Synthesis and Characterization of 3-Bromo-1,4-dihydroxy-2-butanone 1,4-Bisphosphate, a Potential Affinity Label for Enzymes That Bind Sugar Bisphosphates[1]

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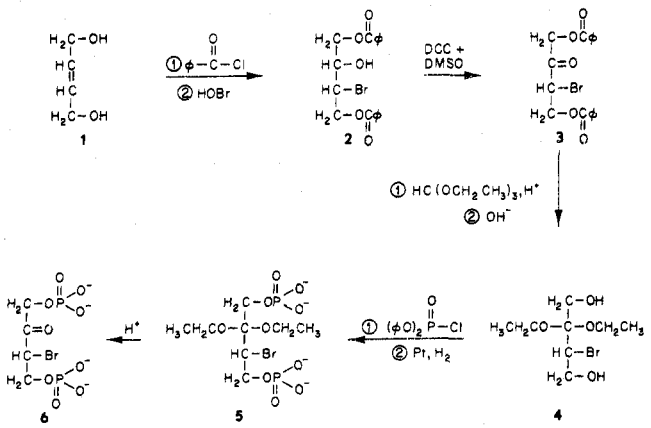
3-Bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, a compound that can be considered a reactive analog of several sugar bisphosphates, has been synthesized as a potential affinity labeling reagent for enzymes whose substrates are sugar bisphosphates. The starting material for the synthesis is the commercially available *cis*-2-butene-1,4-diol. The immediate precursor to the reactive reagent, the corresponding diethyl ketal, is obtained as a crystalline triscyclohexylammonium salt. The stability of 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate at pH 4–10 has been determined by measuring the release of both bromide and P_i. Even at pH 4.0 the reagent is unstable, and at pH 10.0 the release of bromide is complete within 3 hr. Studies with free amino acids, glutathione, and N-α-acetyl-L-lysine indicate that of the functional groups found in proteins, sulfhydryl is the most rapidly modified by bromobutanone bisphosphate. Modification of amino groups by the reagent has also been demonstrated, but at a much slower rate. The compounds resulting from the reactions of glutathione and N-α-acetyl-L-lysine have been reduced with tritiated sodium borohydride and hydrolyzed with 6 N HCl; the hydrolysates were analyzed on an amino acid analyzer to provide chromatographic markers for future studies involving bromobutanone bisphosphate as a protein reagent.

Affinity labeling is recognized as a powerful tool for the selective modification and subsequent characterization of the active sites of enzymes.[2] A major limitation of affinity labeling is the scarcity of reagents for any given enzyme; therefore, application of the technique usually necessitates designing reactive derivatives of the naturally occurring substrate. Reactive sugar bisphosphates should provide potentially versatile affinity labels, since there are a number of enzymes involved in carbohydrate metabolism whose substrates are sugar bisphosphates, e.g., phosphofructokinase, fructosebisphosphate aldolase, fructose bisphosphatase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglucomutase, phosphoglyceromutase, phosphoglycerate kinase, ribulosebisphosphate carboxylase, and phosphopentokinase. To begin the development of a series of reactive bisphosphates, I have synthesized Br-butanone-P_2[3] (**6**) according to Scheme I and studied its stability and reactivity toward functional groups that are present in proteins. In a preliminary study[4] this reagent proved useful as an affinity label for ribulosebisphosphate carboxylase.

Scheme I



The overall yield of **6** was only 11%, but the first three intermediates are readily prepared in large quantities. Since Br-butanone-P_2 is quite unstable and therefore was not isolated from solution, efforts were made to obtain the immediate precursor, the diethyl acetal (**5**), in a highly purified form. Thus, the material to be phosphorylated (**4**) was purified to homogeneity by chromatography on Florisil. This diol is unstable and will decompose (elimination of bromine with tarring) during concentrating in organic solvents. Decomposition does not take place if the temperature is kept at 30° or below during concentration and if exhaustive drying is avoided. The phosphorylated ketal (**5**) was obtained as a crystalline triscyclohexylammonium salt which was slightly contaminated with P_i. Elemental analyses of this compound and the corresponding lithium salt, which was freed of P_i, agreed closely with theory. The formation of a tris- instead of a tetracyclohexylammonium salt of a bisphosphate ester is not without precedent. D-Erythrulose 1,4-bisphosphate also yielded a triscyclohexylammonium salt.[5]

Several lines of evidence confirm the formation of **6** from the free acid of the corresponding ketal upon incubation in aqueous solution. (1) The pure ketal is converted to a new phosphate ester without substantial appearance of P_i or bromide. (2) The phosphate ester formed is a reducing sugar. (3) The phosphate ester formed contains both base-labile bromide and phosphate.

Incubation of Br-butanone-P_2 in base results in the liberation of 1.0 molar equiv of bromide and about 1.15 molar equiv of P_i (Table I). On the basis of the previous observation that D-erythrulose 1-P is much more sensitive than the 4-P isomer to degradation by alkali,[6] it is assumed that the phosphate group in the 1 position (adjacent to the carbonyl) is the one in Br-butanone-P_2 that is base-labile. In the case of erythrulose 4-P, only 50% of the total phsophate was released as P_i, and it was postulated that some phosphate ester was formed in alkali that could not undergo phosphate elimination.[6] A similar phenomenon could account for the release of slightly more than 1 molar equiv of P_i upon incubation of Br-butanone-P_2 in alkali. Both phosphate groups of the bromo reagent were hydrolyzed by 1 N H_2SO_4 at 100° at about equal rates (half-time of 12 min), as was observed for erythrulose 1,4-P_2.[5] Both phosphate groups of D-ribulose 1,5-P_2 were also reported to be acid labile,[7] as confirmed in the present study. However, in contradiction to the earlier report,[7] I found only one of the two phosphate groups to be hydrolyzed by base. Thus, three related bisphosphates (erythrulose 1,4-P_2, ribulose 1,5-P_2, and Br-butanone-P_2) have similar stabilities in acid and base.

Table I
Phosphate and Bromide Analyses on Br-butanone-P_2 and Related Compounds[a]

Compd	Concn, mM					
	By weight	Phosphate				Base-labile bromide
		P_i	Total organic[b]	Acid-labile[b]	Base-labile[b]	
Br-butanone-P_2	25.0	4.1	48.1	43.7[c,d]	27.0	23.4
Br-butanone-P_2 diethyl ketal	25.0	1.1	47.5	47.7[c,d]	0	0
Ribulose 1,5-P_2	25.0	10.8	41.2	40.8	20.5[c]	

[a] Analyses performed as described in Experimental Section. [b] These concentrations obtained after subtraction of the P_i concentration. [c] These values obtained after an incubation period of 2 hr. [d] The half-time of phosphate release during the incubation in acid was 12 min.

The kinetics of P_i and bromide formation from Br-butanone-P_2 at pH 4–10 are complex. It seems apparent from data in Figure 1 that the bromine atom increases the lability of one phosphate group. This conclusion was supported by the detection of tetrulose bisphosphate (a tentative identification based on chemical and chromatographic properties) as a decomposition product after the release of P_i had virtually ceased. It also appears that a phosphate group (see data for pH 6.0 and 8.0 in Figure 1) increases the lability of the bromine atom. This could be a reflection of an intramolecular displacement of bromide by a phosphate anion.

To determine which amino acid residues in proteins would be the most likely sites of modification by Br-butanone-P_2, we studied its reaction with free amino acids. These studies revealed sulfhydryl and amino groups as the most reactive and therefore the reactions of **6** with the sulfhydryl group of glutathione and the amino group of N-α-acetyl-L-lysine were investigated in some detail. Although the products of modification of glutathione and N-α-acetyl-L-lysine by **6** have not been completely characterized, the prime objective of tagging them with a radioactive label that survives conditions used to hydrolyze proteins was achieved by reduction of the carbonyl group with NaB^3H_4. Hydrolysates of the derivatized acetyllysine and glutathione were chromatographed on the amino acid analyzer (Figures 2 and 3). The established elution positions of the cysteinyl and lysyl derivatives will serve as markers in the determination of whether in a given protein sulfhydryl and ϵ-amino groups react with the reagent. Sulfhydryl and

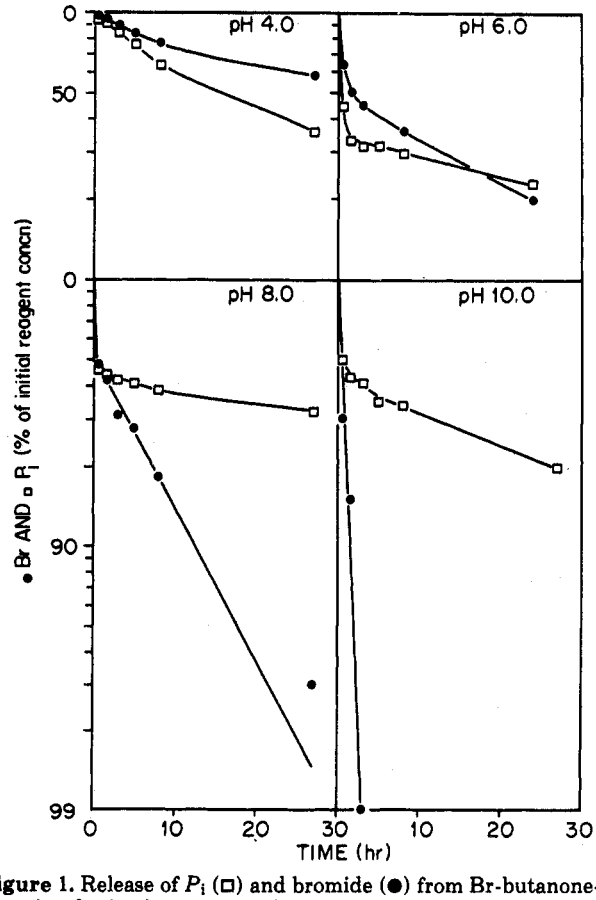


Figure 1. Release of P_i (□) and bromide (●) from Br-butanone-P_2 upon incubation in aqueous solution at pH 4–10.

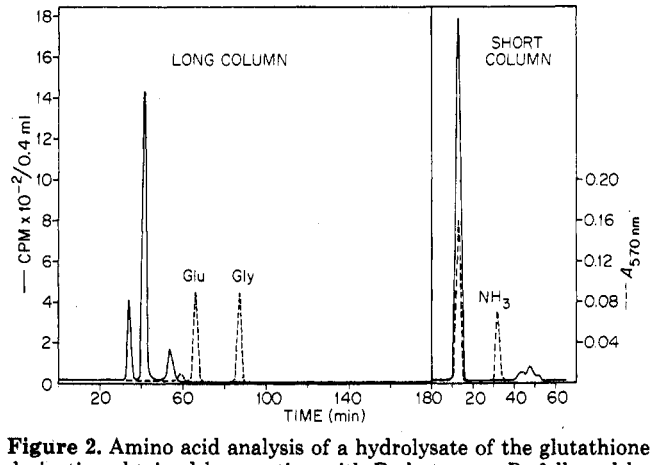


Figure 2. Amino acid analysis of a hydrolysate of the glutathione derivative obtained by reaction with Br-butanone-P_2 followed by reduction with [^{3}H]$NaBH_4$. Fractions (3 min from the long column and 1 min from the short column) were collected, and 0.4-ml aliquots of each were assayed for radioactivity.

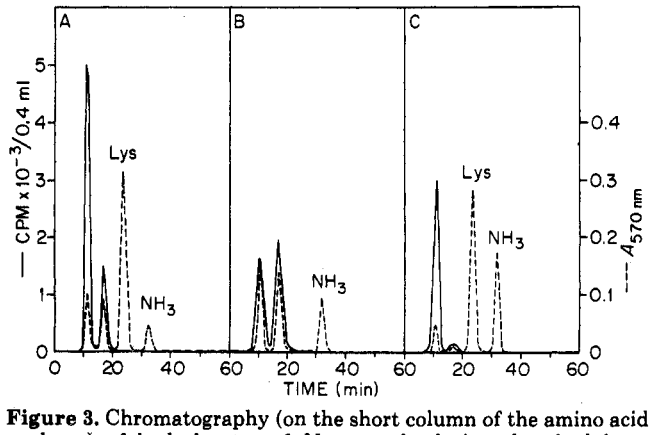


Figure 3. Chromatography (on the short column of the amino acid analyzer) of hydrolysates of N-α-acetyl-L-lysine that had been modified with Br-butanone-P_2 and reduced with [^{3}H]$NaBH_4$. Fractions (1 min) were collected, and 0.4-ml aliquots of each were assayed for radioactivity. (A) Hydrolysate of unfractionated reaction mixture. (B) Hydrolysate of the acetyllysine derivative after purification on Dowex 1. (C) Same hydrolysate as shown in B after incubation for 30 min at room temperature in 0.01 M sodium metaperiodate buffered with 0.1 M sodium bicarbonate (pH 8.6).

amino groups, by virtue of their nucleophilicity, presumably displace the bromine atom of the reagent and are thereby alkylated. However, reactions involving the carbonyl group of the reagent or even nucleophilic attack on the carbon atom bearing the base-labile phosphate group with alkyl–oxygen scission cannot be excluded. A similar reagent, bromoacetol phosphate,[8] is highly reactive toward sulfhydryls and forms a *S*-alkyl derivative of glutathione. Reaction of glutathione with Br-butanone-P_2 gives two major ninhydrin-positive phosphate esters, both of which lack a sulfhydryl group. The structural differences between these two compounds have not been elucidated.

N-α-Acetyl-L-lysine was treated with Br-butanone-P_2 and reduced with tritiated borohydride. After the product(s) was purified by ion-exchange chromatography, acid hydrolysates contained two radioactive components (Figure 3B). One of these could be a decomposition product formed during hydrolysis. Alternatively, two chemically distinct lysyl derivatives (e.g., mono- and dialkylated products) may not have been resolved by the chromatographic procedure used. Both compounds in the hydrolysate can be converted to lysine by periodate oxidation (Figure 3C), as was reported for the related compound, N^6-β-glyceryllysine.[9] This latter lysyl derivative was retarded on the short column of the amino acid analyzer and eluted before lysine in a position close to that observed for one of the derivatives reported here.[10]

The Br-butanone-P_2 synthesized and used in the present studies is a racemic mixture. Furthermore, the reduction of the reagent carbonyl to a hydroxyl group creates a second asymmetric center so that the derivatives of glutathione and lysine that were prepared are mixtures of diastereoisomers. The degree of separation obtained by paper chromatography of the glutathione derivatives and by ion-exchange chromatography of the hydrolyzed lysyl derivatives seems too great to represent resolution of diastereoisomers, but this possibility cannot be excluded.

Experimental Section

Materials. *cis*-2-Butene-1,4-diol, dicyclohexylcarbodiimide, triethyl orthoformate, diphenyl chlorophosphate, and DTNB were obtained from Aldrich Chemical Co. D-Ribulose 1,5-bisphosphate, glutathione, and *N*-α-acetyl-L-lysine were products of Sigma Chemical Co. Florisil was obtained from the Floridin Co., Tallahassee, Fla. Sodium [^{3}H]borohydride (4.3 mCi/mmol) was purchased from Amersham/Searle Corp. and diluted tenfold with unlabeled borohydride before use.

General Methods. Elemental analyses were performed by Stewart Laboratories, Inc., Knoxville, Tenn.

Melting points were taken with a Fisher-Jones apparatus and are uncorrected.

Thin layer chromatography was conducted on plastic sheets coated with silica gel containing a fluorescent indicator (MN-Polygram Sil N-HR sold by Brinkmann Instruments, Inc.). The solvent used was ether–petroleum ether (bp 30–60°) (1:1 v/v). Paper chromatography was conducted by the descending method using Whatman No. 1 paper. The solvent used was *n*-butyl alcohol–glacial acetic acid–water (7:2:5 v/v/v). Some compounds on the thin layer sheets could be visualized under ultraviolet light. Ketones were detected with 2,4-dinitrophenylhydrazine[11] or silver nitrate–sodium hydroxide.[12] Phosphate esters were detected with ammonium molybdate,[13] and amines were detected with ninhydrin–collidine.[14] Periodate–benzidine[15] was used to detect compounds containing vicinal hydroxyl groups. The periodate spray was 0.05% (w/v) sodium metaperiodate rather than a saturated solution of the potassium salt as reported earlier.

Inorganic bromide was measured with a bromide ion electrode (Orion Research Inc.) connected to a Digicord pH-millivolt meter (Photovolt Corp.). Base-labile bromide was determined as inorganic bromide after incubation of the sample for 20 min in 1 *N* sodium hydroxide at room temperature.

Inorganic phosphate was assayed by the method of Marsh.[16] Organic phosphate was determined as P_i after digestion of the phosphate ester for 2 hr with sulfuric acid (initial concentration of 1 *N*) at 180° on a sand bath. After the digestion period, water was added to the sample, which was then heated in a boiling-water bath for 20 min to hydrolyze the phosphoric acid anhydrides formed during digestion. Base-labile phosphate was determined as P_i after incubation of the sample for 20 min in 1 *N* sodium hydroxide at room temperature. Acid-labile phosphate was determined as P_i after incubation of the sample for 5–120 min in 1 *N* sulfuric acid in a boiling-water bath.

Radioactivity was assayed with a Packard 3003 liquid scintillation spectrometer. The scintillation fluid contained 4.6 g of 2,5-diphenyloxazole (PPO) and 115 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) dissolved in 1 l. of toluene–ethanol (4:3 v/v).

Amino acid analyses were performed on a Beckman Model 120C amino acid analyzer according to Spackman et al.[17] Samples were hydrolyzed at 110° for 21 hr in sealed, evacuated (<50 μmHg) tubes with 6 *N* HCl. Before analysis the samples were concentrated to dryness on a rotary evaporator.

***cis*-1,4-Di-*O*-benzoyl-2-butene-1,4-diol.** To a solution of 500 ml of $CHCl_3$ containing 44 g (0.5 mol) of *cis*-2-butene-1,4-diol and 121 ml (1.5 mol) of pyridine that was cooled to −5° in an ice–salt bath was added 140 ml (1.2 mol) of benzoyl chloride. This mixture was left in the ice–salt bath for 2 hr, then transferred to room temperature for 3 hr, at which time 20 ml of water was added. After remaining overnight at room temperature, the solution was washed in succession with two 500-ml portions of 1 *N* H_2SO_4, saturated sodium bicarbonate, and water. The chloroform layer was then dried over anhydrous sodium sulfate and concentrated to dryness at 40° on a rotary evaporator. Crystallization of the residue from 200 ml of ethyl alcohol gave 132 g (90%) of the dibenzoate, mp 64–65°. A value of 65–66° was reported previously.[18] Anal. Calcd for $C_{18}H_{16}O_4$ (296.33): C, 72.96; H, 5.44. Found: C, 73.02; H, 5.57.

1,4-Di-*O*-benzoyl-3-bromo-1,2,4-butanetriol (2). The above dibenzoate (100 g, 0.34 mol) was dissolved in dioxane (300 ml), and to this solution were added 71.2 g (0.4 mol) of *N*-bromosuccinimide and 50 ml of water. The resulting mixture was stirred until homogeneous (about 5 min). Within 1.5 hr the temperature of the reaction mixture rose from 24 to 33°. After the temperature decreased to 30° (total reaction time of 3.5 hr), the dioxane was removed by concentration. The residual liquid was mixed with 200 ml of $CHCl_3$; this solution was washed, dried, and concentrated as described above. The product was crystallized from 100 ml of isopropyl ether to give 56 g (42%) of material with mp 97–99°. Anal. Calcd for $C_{18}H_{17}BrO_5$ (393.25): C, 54.98; H, 4.35; Br, 20.32. Found: C, 55.27; H, 4.24; Br, 20.59.

1,4-Di-*O*-benzoyl-3-bromo-2-butanone (3). A solution containing 500 ml of ether, 20 ml (0.28 mol) of dimethyl sulfoxide, 3 ml (0.037 mol) of pyridine, 50 g (0.24 mol) of dicyclohexylcarbodiimide, and 48 g (0.122 mol) of the dibenzoylbromobutanetriol was cooled to 8°. The oxidation (a method of Pfitzner and Moffatt[19]) was initiated by the addition of 3 ml (0.04 mol) of trifluoroacetic acid. The temperature of the reaction mixture rose rapidly to 33°. Fifteen minutes after initiation, the reaction was terminated by the addition of powdered oxalic acid (15 g, 0.12 mol). The insoluble dicyclohexylurea was removed by suction filtration, and the filtrate was washed, dried, and concentrated as described above. Crystals (39 g, 82%) with mp 83–84° were obtained from 200 ml of isopropyl alcohol. Anal. Calcd for $C_{18}H_{15}BrO_5$ (391.26): C, 55.26; H, 3.86; Br, 20.43. Found: C, 55.28; H, 3.79; Br, 20.42.

3-Bromo-2-butanone-1,4-diol Diethyl Ketal (4). A solution of the above ketone (5 g), ethanol (16 ml), freshly distilled triethyl orthoformate (33 ml), and concentrated sulfuric acid (1.4 ml) was incubated in the dark at room temperature for 7 days. At this time thin layer chromatography showed approximately an 80% conversion of the ketone (R_f 0.40) to the ketal (R_f 0.56) (both compounds visualized under ultraviolet light). The reaction mixture was neutralized with 15 g of sodium bicarbonate. After the addition of 100 ml of ether, the mixture was filtered through Celite and concentrated to dryness at 60°. The residual liquid was dissolved in 150 ml of methyl alcohol, and to the solution was added 35 ml of 1 *N* NaOH. After 1 hr the methyl alcohol was removed by concentration, and the resulting aqueous mixture was extracted twice with 50-ml portions of ether. The extracts were dried and concentrated at 30° to yield 3.0 g of a slightly yellow, thin syrup. Thin layer chromatography showed this material to contain a fluorescent substance (R_f 0.61) tentatively identified as methyl benzoate and two 2,4-dinitrophenylhydrazine-positive (after heating the sprayed sheet at 100° for 5 min) components, a major one with R_f 0.24 and a minor one with R_f 0.47. The mixture was dissolved in 5 ml of cyclohexane and fractionated on a 2.5 × 23 cm column of Florisil

packed in cyclohexane. The column was washed in succession with 300 ml of cyclohexane (which eluted the methyl benzoate), 500 ml of cyclohexane–benzene (1:1) (which eluted the material with R_f 0.47), and 225 ml of benzene–ether (1:1) (which eluted the material with R_f 0.24 assumed to be the 3-bromo-2-butanone-1,4-diol diethyl ketal). Concentration of the benzene–ether washings at 30° gave 1.6 g (49% based on 5 g of the crystalline ketone) of chromatographically pure material as a colorless, slightly viscous liquid.

3-Bromo-1,4-dihydroxy-2-butanone 1,4-Bisphosphate Diethyl Ketal (5). To an ice-cold solution of 3-bromo-2-butanone-1,4-diol diethyl ketal (1.5 g, 5.9 mmol) in a mixture of pyridine (5 ml) and $CHCl_3$ (10 ml) was added diphenyl chlorophosphate (3.8 ml, 18 mmol). The reaction mixture was left overnight at 4°, and then a few chips of ice were added. After an additional 12 hr at 4°, more $CHCl_3$ (100 ml) was added to the mixture, which was then washed (with 1 N H_2SO_4 and saturated $NaHCO_3$), dried, and concentrated. The viscous syrup that was obtained was dissolved in 80 ml of ethyl alcohol; the resulting solution was filtered through Celite and hydrogenated in the presence of platinum black (0.5 g) at 50 psi on a Parr apparatus. Consumption of hydrogen was completed within 3 hr, at which time the catalyst was removed by filtration through Celite. The filtrate was neutralized to pH 8.0 with cyclohexylamine and concentrated to dryness. The residue was slurried in 100 ml of acetone, and the insoluble triscyclohexylammonium salt of Br-butanone-P_2 diethyl ketal (3.4 g, 71%) was collected by filtration. Paper chromatography revealed a single organic phosphate ester (R_f 0.53) and a slight contamination with P_i (R_f 0.36). The salt was recrystallized by dissolving it in 4 ml of 20% (v/v) aqueous cyclohexylamine followed by the addition to this solution of 300 ml of isopropyl alcohol. Crystallization occurred during 24 hr at room temperature to yield 2.6 g of the triscyclohexylammonium salt. Based on a molecular weight of 714, phosphate assays revealed 1.91 molar equiv of organic phosphate and 0.028 molar equiv of P_i. Anal. Calcd for $C_{26}H_{58}BrN_3O_{10}P_2$ (714.63): C, 43.70; H, 8.18; Br, 11.18; N, 5.88; P, 8.67; OCH_2CH_3, 12.61. Found: C, 43.95; H, 8.33; Br, 11.07; N, 5.87; P, 8.59; OCH_2CH_3, 12.35.

A sample (100 mg) of the bisphosphate ester was freed of P_i by subjecting it to anion-exchange chromatography on a 1.2 × 25 cm column of Dowex 1 (Cl^-). The column was equilibrated with 0.05 M LiCl–0.001 N HCl and eluted with a linear gradient consisting of 200 ml of the equilibration solution and 200 ml of 0.5 M LiCl–0.001 N HCl as the limit solution. The bisphosphate eluted at 0.32 M LiCl. The fractions in this region were pooled; the solution was then adjusted to pH 8.0 with 0.1 N LiOH and concentrated to dryness at 40°. Ethyl alcohol (100 ml) was added to the residue, and the insoluble product (49 mg, 88%) was collected by filtration. This material was dissolved in 0.5 ml of water and precipitated with 10 ml of ethyl alcohol. Anal. Calcd for $C_8H_{15}BrO_{10}P_2Li_4 \cdot H_2O$ (458.84): C, 20.94; H, 3.73; Br, 17.42; P, 13.50. Found: C, 20.67; H, 3.87; Br, 17.19; P, 13.46.

3-Bromo-1,4-dihydroxy-2-butanone 1,4-Bisphosphate (6). An aqueous solution (10 ml) containing 179 mg (0.025 M) of the triscyclohexylammonium salt of the diethyl ketal was swirled with 2 g of Dowex 50 (H^+) to remove cyclohexylammonium ions and then filtered. The resulting acidic solution was incubated at 40° for 3 hr. At this time paper chromatography followed by visualization with the molybdate spray revealed an essentially complete conversion of the ketal (R_f 0.53) to the ketone (R_f 0.23). The latter component gave an immediate positive response with the silver nitrate dip, whereas the ketal was not detected. Solutions of Br-butanone-P_2 (the free acid form) were stored in the freezer without appreciable decomposition during several months.

Stability of Br-butanone-P_2. A solution of Br-butanone-P_2 prepared from the corresponding ketal as described in the above paragraph was assayed for P_i, total organic phosphate, acid-labile phosphate, base-labile phosphate, and base-labile bromide (Table I). For comparison, samples of the ketal were subjected to the same analyses. The phosphate assays were also performed on commercial ribulose 1,5-bisphosphate because of the expectation that its phosphate groups might have stabilities similar to those of Br-butanone-P_2. The base labilities of the bromine atom and one of the two phosphate groups in Br-butanone-P_2, in contrast to their stabilities in the ketal, provide convenient methods for quantitating the concentration of the ketone in solution. The appearance of base-labile phosphate was used to follow the hydrolysis ($t_{1/2}$ = 40 min) of the ketal to the ketone. During this hydrolysis, less than 0.1 molar equiv of P_i and bromide are released.

The rates of release of bromide and P_i from Br-butanone-P_2 were determined as a function of pH (Figure 1). At pH 10.0, the release of bromide is rapid and approximates first-order kinetics. The initial rate of P_i appearance at this pH is similar to that of bromide; however, the rate decreases dramatically as the release of bromide appraoches completion and before 1 molar equiv of P_i is liberated. At pH 8 and 6 the kinetics of both bromide and P_i appearance are complex, with less than 1 molar equiv of P_i being released. At pH 4 the reagent is more stable, and the rate of P_i formation slightly exceeds that of bromide.

Decomposition Products of Br-butanone-P_2. The extent of P_i release during incubation of the reagent in 1 N NaOH or at pH 4–10 (1.1 molar equiv with the former and <1 with the latter) suggested that phosphate esters were the end products of decomposition. To verify this assumption, solutions of the reagent, after incubation in 1 N NaOH for 20 min or in 0.2 M $NaHCO_3$ (pH 8.0) for 24 hr, were inspected by paper chromatography. The untreated Br-butanone-P_2 (R_f 0.23) was detectable with both molybdate and silver nitrate but not with periodate–benzidine. After the reagent was treated with base, a single phosphate ester (R_f 0.36) was found that gave positive tests with silver nitrate and periodate–benzidine. On the chromatograms, this compound coincided with P_i but was nevertheless recognizable as a phosphate ester on the basis of color differentiation. P_i gives a yellow spot immediately upon spraying with the molybdate reagent; after drying the chromatogram and exposing it to ultraviolet irradiation, the spot becomes greenish-grey. The mixture of P_i and phosphate ester which coincided gave the expected yellow spot when sprayed, but upon irradiation a definite bluish-green spot was apparent.

The solution of reagent that was incubated at pH 8.0 contained two phosphate esters (R_f 0.25 and R_f 0.18). Both of these compounds were detectable with silver nitrate and periodate–benzidine. The compound with R_f 0.18 is assumed to be tetrulose 1,4-bisphosphate, since on the basis of the less than stoichiometric release of P_i some bisphosphate should remain. A compound that should have similar chromatographic properties, ribulose 1,5-bisphosphate, has an R_f of 0.16 and is detected by all three reagents. The compound with R_f 0.25 is probably tetrulose 4-phosphate. Solutions of ribulose 1,5-bisphosphate, after base treatment, contained a phosphate ester with R_f 0.27. This component was visualized with the periodate–benzidine sprays, but gave a very faint response with the silver nitrate dip.

Reaction of Br-Butanone-P_2 with Glutathione. The rate of reaction between glutathione and Br-butanone-P_2 was determined by measuring the decrease in sulfhydryl concentration with DTNB.[20] Reaction mixtures were prepared with 1.78 ml of 0.2 M buffer [sodium acetate (pH 4.0), Pipes (pH 6.0), potassium phosphate (pH 7.0), or Bicine (pH 8.0), all of which were 1 mM in EDTA], 0.02 ml of 0.1 M glutathione, and 0.2 ml of 0.02 M Br-butanone-P_2. Controls containing all ingredients except Br-butanone-P_2 were also prepared. Periodically, 0.2-ml aliquots of the reaction mixtures and controls were added to cuvettes containing 2.2 ml of 0.2 M potassium phosphate (pH 8.0) and 0.1 ml of DTNB (0.01 M). The sulfhydryl concentration was determined from the increase in A at 412 nm using an $\epsilon_{1\,cm}$ (1%) of 13,600.[20] Calculated second-order rate constants at pH 6.0, 7.0, and 8.0 were 0.68, 1.2, and 1.4 M^{-1} sec^{-1}, respectively. To prepare a quantity of modified glutathione sufficient for characterization studies, glutathione (20 μmol) and Br-butanone-P_2 (50 μmol) were incubated at room temperature for 30 min in 2 ml of 0.2 M $NaHCO_3$–1 mM EDTA (pH 8.0). The reaction was then terminated by acidification to pH 2.0 with 1 N HCl. Paper chromatography showed two ninhydrin-positive components (R_f 0.14 and R_f 0.22) that contained phosphate as demonstrated with the molybdate spray. From the relative intensities of the two components, there appeared to be about three times as much of the compound with R_f 0.14 as of the compound with R_f 0.22. A sample of the reaction mixture (equivalent to 0.2 μmol of glutathione present initially) was chromatographed on the long column of the amino acid analyzer. A major peak (0.17 μmol based on the constant for leucine) eluted essentially with the front (18 min), and a minor peak (0.017 μmol based on the constant for leucine) eluted at 39 min just before the position (42 min) at which glutathione emerges. The sample contained no glutathione but did contain oxidized glutathione (0.017 μmol) that elutes at 50 min. The oxidized glutathione must be considered a reaction product, since a glutathione control that was incubated under the same conditions for the same period of time did not contain oxidized glutathione. The peak that eluted at 18 min represents both ninhydrin-positive phosphate esters visualized by paper chromatography, since this peak was resolved into two by running the sample with the usual citrate buffer (pH 3.25) after adjusting its pH to 2.6 with 12 N HCl.

A sample of the reaction mixture was also hydrolyzed and then

subjected to amino acid analysis. About 5% less glutamic acid than glycine was found, and there was thus an indication of slight reaction of the N-terminal amino group with Br-butanone-P_2. The hydrolysate contained about 0.05 molar equiv of cystine, but did not contain significant quantities of other ninhydrin-positive compounds. Since the derivatives arising from the cysteinyl residue were apparently converted to ninhydrin-negative compounds during the hydrolysis procedure, some of the reaction mixture (1.0 ml) was treated with [^{3}H]$NaBH_4$ (0.1 M at pH 8.0 for 30 min) so as to reduce the carbonyl group of the glutathione derivative and thus provide a radioactive marker. The major glutathione derivative (4.0 μmol, 40%) was separated from the excess reagent on a 1.2 × 25 cm column of Dowex 50 (H^+) that was equilibrated and eluted with 0.05 N HCl. A hydrolysate of the derivative was chromatographed on the amino acid analyzer, and the effluent was monitored for radioactivity (Figure 2).

Reaction of Br-Butanone P_2 with N-α-Acetyl-L-Lysine. A solution of N-α-acetyl-L-lysine (40 μmol) and Br-butanone-P_2 (50 μmol) in 2 ml of 0.2 M $NaHCO_3$ (pH 8.0) was incubated in the dark at room temperature. Periodically, 0.05-ml aliquots were withdrawn and added to 0.95 ml of 0.1 N HCl. One-half of these diluted aliquots was analyzed on the long column of the amino acid analyzer by elution with the second buffer (pH 4.25) only (acetyllysine eluted at 64 min). The percentages of acetyllysine remaining at 0.5, 1, 2, 4, 24, and 48 hr were 94, 87, 77, 63, 51, and 49, respectively. After 48 hr a sample (1.0 ml) of the incubation mixture was reduced with [^{3}H]$NaBH_4$ as described for the glutathione derivative. A portion (0.1 ml) of the reduced reaction mixture was hydrolyzed and then inspected on the amino acid analyzer. The hydrolysate contained ninhydrin-positive components that eluted from the short column at 11 min (0.19 μmol based on the constant for leucine) and 18 min (0.26 μmol based on the constant for leucine) in addition to lysine (0.8 μmol) (Figure 3A). Much of the radioactivity coincident with the ninhydrin-positive peak at 11 min was assumed to be from unreacted reagent. The remainder of the reduced mixture was fractionated on a 1.2 × 25 cm column of Dowex 1 (Cl^-) using a linear gradient of LiCl (0–0.5 M) in 0.001 N HCl. The major radioactive lysyl derivative eluted at about 0.2 M LiCl and was obtained in a 39% yield. Hydrolysates of this material contained both of the ninhydrin-positive peaks found in the hydrolysate of the unfractionated reaction mixture (Figure 3A), and both were radioactive (Figure 3B). Both derivatives were converted to lysine upon treating the hydrolysate with sodium metaperiodate (Figure 3C).

Registry No.—1, 6117-80-2; 2, 55759-07-4; 3, 55759-08-5; 4, 55759-09-6; 5 triscyclohexylamine, 55759-10-9; 5 tetralithium, 55759-11-0; 6, 52084-24-9; *cis*-1,4-di-*O*-benzoyl-2-butene-1,4-diol, 55759-12-1; benzoyl chloride, 98-88-4; N-bromosuccinimide, 128-08-5; triethyl orthoformate, 122-51-0; diphenyl chlorophosphate, 2524-64-3; glutathione, 70-18-8; N-α-acetyl-L-lysine, 1946-82-3; ribuluse 1,5-P_2, 2002-28-0.

References and Notes

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(3) Abbreviations used are: Br-butanone-P_2, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); Bicine, N,N-bis(2-hydroxyethyl)glycine.
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Synthesis of the 2,5-Protoadamantanediols[1]

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Acid-catalyzed conjugate additions to 8,9-dehydro-2-adamantanone provide a general route to 2-exo-substituted 5-protoadamantanones. 2-*exo*-Acetoxy-5-protoadamantanone, thus generated, is shown to be a useful precursor for the synthesis of 2-*exo*-5-*exo*-, 2-*exo*-5-*endo*-, and 2-*endo*-5-*endo*-protoadamantanediol. 2-*endo*-5-*exo*-Protoadamantanediol has been prepared by a reaction sequence which features the Lewis acid catalyzed regioselective ring cleavage of 3-oxatetracyclo[5.3.1.$0^{2,6}$.$0^{4,9}$]undecane. Thus, the four possible 2,5-protoadamantanediols have been synthesized.

The chemistry of protoadamantane (1) and its derivatives has attracted significant attention.[3-5] However, although both 2-substituted[4] and 5-substituted[4a,5] protoadamantanes have been known for some time, no 2,5-disubstituted protoadamantanes have been reported. In principle, substitution of 1 at C-2 and C-5 with different substituent groups such that C-2 and C-5 remain sp^3 hybridized may lead to eight isomeric 2,5-disubstituted protoadamantanes. Of course, if the substituents are identical, only four isomeric 2,5-disubstituted protoadamantanes can be realized (Scheme I). We now wish to report the synthesis of the four 2,5-protoadamantanediols.

Results and Discussion

In terms of molecular architecture, a 2,5-disubstituted protoadamantane may be viewed as a 1,4-disubstituted butane that has been attached to the C-1, C-3, and C-5 axial positions of cyclohexane. Since it is well known that 1,4-disubstituted butanes may be prepared by acid-catalyzed