Facile syntheses of acyl dihydroxyacetone phosphates and lysophosphatidic acids having different acyl groups

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Abstract In this study, we report novel and simple chemical syntheses of acyl dihydroxyacetone phosphate (DHAP) and 1-acyl glycerol-3-phosphate [lysophosphatidic acid (LPA)], key intermediaries in the formation of glycerolipids containing ester and ether bonds. The synthesis of acyl DHAPs involved acylating the dimethyl ketal of DHAP by acid anhydride using 4-pyrrolidinopyridine as the catalyst, and the resulting product was deketalized by HClO4 in acetone to produce acyl DHAP. The acid anhydride was either added directly or generated in the reaction mixture from the corresponding fatty acid using dicyclohexylcarbodiimide as the condensing agent. Using these methods, a number of acyl DHAPs having short-, medium-, and long-chain saturated and unsaturated acyl groups were synthesized, with overall yields from 37% to 75%. The activities of these acyl DHAPs as substrates for guinea pig liver peroxisomal acyl DHAP:NADPH reductase and alkyl DHAP synthase were then determined. Next, starting from these acyl DHAPs, a variety of LPAs were synthesized by chemical reduction of the ketone group. Biological activities of these LPAs were determined by measuring their relative abilities to release intracellular Ca2+ via the LPA receptor. A combined chemical-enzymatic method is also described to prepare the natural LPA from the racemic mixture.—Das, A. K., J. E. Milam, R. C. Reddy, and A. K. Hajra. Facile syntheses of acyl dihydroxyacetone phosphates and lysophosphatidic acids having different acyl groups. J. Lipid Res. 2006. 47:1874–1880.

Supplementary key words acylation • dihydroxyacetone phosphate dimethyl ketal • acyl dihydroxyacetone phosphate reductase • alkyl dihydroxyacetone phosphate synthase • sodium cyanoborohydride reduction

MATERIALS AND METHODS

DHAP-DMK di(cyclohexylammonium) salt, different acid anhydrides, fatty acids, 4-pyrrolidinopyridine (4-PrPy), dicyclohexylcarbodiimide (DCC), NaCNBH3, porcine pancreatic phospholipase A2 (1,000 U/mg protein), and anhydrous pyridine were obtained from Sigma-Aldrich (St. Louis, MO). Unisil (activated silicic acid) was from Clarkson Chemical Co. (Williamport, PA), and AG 50W-X4 (H+ form) resin was from Bio-Rad (Richmond, CA). Dry, alcohol-free chloroform was prepared by

Abbreviations: DCC, dicyclohexylcarbodiimide; DHAP, dihydroxyacetone phosphate; DHAP-DMK, dimethyl ketal of dihydroxyacetone phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PMR, proton magnetic resonance; 4-PrPy, 4-pyrrolidinopyridine.

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Synthesis of palmitoyl DHAP-DMK

**Method A: palmitic anhydride as the acylating agent.** DHAP-DMK was acylated directly by palmitic anhydride to synthesize palmitoyl DHAP-DMK (Scheme 1). The cyclohexylammonium salt of DHAP-DMK was converted to the pyridinium salt by passing its aqueous solution (10 mg or 20 µmol in 1.0 ml) through a column (4 cm × 0.7 cm) of the pyridinium form of the AG 50W cation-exchange resin. Pyridinium salt of AG 50W-X4 (100–200 mesh) was prepared by treating the H⁺ form of the resin with 50% aqueous pyridine. After eluting the pyridinium salt of DHAP-DMK from the column with water (total volume, 3.0 ml), it was lyophilized in a Speed-Vac concentrator and stored over P₂O₅. The dry glassy mass was dissolved in 0.3 ml of anhydrous CHCl₃ and 0.3 ml of anhydrous pyridine. After mixing, N₂ was passed through the mixture to remove traces of water, and the mixture was then stirred magnetically for 3 h at room temperature. When the reaction was complete, the products were examined by TLC (CHCl₃/methanol/acetic acid/water, 100:40:12:4), and two main phospholipid-positive spots having relative mobility (Rf) values of 0.60 and 0.85, along with an occasional minor one (Rf = 0.65), were seen to be present on the chromatogram. The compound having the Rf of 0.60 was identified as the acyl DHAP-DMK (see below), and the two faster moving phospholipids were tentatively identified as the monopalmitoyl and dipalmitoyl mixed anhydrides with the phosphoric group of the same compound (9). The pyridine and chloroform from the reaction mixture were removed by blowing a stream of dry N₂, and the residue was dissolved in 3.0 ml of CHCl₃/methanol (2:1). To the solution, 0.75 ml of 0.5 M HCl was added, mixed, and centrifuged at low speed (600 g, 10 min) to separate the phases. The upper aqueous phase containing most of the catalyst (4-PrPy) was removed, and the lower phase containing the lipids was washed with the acidic theoretical upper phase (CHCl₃/methanol/0.01 M HCl, 3:48:47) to remove the rest of the 4-PrPy. The solvents from the lower phase were evaporated off by blowing a stream of dry N₂, and the residue was transferred onto a 0.5 g Unisil column (0.5 cm inner diameter) with CHCl₃. The column was first eluted with 15 ml of chloroform (fraction 1), which eluted the excess palmitic acid, and the phospholipid was then eluted using 15 ml of 30% methanol in chloroform (fraction 2). TLC analysis, performed as described above, of fraction 2 showed the presence of only a single compound (Rf = 0.60), which demonstrated positive reactions with molybdenum blue spray for phospholipids (16) and primuline spray for lipids (17) and was identified as the palmitoyl DHAP-DMK by NMR analysis (see below). The amount of phospholipid present in this fraction, as determined by phosphate analysis, was 15.6 µmol or 78% of the DHAP-DMK used for the reaction. PMR analysis of this phospholipid in CDCl₃, using tetramethylsilane as the internal standard, showed all of the peaks expected for palmitoyl DHAP-DMK: δ 0.75 (t, 3H for the terminal (C-16) CH₃ of the palmitoyl group), 1.15 (s, sharp 2H associated with C-4 to C-15 of the palmitoyl group), 1.46 (t, 2H for C-3 of the palmitoyl moiety), 2.21 (t, 2H at C-2 of the palmitoyl group), 3.15 (s, 6H of the 2-methoxy groups of the ketal), 3.45 (s, 2H at the carbon atom of DHAP esterified to the phosphoric acid), and 3.85 and 3.95 (each as a singlet for the 2H attached to the carbon atom of DHAP esterified to the phosphoric acid).

**Method B: using a palmitic anhydride-generating system.** The acylation reaction described above can also be carried out by generating palmitic anhydride from palmitic acid in the reaction mixture using DCC as the condensing agent (18), as described below. Twenty micromoles of lyophilized...
The acyl DHAP-DMK, synthesized as described above, was deketalized to the free ketone by treatment with HClO₄ in acetone (Scheme 1). The palmityl DHAP-DMK, eluted from the Unisil column, was converted to the free acid form by dissolving it in CHCl₃/methanol and washing with aqueous HCl as described above. After removing CHCl₃ and methanol, it was dissolved in a mixture of 3.0 ml of acetone and 15 μl of 70% HClO₄ and incubated at 37°C. The time course for this deketalization reaction was monitored by TLC analysis, as described above, in which the parent compound (Rf = 0.60) separated well from the product, palmityl DHAP (Rf = 0.35). After the deketalization was found to be complete (~4 h), 60 μl of 3.0 M sodium acetate was added to the reaction mixture to neutralize the HClO₄, and the acetone was removed from the mixture by blowing a stream of N₂. The residue was suspended in 4.5 ml of CHCl₃/methanol (1:2) and acidified by adding 10 μl of 6.0 M HCl. The insoluble materials were removed by centrifugation, and 2.7 ml of 0.05 M HCl and 1.5 ml of CHCl₃ were added to the extract. After mixing and low-speed centrifugation (600 g, 10 min), the upper aqueous phase was removed and the solvents were evaporated off from the lower phase by blowing N₂. The residue was dissolved in a small volume of CHCl₃ and subjected to Unisil chromatography as described above. The product, palmityl DHAP-DMK, was eluted in fraction 2. From phosphate analysis, the yield of the product was calculated to be 72% of the DHAP-DMK used.

Deketalization

The acyl DHAP-DMK, synthesized as described above, was deketalized to the free ketone by treatment with HClO₄ in acetone (Scheme 1). The palmityl DHAP-DMK, eluted from the Unisil column, was converted to the free acid form by dissolving it in CHCl₃/methanol and washing with aqueous HCl as described above. After removing CHCl₃ and methanol, it was dissolved in a mixture of 3.0 ml of acetone and 15 μl of 70% HClO₄ and incubated at 37°C. The time course for this deketalization reaction was monitored by TLC analysis, as described above, in which the parent compound (Rf = 0.60) separated well from the product, palmityl DHAP (Rf = 0.35). After the deketalization was found to be complete (~4 h), 60 μl of 3.0 M sodium acetate was added to the reaction mixture to neutralize the HClO₄, and the acetone was removed from the mixture by blowing a stream of N₂. The residue was suspended in 4.5 ml of CHCl₃/methanol (1:2) and acidified by adding 10 μl of 6.0 M HCl. The insoluble materials were removed by centrifugation, and 2.7 ml of 0.05 M HCl and 1.5 ml of CHCl₃ were added to the extract. After mixing and low-speed centrifugation (600 g, 10 min), the upper aqueous phase was removed and the solvents were evaporated off from the lower phase by blowing N₂. The residue was dissolved in a small volume of CHCl₃ and subjected to Unisil chromatography as described above. The product, palmityl DHAP-DMK, was eluted in fraction 2. From phosphate analysis, the yield of the product was calculated to be 72% of the DHAP-DMK used. The synthesized acyl DHAPs were tested as substrates for two peroxisomal enzymes, acyl DHAP:NADPH reductase and alkyl DHAP synthase (1). The enzymatic assays were performed as described previously (19, 20), and purified guinea pig liver peroxisomes (21) were used as the source of the enzymes. The specific activities of the enzymes, determined using each of the synthesized acyl DHAPs as the substrate, are shown in Fig. 1A, B.

Synthesis of LPA

Palmityl DHAP was reduced with sodium cyanoborohydride (NaCNBH₃) to make the corresponding LPA (Scheme 1). Five micromoles (2 mg) of the synthetic palmityl DHAP in a screw-top test tube was dissolved in 3.0 ml of methanol, and 1.0 ml of 0.2 M NaCNBH₃ was added, followed by the addition of 0.1 ml of glacial acetic acid. The tube was capped, and the mixture was incubated at 37°C for 4 h. The reaction was stopped by adding 4.0 ml of chloroform and 3.6 ml of 0.2 M HCl to the mixture.

| Acyl DHAP Chain Length | Relative Mobility | Yield % |
|------------------------|------------------|--------|
| 6:0                    | 0.22             | 36.8   |
| 8:0                    | 0.26             | 48.9   |
| 10:0                   | 0.29             | 56.4   |
| 12:0                   | 0.32             | 65.6   |
| 14:0                   | 0.34             | 56.4   |
| 16:0                   | 0.35             | 60.0   |
| 18:1 cis               | 0.40             | 74.6   |
| 18:1 trans             | 0.40             | 72.0   |
| 18:2                   | 0.40             | 50.0   |

DHAP, dihydroxyacetone phosphate. Yield calculations are based upon the dimethyl ketal of DHAP used.
After vortexing and centrifuging at 1,000 g for 10 min, the upper layer was aspirated off and the lower chloroform layer was washed once with 6.5 ml of chloroform/methanol/0.01 M HCl (3:48:47). The solvents from the washed lower layer were removed by blowing a stream of N2. TLC of the product using chloroform/methanol/5% aqueous NaHSO3 as the developing solvent showed the presence of a single primuline- and molybdenum blue-positive spot of Rf = 0.35, the same as authentic LPA, indicating complete reduction of palmitoyl DHAP. In this bisulfite-containing TLC solvent system, acyl DHAP migrates more slowly (Rf = 0.20) than LPA (22).

The synthesized LPA was purified by Unisil column chromatography as described above. LPA was mainly eluted out in the 30% methanol-70% chloroform fraction (fraction 2). Organic phosphate analysis of this purified LPA indicated that the yield was 78% of the palmitoyl DHAP used. On TLC analysis using different solvent systems, as described above, the synthesized LPA always had the same migration rate as authentic LPA. Proton NMR analysis showed all of the peaks expected for 1-palmitoyl glycero-3-phosphate:

1-Oleoyl-rac-glycero-3-phosphate and 1-elaidoyl-rac-glycero-3-phosphate also were synthesized by the NaCNBH3 reduction of the ketone group of the corresponding synthetic acyl DHAPs using the same method described above for the palmitoyl LPA. The yields were between 75% and 80% of the starting compounds.

**Biological activity of the synthetic LPAs**

The identity of the synthetic LPAs was also verified by measuring their biological activity in activating specific cell surface LPA receptors. Both R- and S-(sn-3 and sn-1) isomers of LPA were shown to be equally effective at activating these G-protein-coupled LPA receptors (12). We tested their effect in an established assay of Ca2+ mobilization in human A431 cells. Three synthesized LPAs (oleoyl, palmitoyl, and elaidoyl as their racemic mixture) and one sn-3 (or R-) isomer of LPA obtained from Sigma Co. were used for this purpose. At a saturating receptor concentration (1 μM), all four compounds were equally potent (Fig. 2). However, at a nonsaturating dose (500 nM), we observed a significant reduction in the ability of 1-palmitoyl and 1-elaidoyl LPA to elicit Ca2+ mobilization compared with oleoyl LPA, either synthesized as described here or in a commercially available form (Fig. 2). These results confirm that the 1-oleoyl analog is the most potent biological form of LPA (12).

**Preparation of 1-palmitoyl-sn-glycero-3-phosphate**

The optically active natural sn-3 (R or L-) isomer of the palmitoyl LPA was prepared from the corresponding racemic mixture by first chemically acylating its C-2 hydroxyl group with oleic acid to phosphatidic acid (PA) followed by hydrolysis by pancreatic phospholipase A2 to natural LPA. Phospholipase A2 hydrolyzes only the sn-3 isomer of phospholipids (23).

The free acid form of synthetic 1-palmitoyl-rac-glycero-3-phosphate (2.5 mg, 6.2 μmol) was dissolved in 0.5 ml of
anhydrous chloroform, and 13.6 mg (24.8 μmol) of oleic anhydride and 2.0 mg (13.4 μmol) of 4-PrPy were added. The mixture was magnetically stirred under N\textsubscript{2} at 35°C in a capped Reactivial (Pierce) for 3.5 h. The products were isolated by solvent extraction under acidic conditions as described above for the isolation of acyl DHAPs. TLC examination (chloroform-methanol-acetic acid-water, 25:10:3:1) demonstrated that the LPA (R\textsubscript{f} 0.35) was completely acylated to PA (R\textsubscript{f} 0.75) and also the formation of some phosphoanhydrides of PA (R\textsubscript{f} values between 0.8 and 0.9). The latter compounds were hydrolyzed to PA by dissolving the products in 2.0 ml of acetone containing 20 μl of 6.0 M HCl and incubating the solution at 37°C for 15 min. The incubation was stopped by adding 50 μl of 3.0 M sodium acetate to the mixture. The acetone was removed from the mixture by blowing a stream of N\textsubscript{2}, the residue was dissolved in 3.0 ml of chloroform-methanol (1:1), and 1.35 ml of 0.05 M HCl was added, mixed, and centrifuged at 1,000 g for 10 min. The upper aqueous layer was removed and the lower layer was washed once with 2.5 ml of Bligh and Dyer (24) solvent extraction under acidic conditions as described above. The oleic acid was removed by eluting the column with chloroform, and the PA was then eluted out with 20% methanol in chloroform. Phosphate analysis showed the yield of PA to be 70% of the starting LPA.

A portion (1.0 mg) of the PA was enzymatically hydrolyzed by suspending it in 1.0 ml of 50 mM Tris buffer (pH 7.5) containing CaCl\textsubscript{2} (10 mM), sodium deoxycholate (1.0 mM), and 30 units of porcine pancreatic phospholipase A\textsubscript{2}. The mixture was magnetically stirred under N\textsubscript{2} in a Reactivial at room temperature for 15 h. The phospholipids were isolated from the mixture by Bligh and Dyer (24) solvent extraction under acidic conditions as described above. TLC examination using the same chloroform-methanol-acetic acid-water solvent system showed the presence of two phosphate-positive lipids having the same R\textsubscript{f} values of LPA (0.35) and PA (0.75). Phosphate analysis of these spots showed that 47% of the racemic PA was hydrolyzed to LPA. The LPA and PA were separated from each other as described below.

A solvent partition method (25) was used to separate the LPA formed after hydrolysis from the less polar unhydrolyzed PA. The remaining mixture obtained after phospholipase A\textsubscript{2} treatment was dispersed in 1.2 ml of citrate-phosphate (0.05–0.1 M, pH 4.4) buffer and dissolved in 4.5 ml of CHCl\textsubscript{3}/methanol (1:2) to which 1.5 ml of water and 1.5 ml of additional CHCl\textsubscript{3} were added to make two phases (24). The upper phase containing the LPA was transferred to another tube, and the lower phase was reextracted with 5.0 ml of CHCl\textsubscript{3}/methanol/water (1:12:12). TLC examination of the washed lower layer showed a single molybdenum blue-positive spot having the same R\textsubscript{f} as authentic PA. This crude preparation, contaminated with free oleic acid, was purified by silicic acid (Unisil) column chromatography as described above. The oleic acid was removed by eluting the column with chloroform, and the PA was then eluted out with 20% methanol in chloroform. Phosphate analysis showed the yield of PA to be 70% of the starting LPA.

DISCUSSION

The two-step chemical synthesis of acyl DHAP and its reduction to LPA are summarized in Scheme 1. In the first
step, the acylation of DHAP-DMK by acid anhydrides was complete in a short period of time (3–4 h), in contrast to the significantly longer time (2–3 days) previously reported to be necessary for similar acylation reactions (9–11). This rapid rate of acylation was probably attributable to our use of a pyridine/CHCl₃ mixture as the solvent, in which the reactants and the catalyst dissolved well, and to the use of a better catalyst, 4-PrPy (26), instead of the dimethylaminopyridine used by others (9–11). The reaction rate was slower when the acyl anhydrides were generated in the reaction mixture from free fatty acids and DCC, so a longer reaction time was necessary. The yield of the acyl DHAP-DMK, however, was the same in both methods. This anhydride-generating system will be particularly useful when the anhydride form of a fatty acid is not commercially available. We synthesized both short-chain (octanoyl) and unsaturated (oleoyl) acyl DHAP with good overall yields (50–60%) using this acid anhydride-generating system.

As reported previously (9), we also found that acid anhydrides acylated not only the hydroxyl group of DHAP but also the acidic hydroxyl groups of the phosphate, forming mixed acid anhydrides. Therefore, a large excess (4×) of acid anhydride was found to be necessary for the maximum yield of the desired product. These acyl phosphates are, of course, more unstable than the ester group and decomposed in acidic solution during purification.

The acyl DHAP-DMK was found to be fairly refractory to acid hydrolysis, such as treatment with methanolic HCl (5), so that during such a long hydrolytic process a part of the acyl ester was also hydrolyzed, resulting in a low yield of the product. We found, however, that treatment of the ketol by the strong perchloric acid in acetone resulted in the rapid formation of the ketolipid with little hydrolysis of the ester bond. This rapid deketalization was probably attributable to transketalization of the lipid ketal with little hydrolysis of the ester bond. This rapid deketalization was probably attributable to transketalization of the lipid ketal with acetone (forming 2,2'-dimethoxypropane), which was used as the solvent.

Using the present method (Scheme 1), we synthesized acyl DHAPs having different acyl chain lengths, including unsaturated ones. The relatively lower yield of the short- and medium-chain acyl DHAPs (Table 1) was attributable to their lower hydrophobicity compared with the long-chain DHAPs, so that there were more losses of these compounds during the purification process. Many of these compounds, such as the short and medium chains and the unsaturated acyl derivatives, have not been synthesized previously. These are probably "unnatural" acyl DHAPs, because it has been shown that only the saturated long-chain acyl CoAs can serve as substrates for the enzymatic acylation of DHAP (27, 28). However, these unnatural derivatives are also used as substrates by the two enzymes that metabolize acyl DHAP (Fig. 1). The specificities of the enzymes toward these acyl DHAPs are somewhat different. Whereas alkyl DHAP synthase uses only the long-chain saturated and unsaturated acyl DHAPs with an abrupt cutoff below C-14, acyl DHAP reductase, although preferring the long-chain acyl DHAPs, also uses the medium-chain acyl DHAPs in decreasing activities with the decreasing length of the acyl chain (Fig. 1). Interestingly, although elaaidoyl DHAP was found to be a good substrate for alkyl DHAP reductase, unlike its cis isomer (oleoyl DHAP), we found that it is a poor substrate for alkyl DHAP synthase (Fig. 1).

These synthetic acyl DHAPs provided us substrates for a simple borohydride reduction method to prepare the corresponding LPAs. Previously, we used NaBH₄ to reduce acyl DHAPs to racemic LPA (25). However, because acyl DHAP is labile at high pH but NaBH₄ is not stable at pH < 7.0, we had to use a buffer to keep the pH at ~8.0, which was sometimes difficult to maintain. The use of CNBH₃⁻ as a substrate for guinea pig liver microsomal LPA acyltransferase (data not shown).

The synthetic methods described here for the preparation of acyl DHAPs and LPAs are simple, and all of the starting chemicals are commercially available. The overall yields are higher than those obtained by previously described methods (3–5). Also, many acyl derivatives (e.g., unsaturated ones) that could not be synthesized by previous methods could be successfully prepared using the methods described here. Using the procedures described above, one could also synthesize PAs having two different acyl groups at C-1 and C-2 of the glycerol moiety. These methods should prove valuable not only for making different acyl DHAPs and LPAs as substrates and ligands for different enzymes and receptors but also for making analogs of these physiologically important intermediates, which may act as inhibitors or antagonists of different enzymes and receptors for these lipids (34).

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