Preparation of Acylglycerols and Phospholipids with the Aid of Lipolytic Enzymes[1]

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ABSTRACT

Methods are discussed for the synthesis of some acylglycerols and phospholipids using lipolytic enzymes alone or in combination with chemical procedures. The syntheses are limited to those for which the enzymes are commercially available. The compounds mentioned are tri-, di-, and monoacylglycerols, phosphatidyl choline and ethanolamine, an alkyl-acyl-phosphatidyl ethanolamine, lysoacyl-phospholipids, phosphatidyl glycerol, sphingomyelin, and sphingosine.

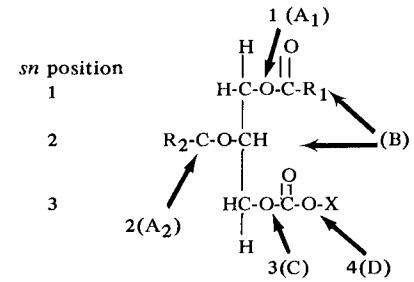
INTRODUCTION

The use of enzymes for synthesis of compounds offers, in general, many advantages over standard chemical methods. Among these are: (a) the substrate need not be pure because of the specificity of the enzyme; (b) reactions can be completed rapidly and always with moderate conditions; (c) the reaction can often to taken to completion, complete conversion of the substrate to the desired product; (d) there are usually few or no side reactions which can produce unwanted compounds complicating purification; (e) one enantiomeric member of a racemic mixture can often be "recognized" by the stereospecificity of the enzyme, reactions which cannot be duplicated by chemical methods; (f) crude enzyme preparations can occasionally be used; (g) many enzyme preparations, both crude and purified, are commercially available; and (h) a pure substrate can be converted completely to the desired product by adding an excess of enzyme thus avoiding purification. Conversely, the enzymes may require purification because of low activity in the original tissue or because competing enzymes produce unwanted compounds. Unfortunately many enzymes become increasingly sensitive as purification usually results in low yields of the enzyme. Finally, because the necessary quantities of enzyme cannot be obtained, only small amounts of the final compound can be produced.

Nevertheless, many have ignored the advantages of enzymes as preparative reagents, probably because, until recently, many of the enzymes have not been readily available in usable form. Today, however, there are several reliable suppliers of enzymes, and, hopefully, more investigators will use selective reagents.

The discussion in our review is limited to the biosynthesis of certain lipids with the aid of lipolytic enzymes. The review is organized around the compounds rather than the enzymes. Nevertheless, a list of the enzymes, substrates, and products produced that we will discuss is presented in Table I (1).

Note that we are using the nomenclature for phospholipases proposed by Brockerhoff and Jensen (1) because it is less confusing than the A_1, A_2, C, and D system in current vogue. These systems are shown below:



Two of the enzymes listed, phospholipases 3 and 4, are phosphohydrolases and thus are not strictly lipases (1). Nevertheless, usage has classified these as lipolytic enzymes, and they are therefore included. Lipolytic enzymes have been reviewed by Brockerhoff and Jensen (1), the synthesis of acylglycerols and some phosphoglycerides has been most recently reviewed by Jensen and Pitas (2), phospholipids in general by Rosenthal (3), and some phospholipids by Slotboom et al. (4), who also comment on the use of enzymes.

We have employed *sn* nomenclature to designate structure and shorthand notation for fatty acids: 16:0 is palmitic, etc. Triacylglycerols are identified by listing the acids horizontally or vertically: 1,3-dipalmitoyl-2-oleoylglycerol would be *rac* 16:0-18:1-16:0. If the prefix is *sn*, the first acid is in the *sn*-1 position.

We are assuming that the reader is familiar with the techniques for dealing with lipids. If not, we recommend the publications of Kates (5) and Perkins (6). Details of the purifications procedures are given in refs. (2,3) and in the cited papers.

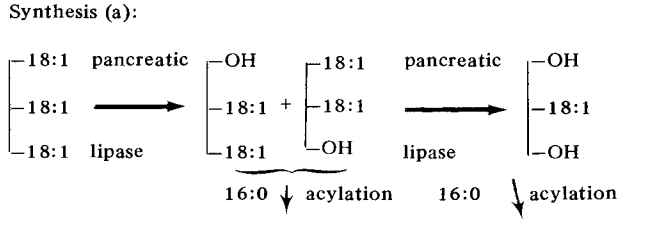
Our discussion will be limited to enzymatic syntheses for which the required enzymes can be purchased or easily obtained from readily available natural materials. These are the lipases from the pancreas and from the microorganisms *Geotrichum candidum* and *Rhizopus delemar,* the phospholipases 1, 2, 3, and 4, and sphingomyelinase. Instructions for the enzymatic procedures are in (1) and in the specific references. Some domestic suppliers of these enzymes are Boehringer-Mannheim, Miles, Sigma, and Worthington.

ACYLGLYCEROLS

Triacylglycerols (TGs)

The specificity of pancreatic lipase for primary esters of acylglycerols produces upon lipolysis of natural TGs or, among others, trioleoylglycerol, 1,2 (2,3)-diacylglycerols (DGs) and 2-monoacylglycerols (MGs) (1). The DGs and MGs can be reacylated with the desired acid to synthesize a *rac*-TG. The synthesis is shown in (a).

Synthesis (a):



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The length of hydrolysis should be limited to no more than 5 min or acyl migration will ensue, resulting eventually in a 1,3/1,2(2,3) DG ratio of 60/40 and a 1-(3)/2-MG ratio of 90/10. In any case, prior to acylation, the DGs and MGs must be purified by boric acid-thin layer chromatography (TLC) which separates the 1,3 from the 1,2 (2,3) DGs and these from 1,(3) and 2-MGs which are also resolved. A prior separation on a regular TLC plate may be necessary.

The time for lipolysis can be reduced to a few minutes if a partially purified preparation of relatively high activity, such as the one available from Worthington Biochemicals employed in our work, is used. Our use of the Worthington product does not imply that preparations from other suppliers are not of equal quality, only that we have not tried them.

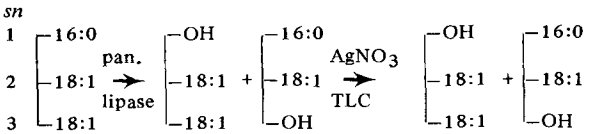
The crude pancreatic lipase powders are remarkably stable retaining activity for years if refrigerated, but some of the products contain a nonspecific lipase which hydrolyzes the 2-position esters (1). The nonspecific enzyme is inhibited by the bile salts generally included in pancreatic lipolysis or is eliminated by using a partially purified enzyme. The digestion mixture should be extracted rapidly without undue exposure to extremes of pH and heat to reduce isomerization.

The DGs or MGs resulting from pancreatic lipolysis can be acylated with the acid chloride (2). The reaction is allowed to proceed at room temperature until complete as judged by TLC.

Natural TGs can be substituted for trioleoylglycerol in synthesis (a) but the acids in the unhydrolyzed positions will be mixed. However, purity can be approached if an oil such as safflower which contains mostly 18:2, unrearranged lard in which the secondary ester is largely 16:0, or cocoa butter where 18:1 is the principal secondary ester, is utilized. We should also mention that a saturated substitute, such as tripalmitoylglycerol, can be hydrolyzed if the incubation temperature is raised to 45 C.

It is possible to synthesize an enantiomeric TG via synthesis (a) even though *rac*-DGs result if an enantiomeric TG containing both saturated and unsaturated acids is digested. The enantiomeric DGs can be resolved as shown in synthesis (b) and acylated with the desired acid. This procedure is redundant since the substrate and product are both enantiomeric and the chemical synthesis of enantiomeric TGs is relatively difficult, starting with D-mannitol. However, if an enantiomeric TG is available and a TG containing different acids is needed quickly, then synthesis (b) is practical.

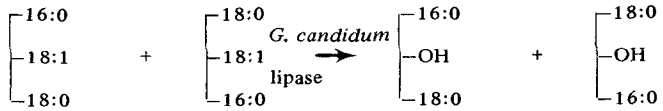
Synthesis (b):



The separated enantiomeric TGs can then be acylated with the desired acid. Acylation of *sn*-16:0-18:1-OH, produced as shown above with a different acid, may be the easiest synthesis of a triacid enantiomeric TG.

Although a lipase specific for the secondary esters of acylglycerols has not been reported, the specificity of the lipase from the microorganism *G. candidum* for cis-9-18:1 and 9,12-18:2 regardless of position within the acylglycerol allows removal of these acids as secondary esters by lipolysis of, for example, the monounsaturated TG fraction of cocoa butter which is largely *rac*-16:0-18:1-18:0 [synthesis (c)]. Hydrolysis of the proper substrate results in 1,2-(2,3) or enantiomeric DGs which can also be acylated as suits the user's needs. The enzyme is not stereospecific and can be obtained from Worthington Biochemicals.

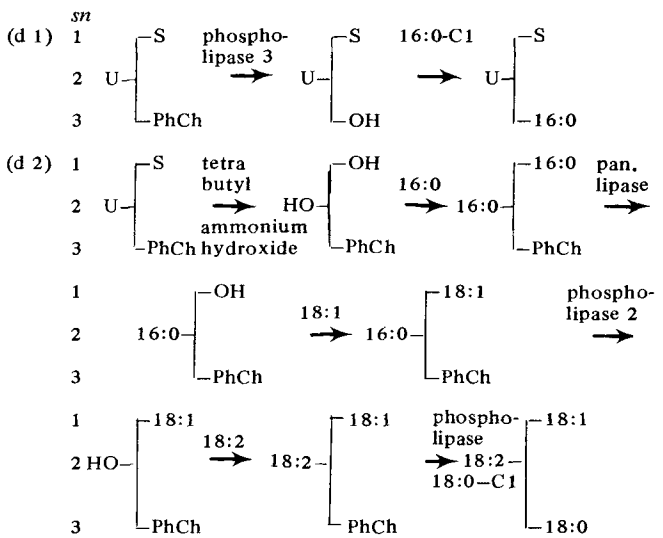
Synthesis (c):



The enzyme does not attack phospholipids (7).

A relatively simple method for the synthesis of the enantiomeric TGs is to utilize phospholipids as the substrate. Of the several phospholipids which contain glycerol, phosphatidyl choline (PC) is probably the simplest to obtain in pure form from a natural source: dried egg yolk (2). This PC, in common with most native PCs, contains mostly saturated acids (S) in the *sn*-1 and unsaturated acids (U) in the *sn*-2 positions. Investigators have several alternatives depending on their needs. These are presented in synthesis (d). PhCh is phosphoryl choline and the suffix Cl added to a fatty acid indicates an acid chloride: 16:0-Cl, 18:1-Cl, etc.

Synthesis (d):



The simplest reaction sequence involves the cleavage of phosphoryl choline (PhCh) moiety from PC with phospholipase 3, then reacylation with the necessary acid. If a particular set of acids is needed, then PC is deacylated with tetrabutylammonium hydroxide, the *sn*-3-glycerol PC reacylated (2), the *sn*-1-acid hydrolyzed with pancreatic lipase and this position reacylated, the *sn*-2-position deacylated with phospholipase 2 (8) and the desired acid attached, the phosphoryl choline removed with phospholipase 3 and the final acid placed on the molecule.

With acylglycerols the traditional method of acylation has been with acyl chlorides and pyridine, but yields are very poor when *sn*-3-glycerol phosphoryl choline or lysoacylphospholipids are acceptors. Yields were much improved when acid anhydrides were substituted for acid chlorides, but heating was required (2,3). Recently Keana and Ertle (9) acylated, among other compounds, *sn*-1-lysoacylphosphatidyl choline with stearoyl-p-toluenesulfonate. The advantages of this reagent are that the reaction is rapid and that it occurs at room temperature. The disadvantage is that it is prepared from stearoyl chloride and silver-p-toluenesulfonate.

Diacylglycerols (DGs)

DGs of the 1,2 type can be readily prepared by the

TABLE I

Lipolytic Enzymes, Substrates, and Products Produced[a]

EC[b]	Systematic name	Trivial name	Substrate	Products
3.1.1.3	Glycerol ester hydrolase	Lipase	Acylglycerols	DGs, MGs, FFAs
3.1.1.4	Phosphoglyceride 2-acyl hydrolase	Phospholipase 2 (A_2)	Phosphoglycerides *sn*-2-acid	Lysophosphoglycerides, FFA
	Phosphoglyceride 1-acyl hydrolase	Phospholipase 1 (A_1)	Phosphoglycerides *sn*-1-acid	Lysophosphoglyceride-FFA
3.1.1.5	Lysophosphoglyceride acyl hydrolase	Lysophosphopholipase	Lysophosphoglycerides	Glycerol phosphoryls FFA
3.1.4.3	Phosphoglyceride diglyceride hydrolase	Phospholipase 3 (C)	Phosphoglycerides	Diacyclycerol, phosphoryl groups
3.1.4.4	Phosphoglyceride phosphatidate hydrolase	Phospholipase 4 (D)	Phosphoglycerides	Phosphatidic acid, ethanolamine, etc.
3.1.4-	Sphingomyelin N-acyl-sphingosine hydrolase	Sphingomyelinase	Sphingomyelins	Sphingosines, phosphoryl groups

[a]Ref. 1.
[b]EC – Enzyme Commission.

action of pancreatic lipase upon an appropriate TG. The proven specificity of the enzyme for the primary esters of acylglycerols results in *rac*-1,2-DGs when TGs are hydrolyzed. In fact, the enzymatic procedure is much simpler and more rapid than chemical synthesis for producing small quantities of these compounds (2), but the length of hydrolysis should be limited to 5 min or less to avoid isomerization of the 1,2 to the 1,3 isomer.

As suggested in synthesis (b), treatment of a racemic TG with pancreatic lipase would produce four individual DGs, and two sets of enantiomers. Digestion of an enantiomeric TG, *sn*-16:0-18:1-18:1 results in *sn* 1,2-and 2,3-DGs, but these can be separated by argentation-TLC (4) [synthesis (b)].

Regardless of the considerable prior effort required to synthesize the TGs in (a) and (b) above for use as substrates, it is still often less arduous to proceed as described than to attempt the total synthesis of small quantities of DGs, particularly the enantiomers. If one desires 1,2 (2,3)-DGs and is not concerned about the fatty acids, trioleoylglycerol is a valuable substrate. Purified olive oil is also useful as a substrate to produce *rac*-1,2-DGs composed mostly of 18:1.

A successful enzymatic synthesis for *sn*-1,2-DGs without specified fatty acid composition involves the hydrolysis of phosphatidy choline (PC) or phosphatidyl ethanolamine (PE) with phospholipase 3 as shown in synthesis (d 1). In (d 1) PC is the substrate of choice since it is easily isolated from dried egg yolk (2).

If a monoacid *sn*-1,2-DG is required, then deacylation of egg yolk PC with tetrabutylammonium hydroxide followed by reacylation with the acid anhydride, or preferably the p-toluenesulfonate, are used as illustrated in (d 2). The phosphoryl choline is then removed from the di-16:0-PC with phospholipase 3.

Mixed acid *sn* 1,2-DGs can be made by following synthesis (d 2). After the acylation with 18:2, phospholipase 3 is applied and we have *sn*-18:1-18:2-OH.

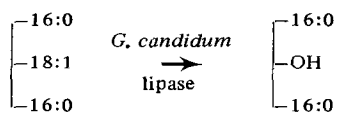
A major problem in dealing with either *sn* 1-or2-lysoacylphospholipids is that the compounds are subject to acyl migration and conditions used must be mild (3).

The *sn*-2,3-DG is probably most easily synthesized by using the appropriate *sn* TG in synthesis (b). As shown, we have a *sn*-OH-18:1-18:1. Obviously, if the enantiomeric DGs are not separable by $AgNO_3$-TLC, the synthesis is not applicable.

A novel and as yet untried enzymatic procedure on a preparative basis for synthesis of DGs would employ the lipase from the mold *G. candidum*, which is highly specific for cis-9-18:1 and cis,cis 9,12-18:2 (1,7). Note in synthesis (c), that 1,2 (2,3), *sn* 1,2 and *sn* 2,3 DGs can be made by substrates containing one hydrolyzable acid, such as *cis*-9-18:1 or *cis,cis*-9,12 18:2 and acids which resist hydrolysis by the lipase such as saturated, elaidic, positional isomers of 18:1 other than -9 and *trans,trans* 18:2. Use of this enzyme on appropriate enantiomeric TGs, e.g., *sn*-18:1-18:0-18:0, would yield *sn*-OH-18:0-18:0, a compound also obtainable by synthesis (b) primarily because pancreatic lipase disproportionately hydrolyzes 18:1 from TGs of this structure as compared to 18:0, thereby leaving relative large quantities of *sn*-OH-18:0-18:0, easily separable by crystallization or $AgNO_3$-TLC (2).

Since *G. candidum* lipase hydrolyzes 18:1 or 18:2 regardless of their positions in acylglycerols, the enzyme could be utilized to synthesize 1,3-DGs as shown in (e). However, compared to 1,2 DGs, the 1,3 isomer is so much easier to synthesize chemically that the enzymatic procedure offers no advantages because the 1,3-DG is the precursor for the TG. Insofar as we know, there are no lipases which are specific for the secondary esters of acylglycerols.

Synthesis (e):



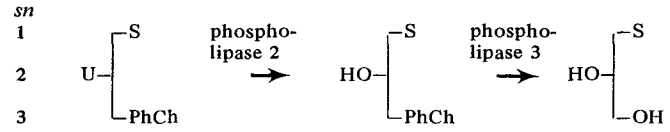
Monoacylglycerols (MGs)

Enzymatic synthesis is by far the easiest method to obtain 2-monoacylglycerols (MGs) because chemical synthesis is very difficult (2). The desired TG, natural or synthetic, is hydrolyzed with pancreatic lipase, for no more than 5 min [synthesis (a)], the lipolysate is extracted, and the 2-MG is purified by boric acid-TLC (2). All of these operations should be carried out promptly since the 2-MGs as mentioned are labile isomerizing to the 1-isomer and eventually reaching a ratio of 90/10 1-MG/2-MG. The 2-isomer can be isolated simultaneously with 1,2-(2,3) DGs since both are products of pancreatic lipolysis.

The *sn*-3-MGs are so easy to synthesize chemically that enzymatic routes do not appear to offer any advantages.

On the other hand, the enzymatic synthesis of *sn* 1-MGs is relatively easy and may be the preferred method. The procedure is depicted in synthesis (f) and involves sequential hydrolysis from PC with phospholipase 2 of the *sn*-2-acid and then removal of the phosphoryl choline moiety with phospholipase 3. The remaining acids can be either saturated, as in egg yolk PC, or individually defined as in a synthetic PC.

Synthesis (f):



There is one lipase, that from *Candida cylindracea,* which approaches random hydrolysis of TGs, thus producing 1-MGs which would require purification by TLC. The enzyme is offered by Worthington Biochemicals.

General Comments: Acylglycerol Hydrolases

There are two groups of lipolytic enzymes which may eventually offer substantial aid in the synthesis of enantiomeric acylglycerols. One consists of lipoprotein lipase which has a partial stereospecificity of about 2/1 for the *sn*-1-position of TGs (10). Although the enzyme has been substantially purified, the "stereospecificities" have not and may not be separable. The other lipase, that from the oral secretions of the rat, is also partially stereospecific but for the *sn*-3 ester (11). If the stereospecific components of these lipases can be separated, then they would be useful for the biosynthesis of enantiomeric acylglycerols.

Immobilized lipolytic enzymes have received little attention and deserve much more, primarily because the immobilized preparations can be used repeatedly. The lipase from *C. cylindracea* was at one time, but is no longer, available as an immobilized preparation linked to an inert copolymer of ethylene and maleic anhydride (1). We studied the preparation and found that it retained activity after repeated use.

Kosugi and Suzuki (12) studied what they designated as an immobilized lipase but which was cell-bound. The "bound" enzyme had 83% of the activity of the soluble lipase which was extracted from *Pseudomonas mephitica.* The major advantages of cell-bound lipases are that extraction and purification of the enzymes can be avoided.

Immobilized enzymes, which should receive much more attention from lipid chemists, are the subject of a volume in "Methods in Enzymology" (13).

PHOSPHOLIPIDS

The applications of the lipolytic enzymes to the synthesis of phospholipids on a preparative basis has been limited to the production of small quantities of materials. In some instances, the enzymatic procedures are superior to chemical synthesis.

Our discussion will not include phospholipases 1 which, although difficult to purify (1), are available from Sigma upon request. More important, however, both purified pancreatic and *Rhizopus delemar* lipases (Miles) remove the *sn*-1 acid from phospholipids. Because of the proven specificities of the lipases above and of phospholipase 2, lysophospholipases would not be particularly helpful in synthesis, especially since they are not offered for sale and are hard to purify (1,2).

A recently published chromatographic method for the purification of phospholipids appears to be superior to older methods. MacDonald and Rempas (14) described a dry column method in which the column was packed with dry gel and the compounds resolved with the same solvents used to separate them on TLC plates. The separated phospholipids were not eluted as in traditional column chromatography but were located on the gel in an openable column, the absorbant removed, and the phospholipid eluted therefrom. The columns developed very rapidly, and separations similar to those on TLC plates were obtainable. Amounts of phospholipids ranging from 10 mg to 10 g were purified. PC, PE, and sphingomyelin were resolved from egg yolk, and most of the components in crude soybean phospholipids were separated.

Glycerol Phosphorylcholine (GPC) and Phosphorylethanolamine (GPE)

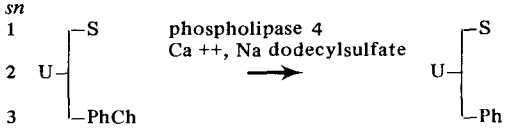
GPC is best obtained by deacylation with tetrabutylammonium hydroxide [reaction (d 2)] but with GPE the ethanolamine group must be protected and deprotected during deacylation.

The lipolytic activity earlier designated as phospholipase B (1 and 2), was probably a mixture of phospholipases 1 and 2 with perhaps some lysophospholipases present (1). Recently, however, Kawasaki et al. (15) purified a phospholipase B from *Penicillium notatum* to homogeneity by disc electrophoresis which hydrolyzed both the *sn*-1 and 2 esters from PC. Although the enzyme offers no advantages over chemical deacylation of PC, it would be useful for the removal of acids from PE, and possibly phosphatidyl serine, since the ethanolamine or other groups would not need protection. Unfortunately, the authors did not test PE as a substrate. Insofar as we know, the purified enzyme is not commercially available.

Phosphatidic Acids

The action of phospholipase 4 (Miles, Sigma, Worthington) upon PC cleaves the phosphate ester bond between the phosphate and choline groups producing phosphatidic acid [synthesis (g)]. If a phosphatidic acid with defined acids is required, then a synthetic PC must be the substrate.

Synthesis (g):



Heller et al. (16) using a phospholipase 4 preparation from peanut seeds obtained optimal rates of hydrolysis of PC when Ca^{++} ions and Na dodecylsulfate were present. The same enzyme mixture also removed the *sn*-1-acid from the *sn*-1-lyso PC (17). A number of other phospholipids are also hydrolyzed by the exzyme (1).

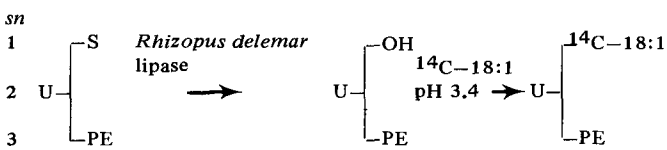
Phosphatidyl Choline and Ethanolamine

Monoacid PCs are most easily prepared by the de- and reacylation of egg yolk PCs as shown in synthesis (d 2). Reacylation should be done with the acid anhydride or p-toluenesulfonate for good yields. The remainder of the reaction sequence in (d 2), excluding the final hydrolysis with phospholipase 3, results in a mixed acid PC. Schulze et al. (18) hydrolyzed egg holk PC with phospholipase 2 and then reacylated with the ^{14}C anhydrides of 16:0, 18:0, 18:1, and 18:2.

Labeled PE has been prepared by treatment with phospholipase 2 (19). Reesterification of the *sn*-2 position was accomplished with rat liver acyl CoA transferase, ^{14}C-18:1, coenzyme A, and ATP. Chemical reacylation of lyso PE would require protection of the ethanolamine group during the reaction.

Brockerhoff et al. (20) introduced ^{14}C 18:1 into the *sn*-1-position of PE by an exchange reaction at low pH (3,4), catalyzed by *Rhizopus delemar* lipase in [synthesis (h)].

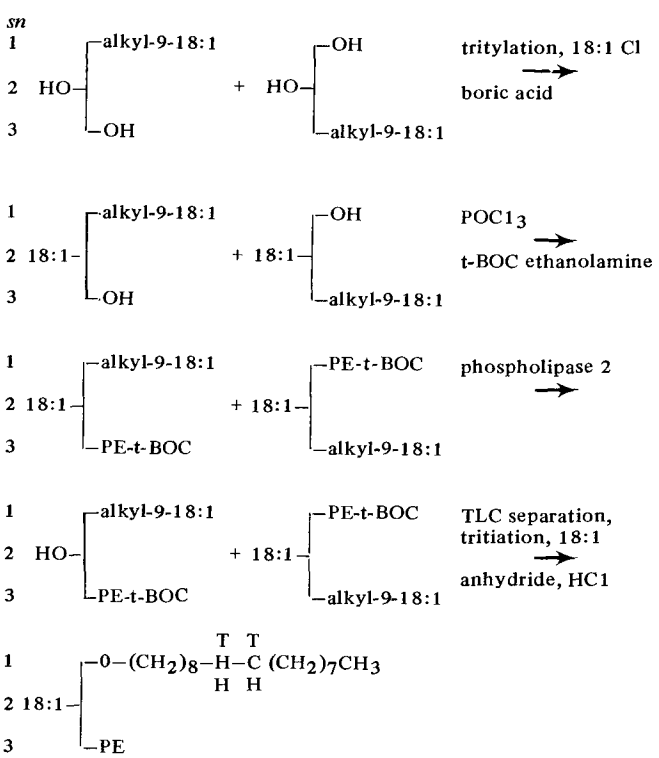
Synthesis (h):



Incorporation of the label was exclusively at *sn*-1 and yields ranged from 14-23%. The enzyme partially hydrolyzed phosphatidyl serine, phosphatidyl inositol, and PC, but the label was not introduced. However, when PC and PE were mixed, some label was found in both compounds. The authors suggested that several other phospholipids might be labeled with the exchange reaction catalyzed by phospholipase 4, for example, ethanolamine against choline. (1).

PCs and other phospholipids can be synthesized by phosphorylation of *sn*-1, 2-DGs or by attachment of the required moiety to *sn*-3-phosphatidic acid (3,21). The synthesis of *sn*-1,2 DGs is shown in synthesis (b) and phosphatidic acids in (g). Paltauf (22), desiring a labeled alkyl unsaturated acyl PE, started with *rac*-1-octadecen-9-ylglycerol and proceeded as in synthesis (i). The primary hydroxyl was blocked with the trityl group, the secondary hydroxyl acylated with oleoyl chloride, and the trityl group removed with boric acid which prevents acyl migration. The free primary hydroxyl was phosphorylated with $POCl_3$, and the ethanolamine with the N function protected by t-butoxycarbonyl (t-BOC) was then attached. Phospholipase 2 then hydrolyzed the *sn*-2-18:1 from the *sn*-3 PE only, thus taking advantage of the stereospecifity of the enzyme. The lysocompound was purified by preparative TLC, the alkyl 18:1 was tritiated, the *sn*-2-position reacylated with 18:1 anhydride, and the *t*-BOC group removed with HCl. This synthesis is an excellent example of using the stereospecificity of a lipase to separate enantiomers and achieve specific labeling.

Synthesis (i):



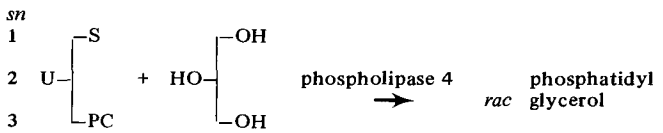
Lysoacylphospholipids

The *sn*-1 and *sn*-2 lysoacylphospholipids present severe challenges to those engaged in organic syntheses (3). The least arduous methods for the preparation of small quantities of these compounds utilize pancreatic or *R. delemar* lipases for the deacylation of the *sn*-1 acid from PC or the *sn*-2 acid by phospholipase 2. These syntheses are presented in (d2), (f), and (h). Once again either natural or synthetic phospholipids can be substrates depending upon the requirements of the investigator.

Miscellaneous Compounds

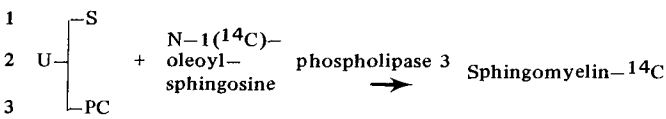
Joutti and Renkonen (23) obtained phosphatidyl glycerol from egg PC and glycerol by transphosphatidylation with phospholipase 4 [synthesis (j)], but analysis of the glycerol phosphate released from the phosphatidyl glycerol by the action of phospholipase 3 revealed that the reaction had resulted in a racemic mixture of phosphatidyl glycerols.

Synthesis (j):



Kanfer and Spielvogel (24) incubated PC and N-1 (^{14}C) oleoylsphingosine with phospholipase 3 to synthesize a labeled sphingomyelin [synthesis (k)]. In this reaction [synthesis (k)]

Synthesis (k):



the phosphorylcholine group is transferred to the labeled sphingosine or ceramide.

A sphingomyelinase, available upon request from Sigma, catalyzes the conversion of sphingomyelin to sphingosine and phosphorylcholine (1).

DISCUSSION

Enzymatic syntheses and combinations of these and chemical syntheses are available for the preparation of many acylglycerols and phospholipids which would otherwise be very difficult to prepare without lipolytic enzymes. Since many of the enzymes can be purchased at reasonable prices, in good purity and relatively large quantities, we hope that more investigators will utilize these selective reagents for the synthesis of lipids otherwise unavailable in the quantities desired.

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