papers on methodology

# Dephosphorylation and benzoylation reactions occurring in the benzoolysis of diacyl-, alkylacyl-, alk-1-enylacyl-, and acyl-lyso-glycerophosphocholines

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**Abstract** Series of benzoolysis experiments with 1,2-dipalmitoyl glycerophosphocholine (GPC), 1-palmitoyl-2-lyso GPC, and 1-O-hexadecenyl-2-oleoyl GPC, as well as a few experiments with 1-oleoyl-2-acetyl GPC and 1-O-hexadecyl-2-acetyl GPC are reported. The results are used to present a consistent picture of the various dephosphorylation and benzoylation reactions occurring in molten benzoic anhydride.—**Gelsema, W. J., and H. van den Bosch.** Dephosphorylation and benzoylation reactions occurring in the benzoolysis of diacyl-, alkylacyl-, alk-1-enylacyl-, and acyl-lyso-glycerophosphocholines. *J. Lipid Res.* 1997. **38:** 1680–1684.

**Supplementary key words** diacyl GPC • alkylacyl GPC • alk-1-enylacyl GPC • acylacetyl GPC • alkylacetyl GPC • diacylglycerol • acyl-lyso GPC • dephosphorylation • benzoylation • isomeric diradylglycerobenzoates • high performance liquid chromatography

In a previous paper (1) we presented a method for the quantitation of the diacyl-, alkylacyl-, and alk-1-enylacyl-subclasses of choline glycerophospholipids (GPC) by chemical dephosphorylation and benzoylation. The method utilizes normal-phase high performance liquid chromatography with UV-detection and is not devised for the assessment of individual molecular species. It appeared that under standard conditions (5 h of heating at 100°C with benzoic anhydride in the presence of boric acid, followed by the addition of DMAP at room temperature) the yield of extracted benzoylated products derived from an alk-1-enylacyl GPC is about half of that from either a diacyl GPC or an alkylacyl GPC. Although a tentative explanation was put forward (1), the matter was not studied in more detail.

In a more recent paper (2) we reported on the acyl migration accompanying the benzoolysis of a diacyl GPC and on the behavior of diacylglycerol under the same reaction conditions. A peculiar similarity of reaction rates and acyl migration of the two processes was found. This led us to suppose that both processes proceed via a common intermediate. In these studies (1, 2), homo-acidic diacyl GPCs were used as model compounds. In this paper, we report on benzoolysis experiments with a mixed-acid GPC and an acyl-lyso GPC. The results of these experiments are discussed and a consistent picture of the various reactions in molten benzoic anhydride is drawn.

## MATERIALS AND METHODS

### Chemicals and apparatus

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine, 1-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine, and 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine were obtained from Sigma, St. Louis, MO, 1-O-hexadecenyl-2-oleoyl-*sn*-glycero-3-phosphocholine was a generous gift from Dr. R.W. Gross, St. Louis, MO. The provenance and purity of all further chemicals and the apparatus used are documented in reference 1.

### Stock solutions

The above lipids were dissolved in chloroform–methanol 2:1 (by volume) at concentrations of about 0.68 μmol · $ml^{-1}$. The exact concentrations were measured by phosphorus determination (see ref. 1). Mixed stock solutions of the same total GPC concentration containing, in known molar ratios, dipalmitoyl GPC + pal-

Abbreviations: DMAP, 4-dimethylaminopyridine; GPC, glycerophosphocholine; HPLC, high performance liquid chromatography.

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mitoyl-lyso GPC and dipalmitoyl GPC + 1-O-hexadecenyl-2-oleoyl GPC were also prepared.

### Benzoolysis

Aliquots (1 ml) of the stock solutions were evaporated to dryness in 10-ml tubes fitted with Teflon-lined screw caps. Benzoic anhydride (250 mg) and boric acid (10 mg) were added and the tubes were heated at 100°C for variable times (generally 5 h). After cooling down to room temperature, 600 µl of a 2.5% (w/v) solution of DMAP in benzene or 600 µl of benzene was added and the mixtures were left at room temperature for 2 h. Then 4 ml of *n*-hexane and 5 ml of 25% (w/v) ammonia were added, the mixtures were thoroughly shaken and left overnight.

### Analysis

HPLC analyses of the lipid products were performed as described in ref. 1. Briefly, aliquots of the hexane–benzene layer were taken to sample vials, evaporated to dryness, re-dissolved in *n*-hexane, and filtered. Samples of the filtrate were eluted under normal phase conditions on a ChromSpher-5Si analytical silica column (250 × 4.6 mm) using *n*-hexane–diethyl ether–ethanol 500:15:0.3 (by volume) at 1 ml · $min^{-1}$ and UV-detection at 230 nm.

## RESULTS AND DISCUSSION

From earlier studies (1, 2) the following picture emerged. During the heating procedure in molten benzoic anhydride in the presence of boric acid a diacyl GPC is dephosphorylated and subsequently benzoylated yielding isomeric diacylglycerobenzoates. In this sequence, the last (benzoylation) step is rate-determining: complete benzoylation is obtained only after about 15 h of heating at 100°C (2), whereas complete dephosphorylation is already reached after 4–5 h (1, 2). It is noted that boric acid is required for reproducible and complete dephosphorylation within the 5-h heating period. However, its exact role is incompletely understood. Therefore, details of the reaction mechanism, as tentatively proposed in ref. 2, are omitted in this paper. In addition, it should be stressed here, that complete benzoylation can be obtained, even after heating times equal to or exceeding 5 h, when only DMAP is added to the extraction medium. Apparently, DMAP catalyzes the benzoylation of intermediates that are dephosphorylated but not yet benzoylated in the molten benzoic anhydride.

The sequence of dephosphorylation and benzoylation reactions is shown in **Fig. 1.** It is seen that (rapid) dephosphorylation leads to cyclic intermediates, which, upon (slow) benzoylation, give rise to three isomeric diacylglycerobenzoates, i.e., 1,2-, 1,3-, and 2,3-diacylglycerobenzoate. Interestingly, cyclic intermediates, albeit in a slightly different form, were, as early as 1966, proposed by Renkonen (3) to explain acyl migration occurring in acetolysis.

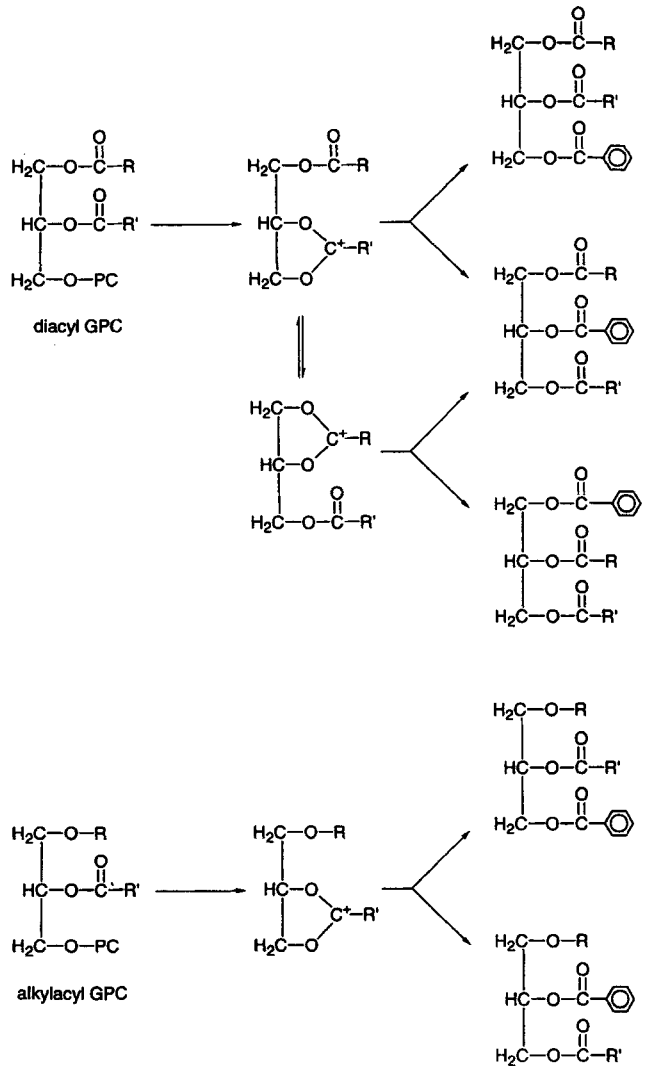


**Fig. 1.** Proposed sequence of dephosphorylation and benzoylation reactions in molten benzoic anhydride during the benzoolysis of diacyl GPC and alkylacyl GPC.

In our previous studies, only homo-acidic diacyl GPCs (R=R′, see Fig. 1) were used. In that case 1,2- and 2,3-diacylglycerobenzoates are not separable by non-chiral chromatography. Consequently, only two peaks, due to 1,2- and 2,3-diacylglycerobenzoates on one hand, and 1,3-diacylglycerobenzoate on the other hand, appeared in the chromatograms obtained with our normal-phase HPLC method (see Materials and Methods). For brevity these peaks were denoted 1,2-diacyl- and 1,3-diacylglycerobenzoate, respectively. This designation is, however, misleading, as is clearly shown in **Fig. 2A,** where the result of a benzoolysis experiment with a mixed-acid GPC,

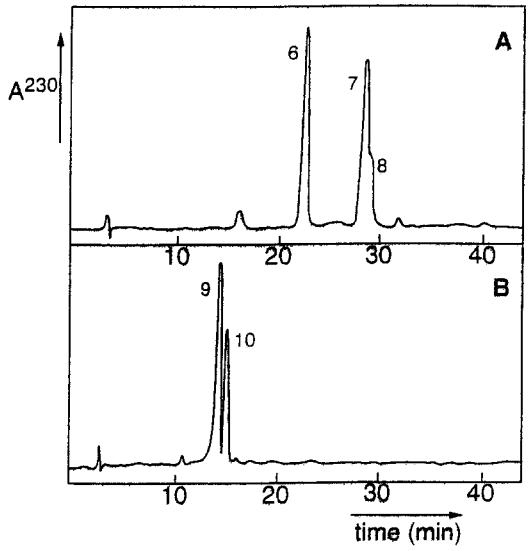


**Fig. 2.** HPLC chromatograms of lipid products obtained in the benzoolysis of 1-oleoyl-2-acetyl GPC (A), and 1-O-hexadecyl-2-acetyl GPC (B). Benzoolysis: 5 h at 100°C in benzoic anhydride in the presence of boric acid (A and B). Extraction of lipid products: in *n*-hexane–benzene 4:0.6 (by volume) in the presence (A) and absence (B) of DMAP. Chromatography: as described in Materials and Methods (A and B). Components: 6, 1-oleoyl-2-acetylglycero-3-benzoate; 7, 1-oleoyl-3-acetylglycero-2-benzoate; 8, 3-acetyl-2-oleoylglycero-1-benzoate; 9, 1-O-hexadecyl-2-acetylglycero-3-benzoate; 10, 1-O-hexadecyl-3-acetylglycero-2-benzoate.

i.e., 1-oleoyl-2-acetyl GPC, is given. The three oleoylacetylglycerobenzoates can be clearly discerned in this chromatogram, their separation being possible by virtue of the large difference of the three acyl groups involved, viz., oleoyl, benzoyl, and acetyl. In Fig. 1, therefore, the formation of three isomers is depicted for the general case (R=R′ and R≠R′).

Whatever the mechanism of the dephosphorylation reaction leading to the cyclic intermediates (and the role of boric acid therein), it appears that the presence of an acyl group at the glycerol *sn*-2 position is essential. As a matter of fact, it was shown that a dialkyl GPC is not dephosphorylated (nor benzoylated) by the benzoolysis procedure (1). On the other hand, an alkylacyl GPC is expected to react in a similar way as a diacyl GPC (see Fig. 1). Indeed, virtually 100% dephosphorylation was found after 4–5 h of heating at 100°C of 1-O-hexadecyl-2-palmitoyl GPC (1). Moreover, benzoolysis experiments with 1-O-hexadecyl-2-acetyl GPC revealed that about 15 h of heating was required for complete benzoylation, yielding 1-O-hexadecyl-2-acetyl- and 1-O-hexadecyl-3-acetyl-glycerobenzoates (see Fig. 2B). Both results, i.e., resistance of di-O-alkyl phosphoglycerides and complete susceptibility of 1-O-alkyl-2-acyl GPC to dephosphorylation, are in agreement with observations made by Kumar, Weintraub, and Hanahan (4) during acetolysis for 5 h at 150°C in a mixture of acetic anhydride and acetic acid.

On the contrary, an alk-1-enylacyl GPC reacts quite differently in molten benzoic anhydride, despite the presence of an *sn*-2 acyl group. In ref. 1 it was shown that isomeric acylglycerodibenzoates are the benzoylated end products in this case. After 5 h of heating at 100°C dephosphorylation was found to proceed to only about 55%. Accordingly, a total yield of acylglycerodibenzoates of only about 55% was then obtained when DMAP was added to the extraction medium. As argued in ref. 1, these results can be explained by supposing that under the acidic conditions in the molten benzoic anhydride the splitting rate of the vinylether bond at the glycerol *sn*-1 position (whereby a fatty aldehyde is released) is much higher than the dephosphorylation rate at the glycerol *sn*-3 position (indeed an addition product of the fatty aldehyde with benzoic anhydride was found in the lipid extract). However, in contrast to the reaction mechanism tentatively proposed in ref. 1, we now tend to think that this reaction leads to 2-acyl-lyso GPC as the first intermediate product (see **Fig. 3**). In theory, then, this species can be imagined to react further in three different ways, visualized in Fig. 3 by reaction routes I, II, and III, respectively. In route I it is dephosphorylated in the same way as depicted in Fig. 1 for an alkylacyl GPC and subsequently benzoylated (twice). This sequence of reactions, however, would hardly explain the hampered dephosphorylation of alk-1-enylacyl GPC as compared to that of diacyl- and alkylacyl GPC. In all cases an *sn*-2 acyl group-assisted dephosphorylation reaction seems equally possible. In route II, the hydroxyl group of 2-acyl-lyso GPC is first benzoylated and the 1-benzoyl-2-acyl GPC thus produced is subsequently dephosphorylated and (once more) benzoylated as depicted in Fig. 1 for a diacyl GPC with two aliphatic acyl chains. Benzoylation of a free hydroxyl group is a rather slow process under our benzoolysis conditions, as was shown in ref. 2 by experiments with diacylglycerols. Thus, although in route II dephosphorylation is preceded by relatively slow benzoylation, the hampered dephosphorylation of alk-1-enylacyl GPC as compared to that of diacyl- and alkylacyl GPC is still not easily explained, due to the presence of an *sn*-2 acyl group to assist in the dephosphorylation.

In route III the plasmalogen-derived 2-acyl-lyso GPC is supposed to rapidly isomerize to 1-acyl-lyso GPC, which subsequently undergoes a sequence of reactions analogous to those in route II. *sn*-2-Acyl group-assisted dephosphorylation via this route III would then only become possible after the slow benzoylation of the free hydroxyl group at the *sn*-2-position and could explain the hampered dephosphorylation as experimentally observed for alk-1-enylacyl GPC.

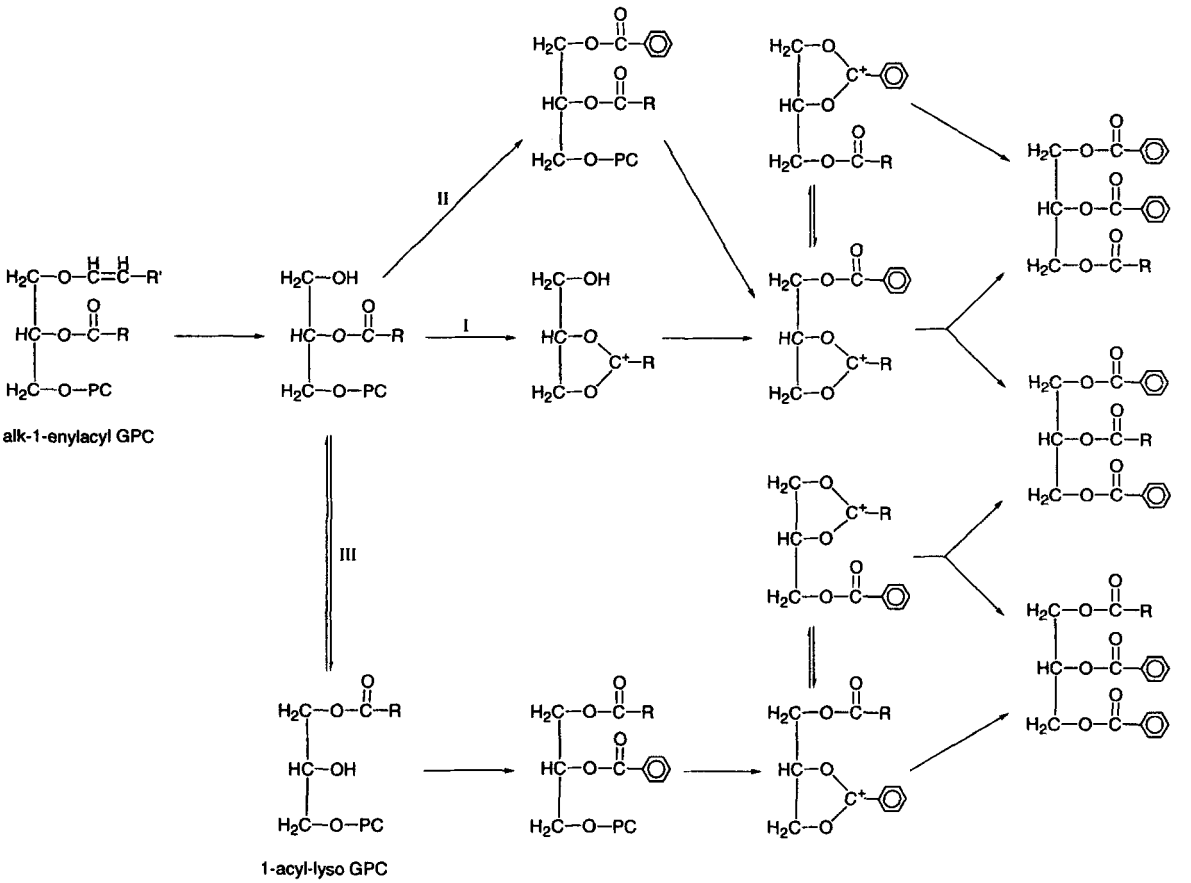


**Fig. 3.** Possible sequences of dephosphorylation and benzoylation reactions in molten benzoic anhydride during the benzoolysis of alk-1-enylacyl GPC and 1-acyl-lyso GPC.

The contribution of each of these routes will depend on the relative rates of dephosphorylation (route I), benzoylation (routes II and III), and isomerization (route III). However, as reaction via routes I and II is not in accord with the experimentally observed hampered dephosphorylation, and as benzoylation presumably proceeds at a smaller rate than isomerization to the more stable 1-acyl-lyso GPC, reaction via route III can be expected to prevail.

In the scheme of Fig. 3 the loss of a fatty aldehyde from the plasmalogen is supposed to occur almost instantly, i.e., at a much higher rate than any of the following events by routes I, II, or III. Therefore, if indeed benzoolysis of a plasmalogen proceeds mainly via route III, benzoolysis of a 1-acyl-lyso GPC (see Fig. 3) should lead by the same reactions to the same end products and the process should also exhibit hampered dephosphorylation.

In order to check this, the extent of dephosphorylation was determined for both processes after a 5-h heating period at 100°C. The 5-h period is an arbitrary choice; it should be mentioned that, for both GPCs, dephosphorylation continues steadily for heating times beyond 5 h (e.g., for 1-O-hexadecenyl-2-oleoyl GPC it increases from about 54 to 67% on prolongation of the heating time from 5 to 10 h, see ref. 1).

As stated before, the extent of dephosphorylation can be found by determining the yield of benzoylated end products upon extraction in the presence of DMAP. Therefore, the latter was measured by applying the procedure to mixtures containing, in known molar ratios, dipalmitoyl GPC and 1-palmitoyl-lyso GPC on the one hand, and dipalmitoyl GPC and 1-O-hexadecenyl-2-oleoyl GPC on the other hand. **Figure 4** shows the HPLC chromatograms obtained. It is evident that acylglycerodibenzoates are formed in both cases, i.e., from acyl-lyso GPC and alk-1-enylacyl GPC, together with dipalmitoylglycerobenzoates from dipalmitoyl GPC. The latter can be considered as an internal standard, as it has been shown (1) that under these conditions its benzoylation is essentially complete. Thus, from the ratio of the total area of the fused acylglycerodibenzoate peaks to that of the diacylglycerobenzoates peak and the known composition of the starting GPC mixture,

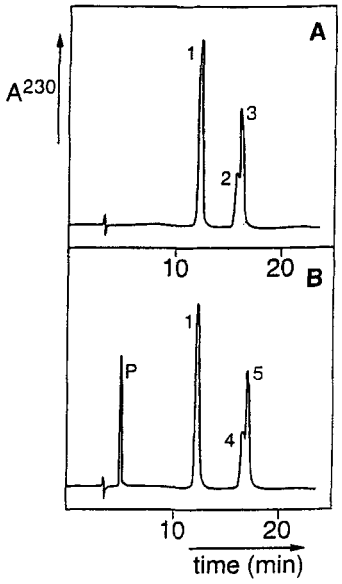


**Fig. 4.** HPLC chromatograms of lipid products obtained in the benzoolysis of a mixture of dipalmitoyl GPC and 1-palmitoyl-2-lyso GPC (A; mol. fraction 1-palmitoyl-2-lyso GPC = 0.471), or a mixture of dipalmitoyl GPC and 1-O-hexadecenyl-2-oleoyl GPC (B; mol. fraction 1-O-hexadecenyl-2-oleoyl GPC = 0.504). Benzoolysis (5 h, 100°C), extraction of lipid products (in the presence of DMAP) and chromatography as described in Materials and Methods. Components: 1, 1,2-plus 2,3- and 1,3-dipalmitoylglycerobenzoate; 2, 2-palmitoylglycerodibenzoate; 3, 1-plus 3-palmitoylglycerodibenzoate; 4, 2-oleoylglycerodibenzoate; 5, 1-plus 3-oleoylglycerodibenzoate; P, addition product of benzoic anhydride and hexadecanal (see ref. 1).

the yield of acylglycerodibenzoates can be found (taking into account a factor of 2 to correct for the 2-fold higher UV absorption of acylglycerodibenzoates compared to diacylglycerobenzoates). The results are given in **Table 1.** Because of its crucial effect as a correction factor in subclass quantitation, the yield of acylglycerodibenzoates from 1-O-hexadecenyl-2-oleoyl GPC was determined (in ref. 1) for a rather high number of individual benzoolysis experiments and in two duplicate series at widely different levels of the relative plasmalogen content. For the sake of comparison, this experimental approach was also adopted for the mixtures containing palmitoyl-lyso GPC.

It is seen in Table 1 that the percentage yield of acylglycerodibenzoates is independent of the molar ratios in the mixtures. Moreover, for both mixtures, the pooled mean yields are identical within experimental error. These results, therefore, indicate that the degree of dephosphorylation (after 5 h of heating at 100°C) is equal for the plasmalogen and the 1-acyl-lyso GPC. This provides strong support in favor of a common reaction path (see Fig. 3, route III).

However, the possibility cannot be excluded that, for both processes, some acylglycerodibenzoates are formed by route I (Fig. 3). In that case, the lipid extract would contain some 3-acylglycerodibenzoate besides 1-acylglycerodibenzoate. Obviously, these two isomers would not be separated by our HPLC method. Moreover, for diacyl GPC it was shown (2), that diacylglycerobenzoate isomer ratios change considerably upon addition of DMAP to the extraction medium. In view of these uncertainties the eventuality of two isomeric acylglycerodibenzoates is left open in the labelling of peaks 3 and 5 in Fig. 4 (see legend).

As the acylglycerodibenzoate peaks in Fig. 4 are not well resolved, the proportion of 2-acylglycerodibenzoate in the isomer mixture cannot be determined exactly. However, integrated peak areas indicate that the mole fraction of 2-acylglycerodibenzoate is essentially the same (about 0.3) for both starting GPCs, i.e., alk-1-enylacyl GPC and 1-acyl-lyso GPC.

TABLE 1. Benzoolysis of mixtures of radyl glycerophosphocholines

| Mixture[a] | X[b] | Yield of Acylglycerodibenzoates |
|---|---|---|
| | | % |
| Palmitoyl-lyso GPC | 0.471 | 53.5 ± 1.7 (10) |
| | 0.183 | 55.8 ± 1.6 (5) |
| Mean | | 54.3 ± 1.3 (15) |
| 1-Hexadecenyl-2-oleoyl GPC[c] | 0.504 | 53.1 ± 1.3 (9) |
| | 0.203 | 53.9 ± 1.2 (8) |
| Mean | | 53.5 ± 0.9 (17) |

Results are given as means ± standard deviation with the number of individual benzoolysis experiments in parentheses.

[a]Mixtures consist of dipalmitoyl GPC and the indicated lipid in mole fractions x as tabulated in the second column.

[b]x represents the mole fraction of palmitoyl-lyso GPC or 1-hexadecenyl-2-oleoyl GPC in the benzoolysis mixture.

[c]Results with the plasmalogen-containing mixtures were reported in ref. 1; for completeness, they are repeated here.

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