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Synthesis and Characterization of 1-*O*-Alkyldihydroxyacetone Phosphates and Derivatives*

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ABSTRACT: This report describes the chemical synthesis of the cyclohexylammonium salt of 1-*O*-hexadecyl-2,2-dimethoxypropane 3-phosphate and its octadecyl analog. The key reaction in the synthetic route is oxidation of benzoylated chimyl or batyl alcohol with dimethyl sulfoxide in the presence of dicyclohexylcarbodiimide and trifluoroacetic acid to the corresponding keto intermediate; the latter compound is subsequently phosphorylated and isolated as the cyclohexylammonium salt. The organically synthesized *O*-alkyldihydroxyacetone phosphates and those synthesized by microsomal enzymes of mammalian cells were found to have identical properties.

The *O*-alkyldihydroxyacetone phosphates are central intermediates in the pathway that produces diacyl-*O*-alkylglycerol, *O*-alkylacylglycerylphosphorylcholine, and *O*-alkylacylglycerylphosphorylethanolamine.

In a recent communication (Piantadosi *et al.*, 1970) we reported preliminary studies directed toward the synthesis of *O*-alkyldihydroxyacetone and its derivatives. Although the biosynthesis of alk-1-enylacyl phospholipids has not been elucidated, Snyder and coworkers (Wykle and Snyder, 1969; Snyder *et al.*, 1969, 1970a–c; Wykle and Snyder, 1970) recently described a microsomal enzyme complex that synthesizes *O*-alkyl ether bonds in normal and neoplastic cells. The intact phospholipids have been synthesized enzymically (Snyder *et al.*, 1970a; Wykle and Snyder, 1970) *via* *O*-alkyldihydroxyacetone phosphate, which is the first ether-containing intermediate formed in the new pathway proposed (Snyder *et al.*, 1969, 1970c); *O*-alkyldihydroxyacetone is also formed by the enzyme system.

The ketone intermediates are reduced enzymically by NADPH and can subsequently serve as acyl acceptors, thereby emphasizing the importance of these new and metabolically important ether-linked keto lipids. The microsomal enzymes synthesize *O*-alkyldihydroxyacetone phosphate from long-chain fatty alcohol and dihydroxyacetone phosphate and require ATP, CoA, and Mg^{2+} as cofactors. The results of similar studies by others (Hajra, 1969, 1970; Kapoulas and Thompson, 1969) have demonstrated the presence of the *O*-alkyl ether synthesizing enzymes in other systems. In this paper, we describe the complete organic synthesis and the characterization of the keto intermediates; the intermediates isolated from enzyme systems have been previously characterized chemically and chromatographically. The organically synthesized compounds are of great importance in confirming the findings of the earlier studies and for use in further metabolic studies.

We have prepared the cyclohexylammonium salt of 1-*O*-octadecyl-2,2-dimethoxypropane 3-phosphate (VIII) and 1-*O*-hexadecyl-2,2-dimethoxypropane 3-phosphate (see Scheme I). In this synthetic route, both 1-*O*-octadecylglycerol and 1-*O*-hexadecylglycerol were used as starting materials. Compound I was benzoylated in the presence of pyridine at $-10°$, resulting in a crude mixture of mono- and dibenzoates (II) in which the monobenzoate predominated approximately 2:1 as determined by thin-layer chromatography. However, II was not isolated but converted into III with Me_2SO and DCC[1] in the presence of trifluoroacetic acid. Compound III was ketalized to afford IV, then hydrolyzed to V with NaOH and subsequently phosphorylated with diphenyl phosphorochloridate; the cyclohexylammonium salt, VIII, was finally isolated. The cyclohexylammonium salt was shaken with dilue HCl or Dowex 50W ion-exchange resin at room temperature to release *O*-alkyldihydroxyacetone phosphate.

We have compared the chemical and chromatographic be-

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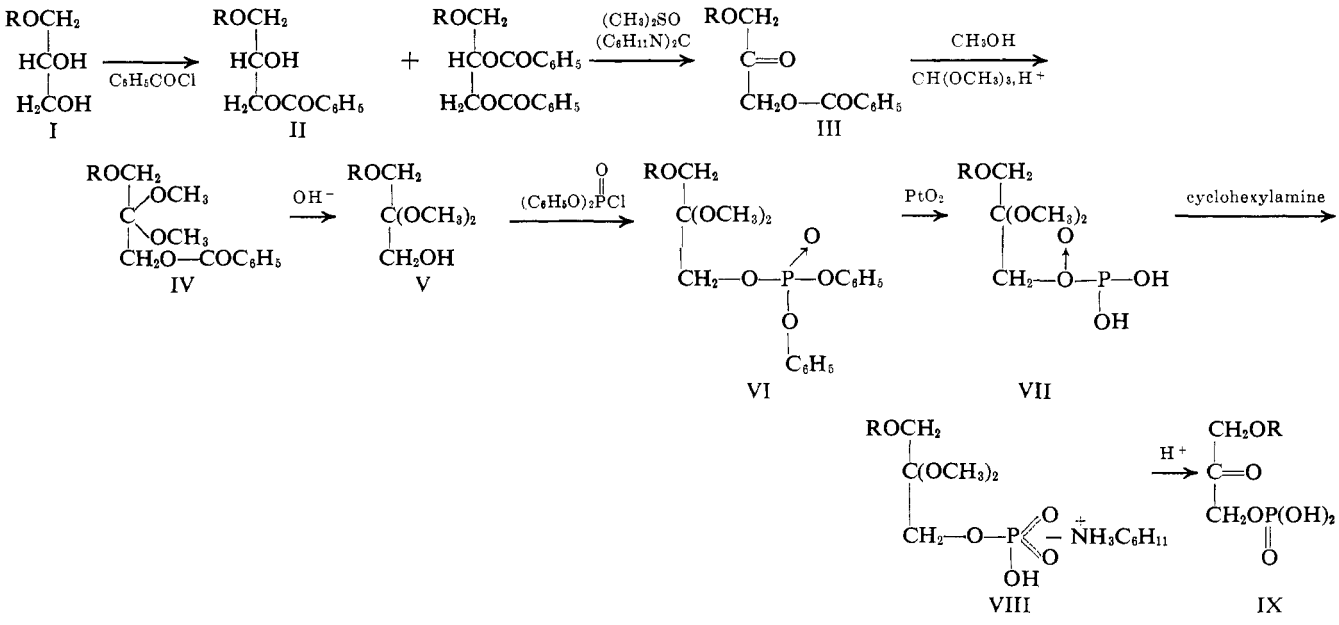
[1] Abbreviation used is: DCC, dicyclohexylcarbodiimide.

SCHEME I



R = $CH_3(CH_2)_{14}CH_2$ and $CH_3(CH_2)_{16}CH_2$

havior of biosynthetic and organically synthesized *O*-alkyldihydroxyacetone phosphate and found that both products have identical properties. This finding verifies the structures of the intermediates synthesized by the enzyme system.

Experimental Section

All melting points are uncorrected and were taken in a Mel-Temp and Fisher-Johns melting point apparatus. The elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga., and Spang Microanalytical Laboratory, Ann Arbor, Mich. The infrared spectra were determined by the Perkin-Elmer 337 and 621. The nuclear magnetic resonance spectra were taken by the Varian HA-100. Chimyl alcohol and batyl alcohol were obtained from Western Chemical Industries, Ltd., Vancouver, Canada (95% pure).

1-O-Hexadecyl-3-O-benzoyl-2-propanone (R = $CH_3(CH_2)_{14}CH_2$) (III). Chimyl alcohol (50 g) was dissolved in 1 l. of dry pyridine in a 2-l. three-necked flask equipped with a mechanical stirrer, thermometer, and a dropping funnel. The solution was cooled to −20° in a Dry Ice bath and 22.2 g of benzoyl chloride was added with rapid stirring during a span of 2.5 hr. During the addition, the temperature of the mixture was kept below −10°. After the addition was completed, the temperature of the mixture was gradually raised to 3° and the stirring was continued for another 4 hr at that temperature. Finally, the mixture was allowed to stir overnight at room temperature. The pyridine was removed under vacuum and the residue dissolved in 600 ml of chloroform; this solution was extracted three times with 500-ml portions of ice-cold 1 N H_2SO_4, once with 300 ml of water, then with a 400-ml portion of saturated $NaHCO_3$ solution, followed by a 300-ml portion of water, and then dried over anhydrous Na_2SO_4. The chloroform was removed under vacuum and the residue dissolved in 200 ml of *n*-hexane and kept at 5° for 1 hr. The chimyl alcohol (3.5 g) that separated was removed by suction filtration. The *n*-hexane was removed under vacuum resulting in 57.5 g of crude products. Thin-layer chromatography using a solvent system of diethyl ether–chloroform (40:60, v/v) revealed over 60% conversion into the monobenzoyl derivative, II. Finally, the crude products (57.5 g) were dissolved in 400 ml of anhydrous diethyl ether and oxidized at room temperature with 20 ml of Me_2SO in the presence of 50.34 g of DCC and 3 ml of pyridine. Trifluoroacetic acid (3 ml) was added with vigorous stirring to the flask in order to initiate the reaction, and within a few minutes the temperature in the reaction flask rose to 38°, then gradually went down to room temperature. Stirring was continued for 5 hr, then 22 g of oxalic acid dissolved in 45 ml of methyl alcohol was added to decompose the excess DCC. After a period of 0.5 hr the dicyclohexylurea was removed by suction filtration and washed thoroughly with diethyl ether. The volume of the ether filtrate was made up to 800 ml, transferred to a separatory funnel, and extracted three times with 200-ml portions of a saturated solution of $NaHCO_3$, followed by two 100-ml portions of water, and finally dried over anhydrous sodium sulfate. The ether was then removed under vacuum and the residue redissolved in 250 ml of ether and kept overnight at 5° in order to remove any residual dicyclohexylurea. The ether was then removed under vacuum, the residue taken up in 70 ml of diethyl ether and kept at 0° for 2.5 hr and finally overnight at 5°. A crystalline product was obtained which was suction filtered, washed thoroughly with ice-cold petroleum ether (bp 30–60°), and air-dried. The crude product (18.7 g) was recrystallized from 55 ml of warm ether, placed at 0° for 6 hr and overnight at 5°. The resulting 10 g of crystalline product was suction filtered, washed thoroughly with ice-cold petroleum ether, and dried under continuous vacuum over drierite and $CaCl_2$. The product has mp 43–44°. Thin-layer chromatography (in *n*-hexane–diethyl ether (80:20, v/v) and diethyl ether–chloroform (40:60, v/v) gave one spot having an R_F value of 0.38 and 0.75, respectively. Another 3.3 g of the compound was obtained from the original filtrate; yield 22% (based on reacted chimyl alcohol).

Anal. Calcd for $C_{26}H_{42}O_4$: C, 74.59; H, 10.11. Found: C, 74.31; H, 10.21.

The infrared spectrum of III (in KBr) showed the expected absorption bands for COC (1085–1140 cm^{-1}), ester and C=O (1718 cm^{-1}, 1738 cm^{-1}), CH_2 and CH_3 (2920, 2940, 1465, and

1380 cm^{-1}), C_6H_5 (1600 cm^{-1}). The 1-*O*-octadecyl analog was prepared in an analogous manner; yield, 11.5 g (21% based on reacted batyl alcohol), mp 50–51°.

Anal. Calcd for $C_{28}H_{46}O_4$: C, 75.29; H, 10.38. Found: C, 75.26; H, 10.29.

1-O-Hexadecyl-2,2-dimethoxy-3-O-benzoylpropane (IV). To 10 g of III dissolved in a mixture of 100 ml of trimethyl orthoformate–absolute methanol (3:2, v/v) was added 0.6 ml of concentrated H_2SO_4 with stirring. The ketalization was allowed to proceed at room temperature and with occasional stirring over a span of 24 hr at which time the mixture was treated with K_2CO_3. The carbonate was removed by filtration, washed with diethyl ether, and the solvent was removed under vacuum, leaving behind a syrupy residue which was taken up in 400 ml of ether. This ether solution was extracted once with a 100-ml portion of saturated $NaHCO_3$ solution, followed by two 50-ml portions of water, and dried over anhydrous sodium sulfate. Removal of the ether under vacuum produced a syrupy residue; the yield was 10 g (90%). Examination by thin-layer chromatography using *n*-hexane–diethyl ether (80:20, v/v) as the solvent system revealed a single product with an R_F of 0.48, indicating that complete ketalization had occurred. The infrared spectrum of IV (as liquid film) showed the expected absorption bands for COC (1085–1140 cm^{-1}), ester (1724 cm^{-1}), CH_2 and CH_3 (2920, 2940, 1465, and 1380 cm^{-1}), and C_6H_5 (1600 cm^{-1}). The 1-*O*-octadecyl analog was prepared in an analogous manner; yield, 10.5 g (95%).

1-O-Hexadecyl-2,2-dimethoxy-3-hydroxypropane (V). Compound IV (10 g) was dissolved in 80 ml of methanol containing 10 ml of 4 N NaOH and the mixture was stirred at room temperature for 24 hr. Approximately 30 ml of methanol was removed and replaced with water to give an aqueous methanol mixture which was then extracted three times with 200-ml portions of ether. The combined ethereal extracts were treated once with 100 ml of water and dried over anhydrous Na_2SO_4. The ether was removed under vacuum and the residue was dried in a vacuum desiccator over drierite–$CaCl_2$ for 24 hr; the yield was 6 g (77%). Examination by thin-layer chromatography in solvent systems of *n*-hexane–diethyl ether (80:20, v/v) and diethyl ether–chloroform (40:60, v/v) revealed the presence of a single spot with R_F values of 0.13 and 0.6, respectively; n^{28}D 1.4518.

Anal. Calcd for $C_{21}H_{44}O_4$: C, 69.94; H, 12.30. Found: C, 69.75; H, 12.31.

The infrared spectrum of V (as liquid film) showed the expected bands for OH (3470 cm^{-1}), CH_2 and CH_3 (2920, 2940, 1465, and 1380 cm^{-1}) and ether COC (1085–1140 cm^{-1}). The 1-*O*-octadecyl analog was prepared in an analogous manner, yielding 7 g (85%), mp 33–34°.

Anal. Calcd for $C_{23}H_{48}O_4$: C, 71.08; H, 12.45. Found: C, 70.82; H, 12.33.

Cyclohexylammonium Salt of 1-O-Hexadecyl-2,2-dimethoxypropane 3-Phosphate (VIII). Compound V (4 g) was dissolved in 20 ml of dry pyridine and the reaction flask was cooled in ice water. To this vigorously stirred solution was added 4 g of diphenyl phosphorochloridate within 7 min. The flask was stoppered and kept at 5° for 25 hr. The excess diphenyl phosphorochloridate was decomposed by the addition of water, the pyridine removed under vacuum, and the residue dissolved in 150 ml of benzene. The benzene solution was extracted once with 50 ml of water, twice with 50-ml portions of ice-cold 1 N HCl, and finally with 50-ml portions of water. Diethyl ether (100 ml) was then added to the benzene solution, and the solution was extracted once with 50 ml of a saturated solution of $NaHCO_3$, twice with 50-ml portions of water, and finally dried over anhydrous sodium sulfate. The solvent was removed under vacuum and the syrupy residue was dried under continuous vacuum for 6 hr, resulting in 6 g of the crude phosphorylated compound. It was dissolved in 100 ml of absolute ethanol containing 0.9 g of platinum oxide and hydrogenated for 3 hr at 15 psi at room temperature to remove the phenyl groups. The platinum oxide was removed by filtration and an excess of cyclohexylamine was added to the filtrate. This mixture was stirred overnight at room temperature, then cooled in ice water for 1 hr, filtered, washed with ice-cold acetone, and dried under continuous vacuum over drierite–$CaCl_2$ for a few hours. This resulted in 6.5 g of a crude product which was recrystallized from a 3:1 mixture of absolute ethanol–acetone, yielding 3.2 g of white crystals, mp 78–80° (53% based on V).

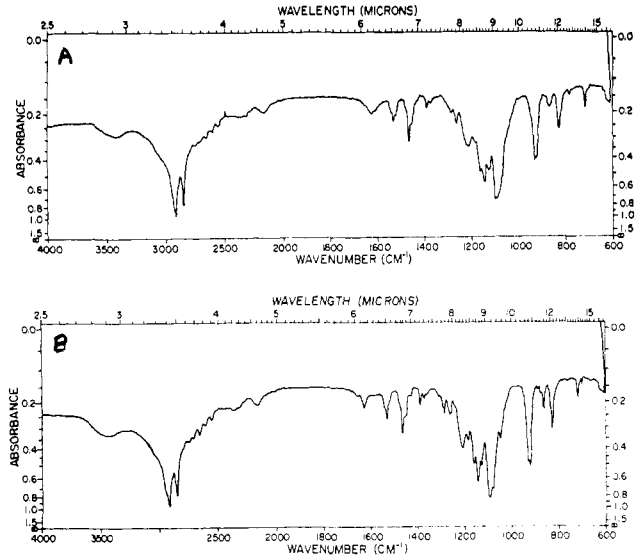


FIGURE 1: Infrared spectra studies in KBr. (A) Of cyclohexylammonium salt of 1-*O*-octadecyl-2,2-dimethoxypropane 3-phosphate. (B) Of cyclohexylammonium salt of 1-*O*-hexadecyl-2,2-dimethoxypropane 3-phosphate.

Anal. Calcd for $C_{27}H_{58}O_7NP$: C, 60.08; H, 10.83; N, 2.59; P, 5.73. Found: C, 60.17; H, 10.87; N, 2.65; P, 5.50.

The infrared spectra of VIII in KBr is shown in Figure 1. The structure of VIII was further confirmed by nuclear magnetic resonance, ($CDCl_3$) δ 3.78 (s, broad, methine proton), 3.45 (t, 4 H, methylene protons adjacent to the dimethoxy functions), 3.25 (s, 6 H, OCH_3), 0.88 (t, 3 H, J = 7 cps, CH_3), 1.26 (s, sharp, 36 H, CH_2), *ca.* 2.0 (s, broad, 4 H, CH_2).

The 1-*O*-octadecyl analog was prepared in an analogous way, yielding 2.7 g, mp 78–80° (53% based on 1-*O*-octadecyl-2,2-dimethoxy-3-hydroxypropane).

Anal. Calcd for $C_{29}H_{62}O_7NP$: C, 61.34; H, 11.00; N, 2.46; P, 5.45. Found: C, 61.38; H, 11.02; N, 2.42; P, 5.52.

1-O-Hexadecyldihydroxyacetone Phosphate. METHOD A. The cyclohexylammonium salt of 1-*O*-hexadecyl-2,2-dimethoxypropane 3-phosphate (25 mg) was dissolved in 6 ml of 0.1 N HCl and shaken at 37°. At the end of 2 hr, the mixture was extracted by the Bligh and Dyer (1959) procedure; the solvent was removed under vacuum and the product dissolved in chloroform. METHOD B. The cyclohexylammonium salt (25 mg) was dissolved in 6 ml of water and 1 g of Dowex 50 W (H^+) (100–200 mesh; 4% cross-linked) ion-exchange resin added. The mixture was shaken at 37° for 1 hr and the products were recovered as in Method A. Thin-layer chromatography of the products obtained by using Methods A and B

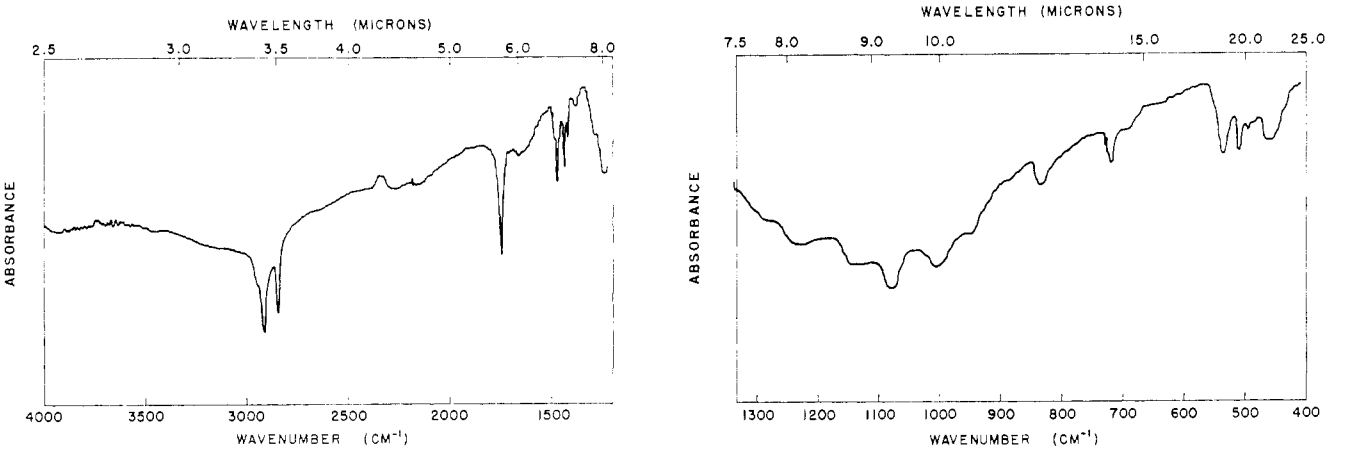


FIGURE 2: Infrared spectra in KBr of 1-*O*-hexadecyldihydroxyacetone phosphate (IX).

revealed a single product having an R_F of 0.42 when developed in a solvent system of chloroform–methanol–glacial acetic acid–0.154 M saline (50:25:8:2, v/v) and an R_F of 0.10 in chloroform–methanol–ammonium hydroxide (65:35:8, v/v). Lysophosphatidic acid was used for R_F comparisons and migrated with *O*-alkyldihydroxyacetone phosphate in both the acidic and basic solvent systems. The infrared spectrum of 1-*O*-hexadecyldihydroxyacetone phosphate IX in KBr is shown in Figure 2.

Chemical and Enzymatic Reactions of O-Alkyldihydroxyacetone Phosphate. Each of the following reactions was carried out with 1 mg of *O*-alkyldihydroxyacetone phosphate according to the procedures reported earlier (Snyder *et al.*, 1970b; Wykle and Snyder, 1970). Reduction by $LiAlH_4$ yielded *O*-alkylglycerol which was identified by thin-layer chromatography and periodate oxidation. Both acid hydrolysis (1 N HCl at 100° for 10 min) and prolonged saponification (2 N ethanolic KOH at 100° for 1 hr) led to the release of fatty alcohol which was identified by thin-layer chromatography. Alkaline phosphatase from *Escherichia coli* (Worthington Biochemical Corp.) removed the phosphate (Wykle and Snyder, 1970; Blank and Snyder, 1971) and resulted in the formation of *O*-alkyldihydroxyacetone which reacted and chromatographed exactly as authentic *O*-alkyldihydroxyacetone prepared by the method described earlier (Piantadosi *et al.*, 1970). Labeled *O*-alkyldihydroxyacetone phosphate was enzymically synthesized from [1-^{14}C]hexadecanol and mixed with the organically synthesized material; the resulting mixture remained homogeneously labeled after thin-layer chromatography in either acid or basic solvent systems. The mixture also behaved as a single compound when reduced by $LiAlH_4$ or hydrolyzed by acid.

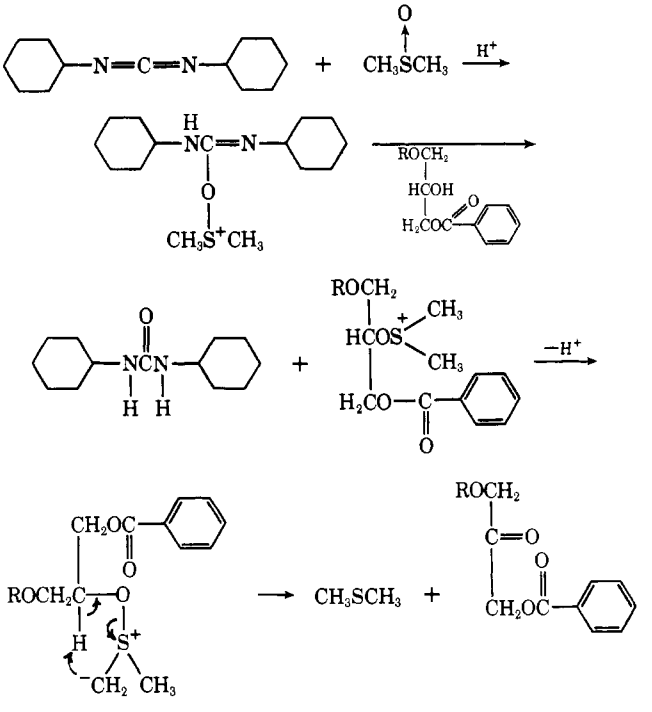
Discussion

The procedure outlined here makes possible the synthesis of *O*-alkyldihydroxyacetone phosphate and *O*-alkyldihydroxyacetone in good yields and high purity. The benzoylated chimyl alcohol was oxidized with Me_2SO in the presence of DCC and trifluoroacetic acid. In this oxidation reaction, the Me_2SO was converted into a labile intermediate which facilitated the attack at the sulfur atom by the β-hydroxy group of chimyl alcohol. Trifluoroacetic acid was used to initiate the reaction, while DCC acted as a polarizing agent. The accepted mechanism for the oxidation of the secondary alcohol to the ketone has been postulated by Pfitzner and Moffatt and it involves the steps shown in Scheme II.

Compound III was ketalized in order to protect the keto moiety during the phosphorylation step. The *O*-alkyldihydroxyacetone phosphates are relatively stable if stored in solvents at low temperatures (<−20°). We prepared the dimethoxy derivative of the ketone lipids to assure they would be stable over long periods of storage.

In the present study, organically and enzymically synthesized *O*-alkyldihydroxyacetone phosphate was shown to have identical chemical and chromatographic properties, thereby confirming it as an intermediate in the biosynthesis of alkylglyceryl ethers. The structure of [^{14}C]*O*-alkyldihydroxyacetone isolated from enzyme systems was similarly confirmed in our earlier study (Piantadosi *et al.*, 1970). The availability of these key compounds in the biosynthesis of alkylglyceryl ethers is

SCHEME II



of great importance for use in further studies of this new pathway. These compounds should also prove very useful for studies on the enzymic conversion of alkylglyceryl ethers to plasmalogens.

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Biosynthesis of 5,6-Dimethylbenzimidazole from 6,7-Dimethyl-^{14}C-8-ribityllumazine*

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ABSTRACT: 6,7-Dimethyl-^{14}C-8-ribityllumazine was added to anaerobically grown cultures of *Propionibacterium shermanii*. After 5 additional days of aerobic growth, the cells were harvested and the vitamin B_{12} was isolated and purified. The 5,6-dimethylbenzimidazole-^{14}C obtained from hydrolysis of this biosynthetic B_{12} represented about 7% of the ^{14}C originally added to the culture. A carbon-by-carbon degradation of the 5,6-dimethylbenzimidazole-^{14}C established that the ^{14}C was entirely confined to carbon atoms C-4(7) and C-8(9).

These results indicate that the 4,5-dimethyl-1,2-phenylene unit of the 5,6-dimethylbenzimidazole was formed by biosynthetic condensation of two molecules of the added 6,7-dimethyl-^{14}C-8-ribityllumazine precursor.

The 1,2-diamino-4,5-dimethylbenzene structural unit is confined in biological materials to riboflavin and its derivatives and to the DBI[1] moiety of vitamin B_{12} (Figure 1). Twenty years ago, Woolley (1951) proposed that this structural relationship between riboflavin and DBI resulted from a common biosynthetic pathway. Woolley found that 1,2-diamino-4,5-dichlorobenzene was toxic to those organisms which did not have a nutritional need for riboflavin and vitamin B_{12}, but that 1,2-diamino-4,5-dichlorobenzene did not retard the growth of those organisms which required exogenous sources of the two vitamins. Woolley postulated that 1,2-diamino-4,5-dimethylbenzene was a biosynthetic precursor of both riboflavin and the DBI moiety of vitamin B_{12}, and that the 1,2-diamino-4,5-dichlorobenzene was acting as an antimetabolite of this common precursor.

Subsequent experiments indicated that 1,2-diamino-4,5-dimethylbenzene was efficiently incorporated into the DBI moiety of B_{12} by *Propionibacterium arabinosum* (Perlman and Barrett, 1958). In the case of riboflavin however, nutritional studies and studies employing labeled precursors both established that the biosynthetic pathway originated within purine metabolism, rather than from 1,2-diamino-4,5-dimethylbenzene derivatives. (For reviews see Plaut (1961) and Goodwin (1970)). The investigations of Plaut (1960, 1963; Harvey and Plaut, 1966) then established that 6,7-dimethyl-8-ribityllumazine was the sole direct precursor of the riboflavin molecule. (For the recent identification of a biosynthetic intermediate between purines and 6,7-dimethyl-8-ribityllumazine see Bacher and Lingens, 1970.) The accumulated experimental evidence, therefore, tended to indicate that the biosyntheses of DBI and riboflavin were unrelated. It was clearly established that riboflavin biosynthesis began with the pyrimidine portion of the molecule and proceeded through 6,7-dimethyl-8-ribityllumazine; while, in contrast, it was interpreted that DBI biosynthesis began with the dimethylphenylene portion of the molecule and proceeded through 1,2-diamino-4,5-dimethylbenzene intermediates (*cf.* Plaut, 1961).

Nevertheless, the results of recent investigations have indicated that the biosyntheses of riboflavin and of the DBI moiety of vitamin B_{12} are connected. It was found that various labeled compounds tested as potential precursors of DBI (Renz and Reinhold, 1967; Alworth *et al.*, 1969, 1970) led to incorporation efficiencies and patterns that closely paralleled the results of earlier investigations into the biosynthetic

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[1] Abbreviations used are: DBI, 5,6-dimethylbenzimidazole; DBAB, 1,2-dibenzamido-4,5-dimethylbenzene; IDC, imidazole-4,5-dicarboxylic acid.