I. Synthesis of *sn*-Glycerol-Cyclic-Phosphodiester Isomers[1,2]

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ABSTRACT

A procedure for the synthesis of stereochemically pure *sn*-glycerol-cyclic-phosphodiesters has been developed. The process involves the following sequence of reactions: benzyl-*sn*-glycerol→benzyl-*sn*-glycerol-cyclic(phenyl)-phosphodiester→ *sn*-glycerol-cyclic-phosphodiester. The following isomers have been synthesized: *sn*-glycerol-2,3-,1,2-, 1,3-cyclic-phosphodiesters and the racemic mixture. The 2,3- and 1,2-cyclic-phosphodiesters of glycerol are optically active antipodes. They are five-membered ring asymmetrical compounds, with specific rotations of $-1.6° \pm 0.1°$ and $+1.6° \pm 0.1°$, respectively. These two enantiomers and their racemate are thick liquids and are unstable; therefore they were converted into Ba(glycerol-cyclic-phosphodiester)$_2$ salts, which can be better stored. The six-membered ring *sn*-glycerol-1,3-cyclic-phosphodiester is a crystalline, stable compound. The physical and chemical properties of these cyclic-phosphodiesters of glycerol are described and their chemical analyses are reported.

INTRODUCTION

Half a century ago, Octave Bailly (1) reacted sodium phosphate with epichlorohydrin and claimed during reaction the formation of a six-membered ring cyclic-phosphodiester of glycerol takes place. About 20 years later, Verkade and coworkers (2) studied the acid-catalyzed phosphate group migration of glycerol-phosphoric-acid-ester and postulated a mechanism by way of cyclic-phosphodiester. Later, Chargaff (3) demonstrated that this rearrangement indeed involves an intramolecular migration of the phosphate group. Baer and Kates (4) studied phosphate group migration of synthetic *sn*-glycerol-3-phosphoryl-choline, in acidic and alkaline media, and also postulated the formation of the cyclic-phosphodiester of glycerol as an intermediate during the migration.

A similar phosphate group migration occurs during the hydrolysis of nucleic acid. Markham and Smith (5) have isolated and identified cyclic-2′,3′-nucleotides as intermediates in the hydrolysis of ribonucleic acid, and have shown that the formation of a cyclic-phosphodiester is responsible for the migration of the phosphate group from the 3′- to the 2′-hydroxyl of the ribose. Other cyclic-phosphodiesters have also been described, i.e., pantetheine-2′,4′-cyclic-phosphodiester (6), glucose-cyclic-phosphodiester (7) and riboflavine-4′,5′-cyclic-phosphodiester (8).

The possibility of isolation of a cyclic-phosphodiester of glycerol was investigated by Ukita et al. (9). They found that no evidence for accumulation of the cyclic-phosphodiester of glycerol during the hydrolysis of lecithin, but synthesized the cyclic-phosphodiester of glycerol by intramolecular cyclization of the *sn*-glycerol -2-phosphoric-acid-ester catalyzed with trifluoroacetic anhydride, according to the procedure of Brown and coworkers (10). Later, Maruo and Benson (11) prepared a radioactive glycerol-phosphate-ester by the method of McMurray et al. (12) and then cyclized this product by intramolecular phosphorylation (term introduced by Khorana et al. [13]) using dicyclohexylcarbodiimide (DCC) as catalyst, according to the procedure of Khorana and coworkers (13).

However no direct synthesis of isomerically pure cyclic-phosphodiesters of glycerol has been reported.

This paper describes the synthesis of the stereo- and positional isomers of *sn*-glycerol-cyclic-phosphodiester.

EXPERIMENTAL PROCEDURES

Benzyl Glycerol Ethers

Reaction of 2,3-isopropylidene-*sn*-glycerol and 1,2-isopropylidene-*sn*-glycerol with 50% sodium hydroxide yielded the sodium alkoxides as described by Kaufmann and Förster (14). Without isolation these were reacted with benzyl chloride and hydrolyzed according to the method of Sowden and Fischer (15) to produce 1- and 3-benzyl-*sn*-glycerols. 2-Benzyl-*sn*-glycerol was prepared from 1,3-benzylidene-*sn*-glycerol. The latter was prepared by the method of Hibbert and Carter (16), but with the modification introduced by Verkade and van Roon (17). 2-Potassium-1,3-benzylidene-*sn*-glyceroxide was prepared according to the method described by Gupta and Kummerow (18), and this, without isolation, was reacted with benzyl chloride to

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[2] The nomenclature used in this communication is that adopted by IUPAC-IUB Commission.

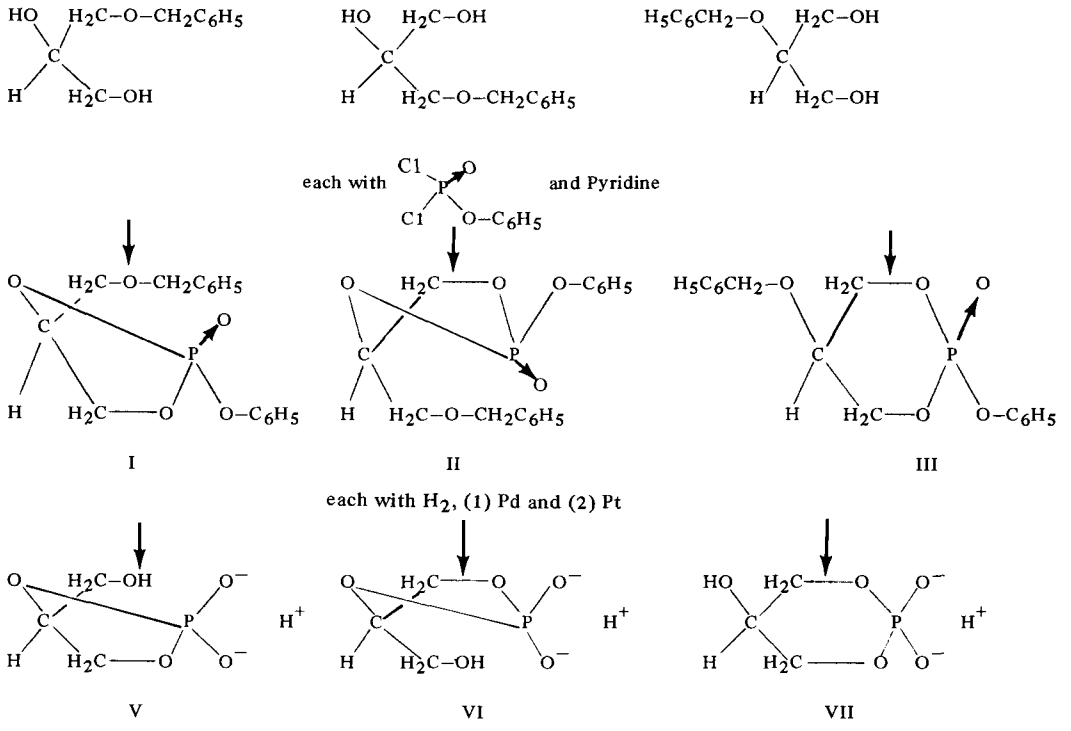


FIG. 1. Synthesis of *sn*-glycerol-cyclic-phosphodiester isomers.

produce 2-benzyl-1,3-benzylidene-*sn*-glycerol, which on hydrolysis of the benzylidene group with 10% acetic acid yielded 2-benzyl-*sn*-glycerol.

Benzyl chloride and phenylphosphoryl dichloride were certified reagents, and both were redistilled before use. Pyridine was dried over calcium hydride and toluene with sodium wire. Both solvents were certified spectroanalyzed.

Phosphocyclization

The phenylphosphoryl dichloride was used as phosphocyclizing reagent, as shown in the reaction scheme (Fig. 1). The position of the benzyl-protective group in the glycerol moiety dictates the cyclization position of the phosphodiester residue.

The phosphocyclization was carried out in a 500 ml three-necked, round-bottom flask fitted with a magnetic stirrer, two dropping funnels and a calcium chloride tube. The reaction flask was kept in a cold bath of ice and salt (-10 to -15 C). An appropriate benzyl-*sn*-glycerol (18.2 g, 0.1 mol) was dissolved in 100 ml dry pyridine (used as solvent and also as base to neutralize hydrogen chloride, which develops during phosphocyclization) and placed in one of the dropping funnels. An equimolar amount of phenylphosphoryl dichloride (21.0 g, 0.1 mol) was dissolved in dry toluene and brought to the same volume as the benzyl-*sn*-glycerol and pyridine mixture. The mixture of phenylphosphoryl dichloride and toluene was placed in the second dropping funnel.

Both reagents were added drop by drop at the same rate with stirring at -10 to -15 C in the reaction flask. The addition of the reagents was finished after ca. 30 min. The reaction mixture was stirred further for 2 hr at -10 C, and for 12 hr at room temperatue (20-25C). The reaction product was freed of the solvents, toluene and pyridine, by distillation in high vacuum at a bath temperature of 30-35 C. The residue, consisting of benzyl-*sn*-glycerol-cyclic(phenyl)-phosphodiester and pyridine hydrochloride, was dissolved in methanol or ethanol, and the alcoholic solution was then passed through an ion exchange column of Rexyn-101 (H^+) in order to remove the pyridine hydrochloride from the reaction product. The column was 40 cm long, 4.5 cm wide and contained 400 g Rexyn-101 (H^+).

The column was washed with either methanol or ethanol until the effluent was free of solute. The eluate was then concentrated to dryness under reduced pressure at a bath temperature of 30-35 C. The benzyl-*sn*-glycerol-cyclic(phenyl)-phosphodiester was recovered,

and, without further purification, showed on thin layer chromatography a major spot at R_f 0.7 and a very tiny spot at the origin, with benzene-diethyl ether 8:2 v/v used as developing solvent.

Two of the intermediate isomers (Fig. 1, structures I and II) and the racemic mixture, designated IV, were obtained in relatively high yields, i.e., over 90% of the theoretical yield. However another isomer (Fig. 1, structure III) was found to consist of two different compounds, which existed in a ratio of ca. 1:1, with total yield, as a mixture, of 88% of theory. The two compounds differed in their melting points, R_f values by thin layer chromatography and solubilities.

It seems reasonable to assume that the 2-benzyl-*sn*-glycerol-1,3-cyclic(phenyl)-phosphodiester appears in two different forms. The compound with high melting point (144-145 C) is suggested to be *trans*-form (IIIa), and the compound with lower melting point (71-72C) is suggested to be the *cis*-form (IIIb). Both 2-benzyl-*sn*-glycerol-1,3-cyclic(phenyl)-phosphodiester forms were crystalline, and were easily separated. Form IIIa crystallized from diethyl ether at 0 C, and form IIIb crystallized from petroleum ether (bp 30-60 C) at -6 C. These two forms could also be separated by column chromatography on silicic acid with benzene-diethyl ether 8:2 v/v as solvent, compound IIIa preceding compound IIIb in the elution.

Tentatively the structures, which are still under investigation are shown in Figure 2.

The analytical values of the stereoisomers and of the racemic mixture of benzyl-glycerol-cyclic(phenyl)-phosphodiester are listed below:

1-Benzyl-sn-glycerol-2,3-cyclic(phenyl)-phosphodiester (I): Specific rotation: $[\alpha]_D^{25}C + 7.5° \pm 0.1°$, in chloroform, c,10. Analysis calculated for $C_{16}H_{17}O_5P$(320): C, 60.00; H, 5.45; P, 9.67. Found: C, 60.12; H, 5.52; P, 9.60.

3-Benzyl-sn-glycerol-1,2-cyclic(phenyl)-phosphodiester (II): Specific rotation: $[\alpha]_D^{25}C$ $-7.5° \pm 0.1°$, in chloroform, c,10. Found: C, 59.89; H, 5.52; P, 9.84.

2-Benzyl-sn-glycerol-1,3-cyclic(phenyl)-phosphodiester (III): (a) *trans*-Form: mp 144-145 C. Found: C, 60.17; H, 5.84; P, 9.78. (b) *cis*-Form: mp 71-72 C. Found: C, 60.18; H, 5.35; P, 9.80.

Racemic benzyl-glycerol-cyclic(phenyl)-phosphodiester (IV): Found: C, 60.27; H, 5.44; P, 9.81.

The above isomers of benzyl-glycerol-cyclic(phenyl)-phosphodiester are readily soluble at room temperature in chloroform, ethanol, methanol, benzene and toluene, moderately soluble in diethyl ether and petroleum ether, and insoluble in water. At 20 C, I, II and IV are thick viscous liquids.

Catalytic Hydrogenolysis

Removal of benzyl-protective group with palladium black catalyst: Eleven grams of benzyl-glycerol-cyclic(phenyl)-phosphodiester was dissolved in 160 ml absolute ethyl alcohol by warming to 50 C. The clear solution, to which 2.5 g palladium black was added, was shaken in an all-glass reduction vessel in an atmosphere of pure hydrogen at room temperature and at a water pressure of 50 cm until the absorption of hydrogen ceased. The catalytic hydrogenolysis of the benzyl-protective group was completed at the end of ca. 1 hr, with enough uptake of hydrogen for ca. 100% removal of the benzyl-protective group. After replacing hydrogen by nitrogen, the reduction product was separated from the catalyst (palladium black) by centrifugation, and the catalyst was extracted three times with 25 ml portions of ethyl alcohol.

Removal of phenyl-protective group with platinum catalyst: The mother liquor, combined with ethyl alcohol extracts, together with 1.7 g platinum oxide, was placed in an all-glass reduction vessel, and the reductive cleavage was carried out as described above. The catalytic hydrogenolysis of the phenyl-protective group was completed at the end of ca. 3 hr, with consumption of ca. 4000 ml hydrogen (uncorrected). The hydrogen was then replaced with nitrogen, and the reduction product was separated from the catalyst by centrifugation. The catalyst was then washed three times with 25 ml portions of ethyl alcohol, and the combined solutions were brought to dryness by distillation under reduced pressure at a bath temperature not exceeding 35 C. The residue was then kept in high vacuum until a constant weight was reached. In each case ca. 96% of glycerol-cyclic-phosphodiester was recovered from hydrogenolysis. Thin layer chromatography, with chloroform-methanol-acetic acid 50:48:2 v/v as developing solvent, showed in each case a major spot at R_f 0.21, which was the cyclic-phosphodiester of glycerol, and a very tiny spot at the origin, which was not identified.

After removal of the protective benzyl and phenyl groups, all three *sn*-glycerol-cyclic-phosphodiesters (Fig. 1, structures V-VII) and the racemic mixture (VIII) exhibited a very pleasant aromatic, apple-like aroma. Two of these isomers (Fig. 1, structures V and VI) are optically active enantiomers. The carbon atom in position 2 of the glycerol moiety of V and VI is asymmetric, and the asymmetry of this carbon atom is maintained throughout the synthesis.

TABLE I

Consumption of Periodate in Mole Per Cent by Vicinal Hydroxyl Groups

Glycerol-cyclic-phosphodiester	Periodate consumption, %	Purity of cyclic-phosphodiester, %
V	2.2	97.8
VI	1.3	98.7
VIIa	0.2	99.8
VIIb	0.1	99.9
VIII	12.0	88.0

The analytical values of the glycerol-cyclic-phosphodiesters, after the removal of the protective benzyl and phenyl groups, are listed below.

sn-Glycerol-2,3-cyclic-phosphodiester (V): Specific rotation: $[\alpha]_D^{25}$ C -1.6° ± 0.1°, in ethanol solution, c, 10 (in water, exhibits no rotation). Analysis calculated for $C_3H_7O_5P$ (154): C, 23.39; H, 4.58; P, 20.11. Found: C, 23.41; H, 4.62; P, 20.04.

sn-Glycerol-1,2-cyclic-phosphodiester (VI): Specific rotation: $[\alpha]_D^{25}$ C + 1.6° ± 0.1°, in ethanol solution, c, 10 Found: C, 23.50; H, 4.60; P, 20.09.

sn-Glycerol-1,3-cyclic-phosphodiester (VII): Once the blocking groups, benzyl and phenyl, from IIIa and IIIb were removed, and the acid form of these two compounds was restored, their *trans*- and *cis*-characteristics disappeared and they became identical, i.e., *sn*-glycerol-1,3-cyclic-phosphodiester. Compound VII from IIIa or b had mp 137-138 C, with no melting point depression on mixing. NMR and IR spectra and R_f values of the two preparations were found to be identical. The elementary analyses found: (from a) C, 23.45; H, 4.67; P, 20.03 and (from b) C, 23.95; H, 4.89; P, 19.88.

Racemic glycerol-cyclic-phosphodiester (VIII): Found: C, 23.45; H, 4.67; P, 20.00. At 20 C, of V, VI and VIII, were very viscous liquids. All the isomers were readily soluble at room temperature in water, methanol and ethanol, and insoluble in other common organic solvents.

The purity of glycerol-cyclic-phosphodiester was checked by oxidation with periodic acid for the detection of any free vicinal hydroxyl groups. Results are shown in Table I.

Once the blocking groups from glycerol-cyclic-phosphodiesters were removed, the five-membered ring compounds (V, VI and VIII) were less stable, and slow deterioration of the five-membered ring occurred. To prevent the decomposition of the five-membered ring, isomers V, VI and VIII were converted into their barium-phosphodiester salts.

Preparation of Ba(Glycerol-Cyclic Phosphodiester)$_2$ Compounds of V, VI and VIII

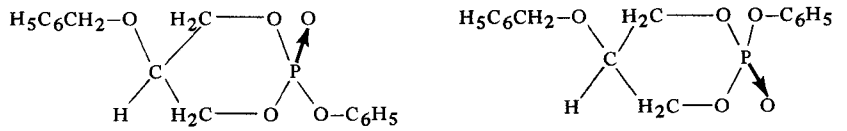
One hundred and fifty grams of Amberlite IRC-50 was suspended in 500 ml of a saturated barium hydroxide solution. The mixture was stirred for 30 min, and the slurry was poured into a glass column, 60 x 4.5 cm, with closed outlet. The outlet was opened and the column tapped occasionally with a rubber hammer to promote uniform settling of the amberlite particles. The excess of barium hydroxide was eluted with distilled water until the effluent was free of barium ions.

The glycerol-cyclic-phosphodiester (7.5 g) was dissolved in 100 ml 80% ethanol or methanol, and the solution was passed through a freshly prepared Amberlite IRC-50 (Ba^{++}) ion exchange column. The column was eluted with ethanol or methanol until the effluent was free of solute, Ba (glycerol-cyclic-phosphodiester)$_2$. The eluate was then concentrated under reduced pressure at a bath temperature of 30-35 C. The concentrated barium salt of cyclic-phosphodiester was freed of the rest of solvent by keeping it in high vacuum until a constant weight was reached. The recovery of Ba (glycerol-cyclic-phosphodiester)$_2$ from the ion exchange column was between 93 and 97% of theory (10-10.5 g).

The following analytical values were found for *Ba (glycerol-cyclic-phosphodiester)*$_2$ of V, VI and VIII: Analysis calculated for $Ba(C_3H_6O_5P)_2$ (443): Ba, 30.97; P, 13.97. Found: V = Ba, 30.93; P, 13.88; VI = Ba, 30.91; P, 13.17 and VIII = 31.05; P, 13.75.

On heating, the barium salt of *sn*-glycerol-2,3-cyclic-phosphodiester (V), softened at 155-160 C and decomposed at 180 C. The isomer 1,2-cyclic-(VI), on heating to 220 C, did not change its appearance. The racemic barium salt (VIII), on heating, behaved similarly to the salt of isomer V. The barium salts of glycerol-cyclic-phosphodiesters were soluble, like free diesters, in water, ethanol, methanol and insoluble in other common organic solvents.

The six-membered ring *sn*-glycerol-1,3-cyclic-phosphodiester (VII) was crystalline and



trans-Form (IIIa) *cis*-Form (IIIb)

FIG. 2. Proposed structures of 2-benzyl-*sn*-glycerol-1,3-cyclic (phenyl)-phosphodiester in *trans*- and *cis*-forms.

quite stable; therefore it was not necessary to convert it into its barium salt.

The success of the synthetic phosphocyclization of the benzyl-*sn*-glycerol with phenylphosphoryl dichloride to produce *sn*-glycerol-cyclic-phosphodiester depends largely on the following experimental conditions: (a) High purity of benzyl-*sn*-glycerol and phenylphosphoryl dichloride is imperative. (b) Solvents pyridine and toluene must be certified spectroanalyzed or equivalent grade and absolutely dry. (c) Phosphocylization of benzyl-*sn*-glycerol with phenylphosphoryl dichloride must be carried out at low temperature, under strictly anhydrous conditions. (d) Reagents must be mixed together simultaneously and in equal portions.

DISCUSSION

As stated in Experimental Procedures, 2-benzyl-*sn*-glycerol-1,3-cyclic(phenyl)-phosphodiester appears in two different forms—one with a high melting point (144 C), considered to be a *trans*-form, and the other with low melting point (71 C), considered to be a *cis*-form. After removal of the benzyl and phenyl blocking groups only one form is found. Identity of the preparations results from loss of configuration due to ionization of the acidic phosphate proton. The phosphorus atom in benzyl-*sn*-glycerol-1,2- and -2,3-cyclic(phenyl)-phosphodiesters is asymmetric. Once the phenyl blocking group is removed the proton is ionizable, and the enantiomeric forms become unresolvable due to ionization and resonance in the ionic species. This postulation is confirmed by the equivalence of preparations of *sn*-glycerol-1,3-cyclic-phosphodiester in acid form from presumed *cis* and *trans* sources.

This study is being extended to the synthesis of acyl-*sn*-glycerol-cyclic-phosphodiesters and -cyclic-phosphosphotriesters. In the latter, the hydroxyl group in the -cyclic-phosphoric acid residue is substituted with choline, ethanolamine or serine, respectively.

Cyclic-phosphodiesters and cyclic-phosphotriesters of glycerol have never been isolated from natural sources. In view of the ubiquitous occurrence of phosphosdiesterases and the lack of appropriate standards, it may have been an impossible task.

Dawson and Clarke (19) have recently revised the structure of myo-inositol phosphate released from phosphatidyl inositol on enzymatic hydrolysis to myo-inositol-1,2-cyclic-phosphodiester.

There is evidence that the cyclic-phosphodiesters of glycerol might serve as intermediates in chemical and enzymic transformation of phospholipids. Preliminary experiments (A. Kuksis, private communication) have shown that the *sn*-glycerol-1,3-cyclic-phosphodiester can serve a substrate of the purified 3′,5′-nucleophosphodiesterase, yielding *sn*-glycerol-3-phosphoric acid ester. Kuksis and O'Doherty (manuscript in preparation) have shown that *sn*-glycerol-1,3- and 2,3-cyclic-phosphodiesters are effective precursors of glycerophospholipids and triglycerides when incubated with microsomes from rat liver and intestinal mucosa in the presence of the diesterase.

Possibly the cyclic-phosphodiesters of glycerol are biochemically important compounds.

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