme. In the presence of the 1.5 M Tris buffer, the enzyme catalyzes the transfer of the phosphate group from the substrate to the buffer via the serine phosphoenzyme characteristic of alkaline phosphatase. The ratio of hydrolysis to transphosphorylation illustrated in Figure 1 is 4 to 1. If the concentration of the Tris buffer is lowered to 0.1 M, then the hydrolysis reaction is readily observed but transphosphorylation does not occur to a detectable degree. Phosphate (oxygen)–water exchange is catalyzed by this enzyme,[28] but under the reaction conditions

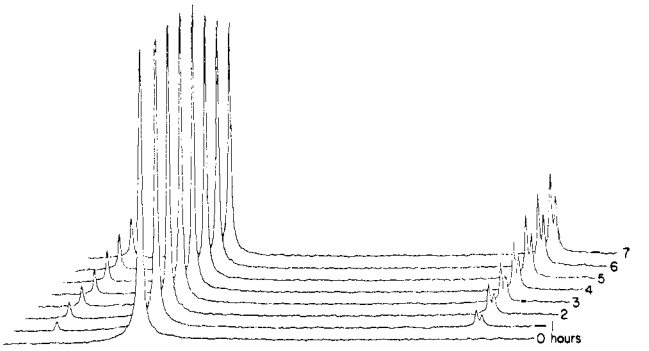


Figure 1. The P–O bond of benzyl phosphate is broken in the transphosphorylation and hydrolysis reactions catalyzed by alkaline phosphatase from *E. coli*. The ^{31}P NMR spectra acquired during the incubation of the enzyme with benzyl phosphate in 1.5 M Tris buffer in 40% [^{18}O]water at 18 °C show resonance signals (left to right) for Tris-phosphate, benzyl phosphate, and inorganic [$^{16}O_4$]- and [$^{16}O_3{}^{18}$O]phosphate. Upon hydrolysis, one ^{18}O is incorporated from the solvent into the hydrolysis product, inorganic phosphate, at the same isotopic composition as the water; the isotope-induced shift is 0.020 ppm upfield. The ratio of hydrolysis to transphosphorylation is 4 to 1.

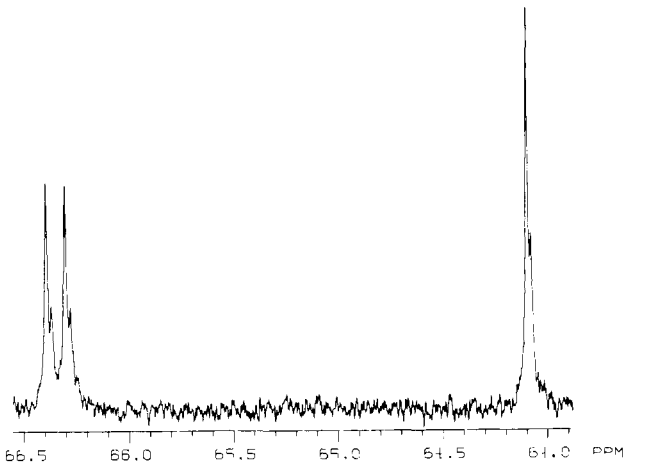


Figure 2. ^{13}C NMR spectrum showing that the ^{18}O isotopic composition of the substrate, [α-^{13}C,*ester*-^{18}O]benzyl phosphate, and the product, [α-^{13}C,^{18}O]benzyl alcohol, are the same following hydrolysis catalyzed by homogeneous human prostatic acid phosphatase. The reaction occurs with P–O bond cleavage. This ^{13}C NMR spectrum of the hydrolysis reaction mixture was obtained at 50% hydrolysis and shows the phosphate ester at 66.35 ppm ($^{2}J_{P-C}$ = 4.5 Hz) and the alcohol at 64.10 ppm. The ^{18}O isotope shifts are 0.024 ppm upfield in the ester and 0.019 ppm upfield in the alcohol.

used here it does not occur to an observable degree. Simultaneously with the bond-cleavage determination, additional characteristics of the enzyme-catalyzed reaction could be established: the course of the reaction, a transphosphorylation reaction not involving the intermediate formation of free phosphate ion, and the ratio of hydrolysis to transphosphorylation. Modifying the experimental parameters would allow further evaluation of the phosphate (oxygen)–water exchange reaction and of the phosphorylation reaction catalyzed by the enzyme.

Upon incubation of [α-^{13}C,*ester*-^{18}O]benzyl phosphate with homogeneous human prostatic acid phosphatase, [α-^{13}C,^{18}O]benzyl alcohol and inorganic phosphate ion are formed, consistent with hydrolysis occurring via cleavage of the P–O bond; the ^{18}O enrichment of the benzyl alcohol is the same as in the ester substrate (Figure 2). An independent confirmation of P–O bond scission was obtained by using ^{31}P NMR and the same sample. Further verification was obtained from the complementary reaction involving unlabeled benzyl phosphate in [^{18}O]water with the enzyme. (The phosphate (oxygen)–water exchange reaction[29] is much too

(28) Bock, J. L.; Cohn, M. *J. Biol. Chem.* **1978**, *253*, 4082–4085. Eargle, D. H., Jr.; Licko, V.; Kenyon, G. L. *Anal. Biochem.* **1977**, *81*, 186–195.

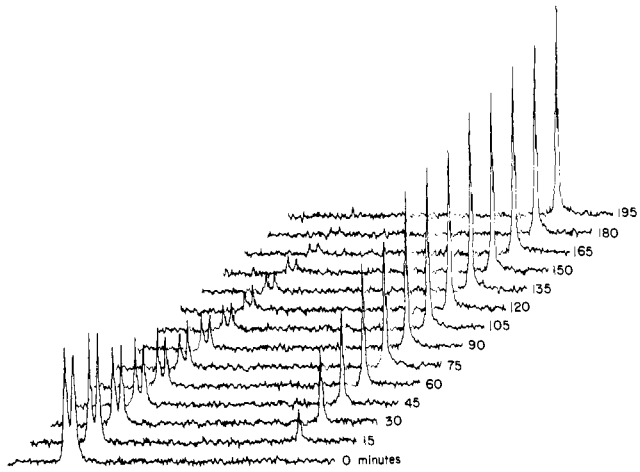


Figure 3. The incubation of [α-^{13}C,*ester*-^{18}O]benzyl phosphate with a homogeneous isoenzyme of human liver acid phosphatase[22] proceeds with P–O bond cleavage to yield [α-^{13}C,^{18}O]benzyl alcohol of the same isotopic composition. The ^{13}C NMR spectra were recorded at the indicated times during the reaction and show the ^{13}C resonance signals corresponding to the phosphate ester ($^2J_{P-C}$ = 4.5 Hz) and the alcohol.

slow and could not be observed under the conditions of the present experiments.) The enzyme was saturated in these reactions (K_m = 0.5 mM with a V_{max} = 501 μmol min^{-1} mg^{-1}). The analysis of the alcohol isolated from the enzyme-catalyzed hydrolysis reaction of (*S*)-(+)-[α-^{2}H]benzyl phosphate (discussed below) showed no change in the chirality (the ee remained 70% *S*), as would be expected for a reaction involving P–O bond scission and proceeding via a phosphoenzyme[2c] intermediate formed by attack at phosphorous.[30]

In order to determine the site of bond cleavage in the hydrolysis reaction which was catalyzed by a homogeneous isoenzyme of human liver acid phosphatase,[26] products from the reaction of [α-^{13}C,*ester*-^{18}O]benzyl phosphate were analyzed by ^{13}C NMR (and by ^{13}P NMR utilizing the same sample in order to confirm the ^{13}C NMR observation), as well as for the complementary reaction system involving benzyl phosphate in [^{18}O]water plus enzyme. The enzyme catalyzes the hydrolysis reaction by P–O bond fission as illustrated in Figure 3. The isotopic composition of the product, [α-^{13}C,^{18}O]benzyl alcohol, does not change during the incubation.

Thus *E. coli* alkaline phosphatase, human prostatic acid phosphatase, and an isoenzyme of human liver acid phosphatase all catalyze the hydrolysis of benzyl phosphate by fission of the P–O bond and the ^{18}O isotope effects on ^{13}C and ^{31}P NMR permit the convenient, direct analyses of such reactions. This is the first application of the two complementary NMR analyses in the determination of the site of bond cleavage. In addition to the delineation of the scissile bond, information on other aspects of the reactions such as reaction rates may be obtained simultaneously. The ^{18}O isotope effect in ^{31}P NMR has been used to demonstrate that alkaline phosphatase from calf intestine and acid phosphatase from potato catalyze the hydrolysis of α-D-ribofuranose 1-[$^{18}O_4$]phosphate by P–O bond fission,[31] whereas purine nucleoside phosphorylase from calf spleen[32] and from human erythrocytes and *Escherichia coli*[33] catalyze the hydrolysis of α-D-ribofuranose 1-[$^{18}O_4$]phosphate by C–O bond fission. In terms of isotope cost, instrument accessibility, and much higher precision

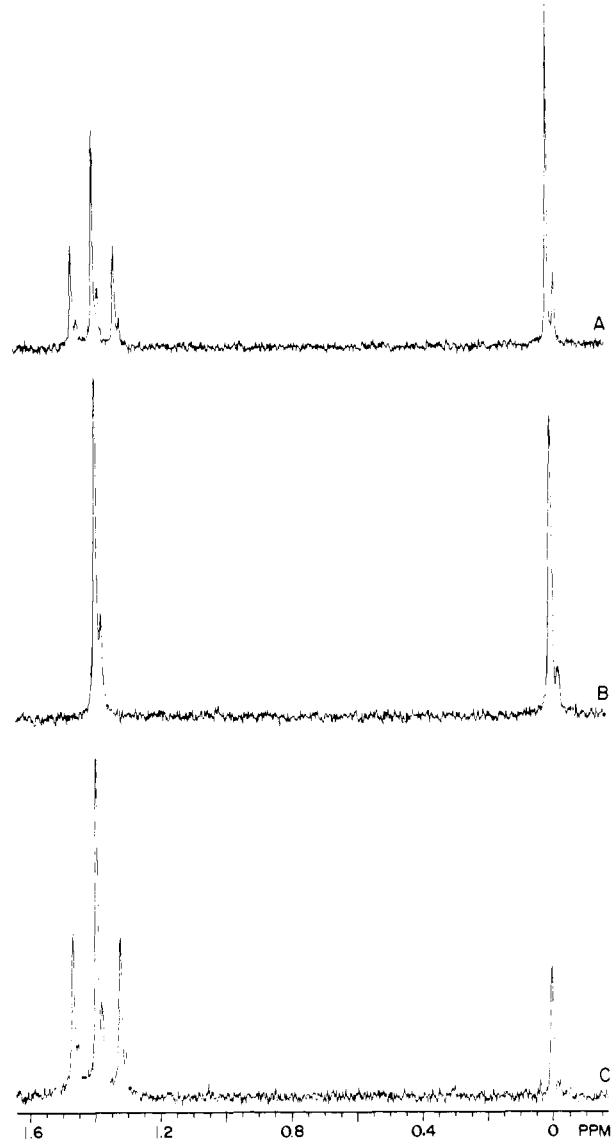


Figure 4. ^{31}P NMR spectra illustrating the hydrolysis reactions of [^{18}O]benzyl phosphate in acidic aqueous solutions. The ^{18}O isotope shifts are 0.016 ppm upfield in the phosphate ester and 0.020 ppm upfield in inorganic phosphate. (A) Following reaction in 1 M perchloric acid, the ^{18}O isotopic composition of [^{18}O]benzyl phosphate and inorganic [$^{18}O^{16}O_3$]phosphate are the same, indicative of C–O bond fission. The NMR signals correspond to proton-coupled [^{18}O]benzyl phosphate ($^3J_{P-H}$ = 5.5 Hz) and inorganic [^{18}O]phosphate. (B) Following reaction at pH 1.9, the ^{18}O isotopic composition of the inorganic phosphate is approximately 50% that of the starting phosphate ester, resulting from concurrent P–O and C–O bond fission. The ^{31}P NMR signals correspond to proton-decoupled [^{18}O]benzyl phosphate and inorganic [^{18}O]phosphate. (C) The absence of an ^{18}O isotope shift in the ^{31}P NMR signal of the inorganic phosphate product indicates that the hydrolysis reaction at pH 2.0 proceeds by P–O bond cleavage. The NMR signals correspond to proton-coupled [^{18}O]benzyl phosphate ($^3J_{P-H}$ = 5.9 Hz) and inorganic phosphate.

of quantitation, these techniques offer significant advantages over the use of ^{17}O NMR, although that technique has been used to establish P–O bond fission in the hydrolysis reaction of *p*-nitrophenyl phosphate catalyzed by a metal ion-containing acid phosphatase from sweet potato tubers.[34]

Hydrolysis in Acidic Aqueous Solutions. After incubation at 75 °C for varying lengths of time, the perchloric acid-containing reaction mixtures were cooled to room temperature and neutralized with aqueous sodium hydroxide. Both proton-coupled and proton-decoupled ^{31}P NMR spectra of each solution were obtained.

(29) Van Etten, R. L.; Risley, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4784–4787. Van Etten, R. L.; Saini, M. S. *Arch. Biochem. Biophys.* **1982**, *219*, 155–162.
(30) Saini, M. S.; Buchwald, S.; Van Etten, R. L.; Knowles, J. *J. Biol. Chem.* **1981**, *256*, 10453–10455. Buchwald, S. L.; Saini, M. S.; Knowles, J. R.; Van Etten, R. L. *J. Biol. Chem.* **1984**, *259*, 2208–2213.
(31) Jordan, F.; Kuo, D. J.; Salamone, S. J.; Wang, A. L. *Biochim. Biophys. Acta* **1982**, *704*, 427–436.
(32) Jordan, F.; Patrick, J. A.; Salamone, S., Jr. *J. Biol. Chem.* **1979**, *254*, 2384–2386.
(33) Salamone, S. J.; Jordan, F.; Jordan, R. R. *Arch. Biochem. Biophys.* **1982**, *217*, 139–143.
(34) Kawabe, H.; Sugiura, Y.; Tanaka, H. *Biochem. Biophys. Res. Commun.* **1981**, *103*, 327–331.

Table I. Proportion of the Benzyl Phosphate Hydrolysis Reaction in Acidic Aqueous Solutions at 75 °C Occurring by C–O and by P–O Bond Scission

pH	time, h	% hydrolysis	bond cleavage, % C–O	P–O
(1 M)	3	100	100	
1.00	15	35	100	
1.30	100	89	100	
1.51	100	66	85	15
1.71	100	49	75	25
1.91	100	44	60	40
2.01	30	10		100
3.00	32	8		100
4.00	31	8		100

The ^{18}O isotopic enrichments in the ester and in the inorganic phosphate were quantitated and the extents of C–O bond fission and of P–O bond fission were calculated. The ^{31}P NMR spectra from the hydrolysis reaction in 1 M perchloric acid, at pH 1.9, and at pH 2.0 are shown in Figure 4 and the results of experiments are tabulated in Table I. These data indicate that at pH $\leq$1.3 hydrolysis of the benzyl phosphate ester proceeds exclusively by C–O bond fission; the ^{18}O label is clearly retained in the product inorganic phosphate at the same isotopic enrichment as in the starting ester. At 1.3 < pH < 2.0 there is concurrent C–O and P–O bond fission; as the pH is raised within this narrow range, progressively more P–O bond fission is observed (Table I). The reaction at pH $\geq$ 2.0 proceeds exclusively by P–O bond fission; the ^{18}O label is retained in the product, benzyl alcohol. These results were confirmed by incubation of [α-^{13}C,*ester*-^{18}O]benzyl phosphate in both 1 M perchloric acid and at pH 1.9, with subsequent analysis of the reaction mixtures by ^{13}C NMR. For the reaction conducted in 1 M acid, although an ^{18}O shift was observed in the phosphate ester, none was present in the product benzyl alcohol (that is, exclusive C–O bond fission occurred). In contrast, ^{18}O shifts were observed in the ^{13}C NMR signals of both the phosphate ester and the benzyl alcohol product obtained after hydrolysis at pH 1.9; the ^{18}O isotopic enrichment in the phosphate ester was approximately twice the enrichment in the benzyl alcohol (C–O *and* P–O bond fission).

Two control reaction mixtures of [α-^{13}C,*ester*-^{18}O]benzyl alcohol and potassium [$^{18}O_4$]phosphate were prepared to investigate the possibility of acid-catalyzed oxygen exchange between solvent and the hydrolysis reaction products. Upon analysis by ^{13}C NMR and by ^{31}P NMR, no change in the ^{18}O isotopic composition was detected during the time needed to conduct and to analyze the hydrolysis reactions.

Further details of the acid-catalyzed hydrolysis reaction were obtained by using benzyl phosphate synthesized from (*S*)-(+)-[α-^2H]benzyl alcohol (ee 70%). The chirality of the alcohol did not change during synthesis of the phosphate ester. The chiral alcohol was incubated in perchloric acid as a control reaction, and the hydrolysis reaction of the chiral phosphate ester catalyzed by human prostatic acid phosphatase also served as a control reaction. The ester was hydrolyzed in aqueous perchloric acid solutions under conditions of C–O bond fission, P–O bond fission, and concurrent C–O and P–O bond fission. Following incubation, the solutions were neutralized with sodium hydroxide solution and the [α-^2H]benzyl alcohol was isolated. The addition of Eu(hfc)$_3$ chiral shift reagent to a deuteriochloroform solution of [α-^2H]-benzyl alcohol, at a 0.5–0.6 mol ratio of shift reagent to alcohol, shifts the ^1H NMR signal of the benzylic proton of the *R* enantiomer of the chiral alcohol 0.15 ppm farther downfield compared to that of the *S* enantiomer.[35] The areas under the peaks were measured and the enantiomeric excess was calculated. The results of these experiments are listed in Table II. No change in the chirality of the alcohol is found under conditions where P–O bond scission occurs, or in the control reactions. Under conditions of

Table II. Enantiomeric Excess of the [α-^2H]Benzyl Alcohol Recovered from Hydrolysis of (*S*)-(+)-[α-^2H]Benzyl Phosphate

reaction	ee, %
[α-^2H]benzyl alcohol (control)	70 *S*
human prostatic acid phosphatase catalyzed	70 *S*
pH 2.50	70 *S*
pH 1.90	30 *S*
1.0 M perchloric acid	20 *R*

C–O bond cleavage the chirality of the [α-^2H]benzyl alcohol changes from 70% *S* to 20% *R*. At pH 1.9 where concurrent C–O and P–O bond fission is observed, the chirality of the alcohol changes from 70% *S* to 30% *S*.

In previous studies, considerable difficulties were often encountered in the isotopic analysis of the products of hydrolysis reactions in acidic aqueous solutions. Consequently, conclusions about the identity of the scissile bond were sometimes a matter of deductive reasoning rather than direct experimental evidence. Recently, this situation has been rectified by the use of new NMR methods. Thus, the ^{18}O-isotope effect in ^{31}P NMR was used[31] to determine that in 50% aqueous formic acid solution, the C–O bond of α-D-ribofuranose 1-[$^{18}O_4$]phosphate was cleaved in the hydrolysis reaction; the result was verified by mass spectrometry. As illustrated here, elucidation of the scissile bond is also conveniently done by utilizing the ^{18}O-isotope effect in ^{13}C NMR as well as that in ^{31}P NMR. Not only have we confirmed experimentally the correctness of assertions that in the hydrolysis of benzyl phosphate there is scission of the P–O bond during reaction of the monoanion and of the C–O bond during reaction of the neutral species in strong acid solutions but we were also able to show a definite transition region from C–O to P–O bond fission. Although concurrent bond cleavage reactions have been observed[5,6] in other phosphate ester hydrolysis reactions such as methyl phosphate in strong acid solutions, to our knowledge the hydrolysis reaction of benzyl phosphate between pH 1.3 and 2.0 is the first experimental demonstration of such a progression in the bond scission process.

A general mechanism proposed for the hydrolysis reaction of the monoanion of monoalkyl phosphate esters involving the formation of an intermediate monomeric metaphosphate ion was applied[8] to the monoanion of benzyl phosphate, with the additional criterion that the reactivity of the neutral species of benzyl phosphate was due specifically to the benzyl group, which forms stable carbocations and readily undergoes displacement reactions.[10] Although the involvement of an intermediate monomeric metaphosphate ion in the hydrolysis reactions of monoalkyl phosphate ester monoanions often seemed compelling, that dissociative mechanistic pathway may now be regarded as irrelevant, on the basis of a definitive study of the stereochemistry of the methanolysis of phosphate monoesters.[11] The methanolysis of two chiral phosphate monoester ions resulted in complete inversion of configuration at phosphorus. The results appear to preclude a dissociative mechanistic pathway and an alternate explanation involving a "preassociative" mechanistic pathway was proposed.[36]

Both ^{13}C NMR and ^{31}P NMR data clearly demonstrate C–O bond fission in the hydrolysis reaction of benzyl phosphate in strong (1 M) acid where the neutral species and its conjugate acid predominate. However, whether the benzyl group participates in the reaction as a carbocation or via a displacement reaction cannot be determined from the bond scission data. The mechanistic pathway for this reaction is more clearly revealed in the ^1H NMR studies conducted with the phosphate ester possessing a chiral benzyl group. Should the hydrolysis reaction of the *S* ester proceed by hydration of a carbocation intermediate (A-1, S_N1), a mixture of racemization and inversion of configuration of the alcohol (in any proportion) would be expected upon hydration.[37] On the other hand, complete inversion of the enan-

(35) Reich, C. J.; Sullivan, G. R.; Mosher, H. S. *Tetrahedron Lett.* **1973**, 1505–1508.

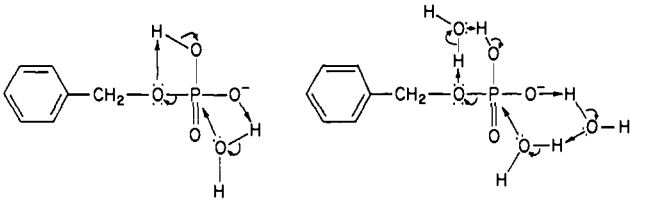
(36) Jencks, W. P. *Acc. Chem. Res.* **1980**, *13*, 161–169. Jencks, W. P. *Chem. Soc. Rev.* **1981**, *10*, 345–375.

(37) Ingold, C. K. "Structure and Mechanism in Organic Chemistry", 2nd ed.; Cornell University Press: Ithaca, NY, 1969; pp 520–531.

tiomeric ratio of the alcohol (to 70% *R* ee) would be expected if the reaction were to be a displacement reaction (S_N2) involving solvent attack at the benzyl carbon atom. We found a 20% *R* ee in [α-^{2}H]benzyl alcohol following hydrolysis in 1 M perchloric acid solution, which may be interpreted to mean that the benzyl phosphate ester is subjected to hydrolysis by an A-1 (S_N1) mechanism involving carbocation formation. The configuration of the product formed during reaction in 1 M $HClO_4$ at 75 °C is consistent with 70% racemization and 30% inversion.

The hydrolysis of benzyl phosphate in the region $1.3 < pH < 2.0$ is effected through concurrent C–O and P–O bond fission. As the pH is raised, progressively more ester P–O bond scission is observed with the transition half-way complete between pH 1.9–2.0. When chiral benzyl phosphate was hydrolyzed at a pH (1.9) where fission of the C–O bond and of the P–O bond were approximately equivalent, the isolated [α-^{2}H]benzyl alcohol had ee 30% *S*. An ee of 25% *S* may be calculated for a reaction with equivalent concurrent C–O and P–O bond fission. This calculation is based on the assumption that the reactions for scission of the C–O bond and of the P–O bond are independent and that the mechanism for C–O bond fission is A-1 (S_N1) involving 70% racemization and 30% inversion. This calculation is well within the experimental error and agrees with the experimentally measured value. This result implies that the bond cleavage reactions are independent and the mechanisms are preserved. The change in the scissile bond is quite dramatic, with the transition taking place within ±0.4 pH units of the pK_{a_1} (1.6) for the ester.

Thus, the experimental observations regarding the hydrolysis reactions of benzyl phosphate are accommodated in the following mechanisms. An A-1 (S_N1) mechanism—formation of a benzyl carbocation—operates in the hydrolysis of the neutral species and is accompanied by C–O bond scission. Formation of the conjugate acid of the neutral ester species in strongly acidic solution only enhances the reactivity of the benzyl group and renders the phosphate ester susceptible to hydrolysis by this mechanism. The hydrolysis mechanism involving C–O bond cleavage continues to be favored as the pH of the acidic solution passes through the pK_{a_1} of the benzyl phosphate ester. However, the monoanion of the ester undergoes hydrolysis by another mechanism. For the monoanion of benzyl phosphate, the hydrolysis reaction resulting in scission of the P–O bond proceeds by an intramolecular concerted general acid–general base mechanistic pathway. The same properties that enable the benzyl group to form a relatively stable carbocation and that facilitate its participation in displacement reactions also make the oxygen atom in the ester more basic, as does the formation of the monoanion of the phosphate ester, and thus the benzyl phosphate is subject to hydrolysis by this mechanism. Intramolecular concerted general acid–general base hydrolysis thus proceeds by the intramolecular proton transfer from the phosphate ester monoanion to the ester oxygen concerted with the phosphate ester monoanion-catalyzed attack by the solvent, water:



Hydrolysis by this mechanism is consistent with a proposed preassociative mechanistic pathway which results in inversion at the phosphorus atom.[11]

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Acyclic Stereoselection. 23. Lactaldehyde Enolate Equivalents†[1]

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Abstract: A number of lactate esters have been synthesized and stereochemistry of the reactions of their enolates with aldehydes examined. Dioxolanones **3** and **4** and oxazolanone **10** show low stereoselectivity (Table I). Methyl esters of various O-alkylated lactic acids (**17–19**) show generally higher stereoselectivity (Table II). Of these reagents, the best is methyl 2-methoxypropanoate (**17**), which shows exceedingly high selectivity with aliphatic aldehydes that are branched at C-2. For example, it gives a single adduct with isobutyraldehyde and pivalaldehyde and shows comparable simple diastereoselectivity with the chiral aldehydes **29** and **30**. Hindered aryl esters of *O*-benzyllactic acid (**37–39**) show complex behavior (Table III), with the sense and magnitude of stereoselectivity clearly being associated with the steric bulk of the aryl group (Table IV). The most useful member of this series of compounds is 2,6-di-*tert*-butyl-4-methylphenyl (butylated hydroxytoluene, BHT) *O*-benzyllactate (**39**), which gives only one isomer in its reactions with isobutyraldehyde and benzaldehyde. Ester **39** also shows useful stereoselectivity with chiral, β,γ-unsaturated aldehydes (**73** and **76**). The stereoselectivities observed in this study may be understood in terms of the transition-state models presented in Figure 2. It is argued on the basis of circumstantial evidence that the lactate esters give enolates of the *Z* configuration (eq 8 and 15). As shown in Figure 2, it is proposed that the dihedral angle between the carbonyl and enolate double bonds is approximately 90° and that the two stereoisomers in each case arise from transition states A and B. When the R″ group is small (methyl), then transition state A is preferred, leading to the sense of stereoselectivity shown by esters **17–19**. However, when R″ is large (BHT), transition state B predominates. The DMP and DIPP esters show intermediate behavior. The studies reported in this paper are the first that demonstrate aldol stereoselectivity with fully substituted enolates.

In previous papers in this series[3] we have outlined a strategy for the synthesis of macrolides and other polyketide natural products wherein the crucial carbon–carbon bond constructions would be made by stereoselective aldol addition reactions. In fact, an elegant synthesis of 6-deoxyerythronolide B (**1**), proceeding

† Dedicated to the memory of R. V. Stevens, 1941–1984.

(1) For part 22, see: Heathcock, C. H.; Kiyooka, S.-I.; Blumenkopf, T. A. *J. Org. Chem.*, in press.

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