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A rapid and efficient method for migration-free acylation of lysophospholipids: synthesis of phosphatidylcholines with *sn*-2-chain-terminal reporter groups

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Abstract—A rapid and efficient method for migration-free acylation of lysophosphatidylcholines has been developed using ultrasound for agitation of the reaction mixture and glass beads for increasing the surface in the reaction vessel. The products were obtained in good yields and short reaction times. The method has been applied for the preparation of a variety of substituted phospholipid substrates.

Glycerophospholipids, long recognized as biologically important compounds for their contribution to the construction of biological membranes,[1] have recently emerged as essential molecules for the regulation of cell functions by hormones, neurotransmitters, growth factors, and inflammatory cytokines.[2] Specifically, membrane phospholipids are precursors of lipid metabolites with second messenger functions, serving as substrates of a series of phospholipases, lipid kinases, and phosphatases that generate signaling lipid molecules.[2,3] Naturally occurring phospholipids have a wide variety of structures,[1] and some of the most potent compounds are present at low concentrations in the cell, such that studies for establishment of structure–activity relationships require preparation of synthetic phospholipid derivatives.[2–4]

Acylation of lysophospholipids is a commonly used synthetic method for the preparation of mixed-chain phospholipids, since the necessary intermediates are readily available either from enzymatic deacylation of symmetrically substituted diacyl phospholipids (e.g., at the *sn*-2-position with phospholipase A_2),[5] or using one of the recently developed syntheses that afford the desired lysophospholipids directly.[6] However, there are two problems associated with this method: (1) lysophospholipids have low solubility in nonprotic solvents that are required for the acylation step, and (2) acyl migration of the ester group under the reaction conditions often results in a mixture of regioisomers in the products.[5,7] Despite numerous attempts to overcome these problems by using large excess of acylating agents, and avoid heating the reaction mixture to minimize acyl migration, difficulties remain as the reaction times are long, and the yields are modest.[5,7d] These problems are of particular concern when structurally well-defined phospholipids are needed that carry site-specific fluorescent, paramagnetic, or photoactivable probes at the respective *sn*-1- and/or *sn*-2-positions.[8] These compounds are required for physicochemical studies of phospholipid self-assemblies (i.e., monolayers, vesicles, micelles), and investigations of protein–lipid interactions,[9] including mechanistic elucidation of lipolytic enzymes.[8b]

In the present communication we report a rapid and efficient method for acylation of lysophosphatidylcholine, applicable to the synthesis of a wide series of diacyl glycerophosphocholines, including phospholipid derivatives with substituted *sn*-2-chains that carry spectroscopic labels,[8] metal binding functions,[10] as well as electrochemically active reporter groups.[11] The key features of our synthesis are based on the proposition that the reaction between lysophosphatidylcholine and the acylating agents occur at the surface, and that intramolecular acyl migration is decreased as the temperature is

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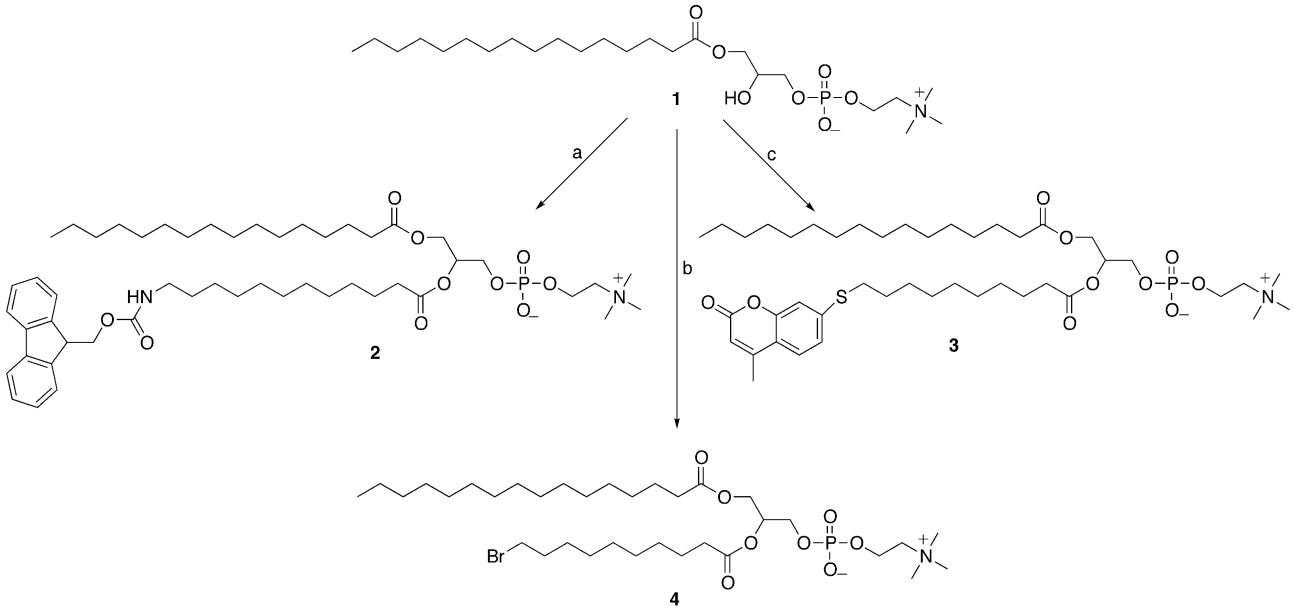
lowered. Thus, we used sonication rather than stirring the reaction mixture, added glass beads to increase the glass surface in the reaction vessel, and maintained the temperature at 25 °C during the course of reaction. Under these conditions acylation of lysophosphatidylcholine occurred much faster, and the target phospholipids were obtained in good yield, and in high regioisomeric purity.

In developing the method we focused on the synthesis of a series of functionalized fatty acid compounds, since (1) phospholipids incorporating spectroscopically labeled fatty acid derivatives are important as membrane probes, as well as phospholipase substrates in their own right, and (2) acylations of lysophospholipids with carboxylic acids carrying chain-terminal functional groups have been reported to be more difficult than similar reactions using unsubstituted substrates.[12] We implemented the synthesis of these derivatives using a two-prong approach: in the first sequence we used substituted fatty acids prepared prior to the acylation step (Scheme 1), while in the second scheme we relied on elaboration of the desired functional group after the appropriate fatty acid precursor has been introduced (Scheme 2).

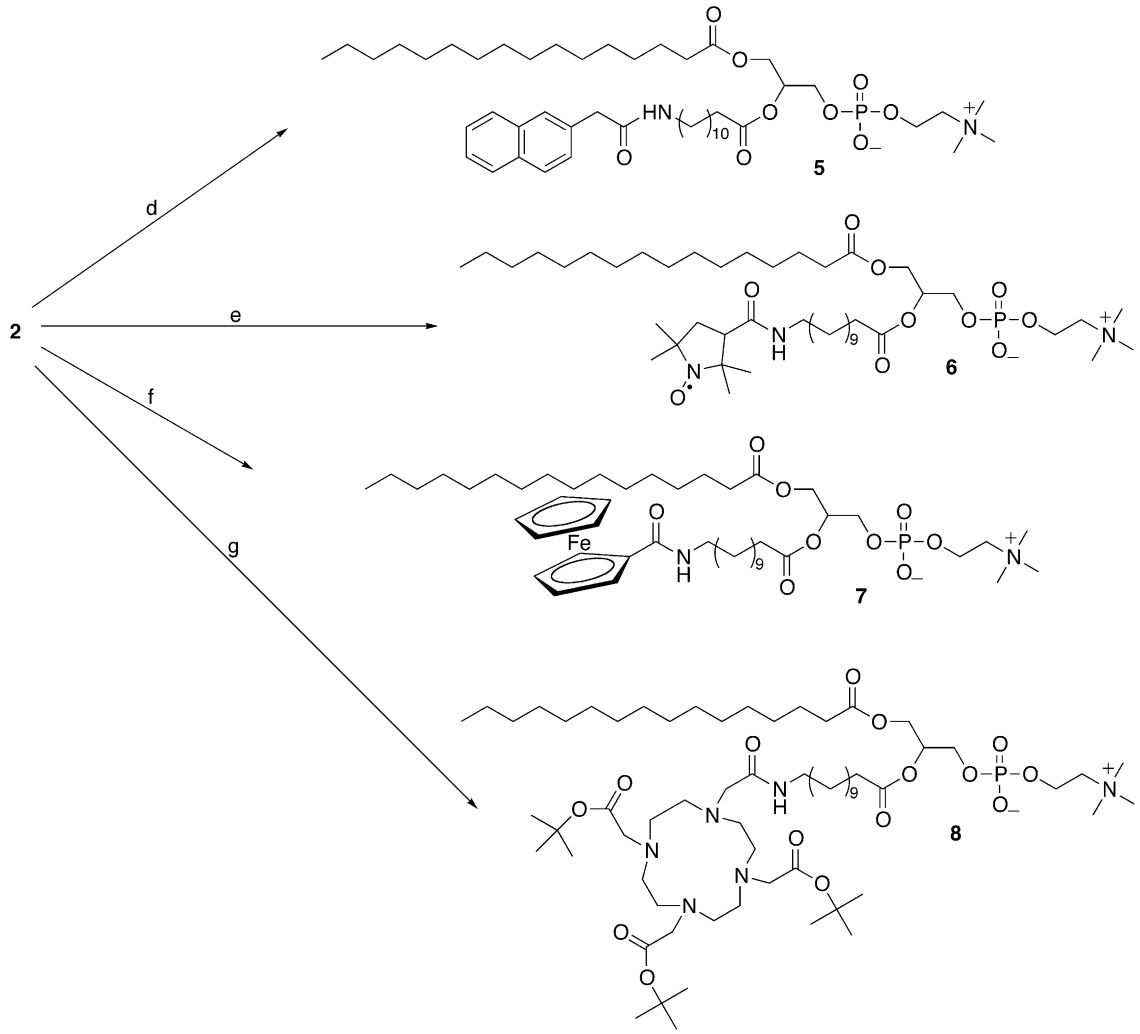
For the synthesis of compound **2** a suspension of 1-palmitoyl-2-hydroxy-*sn*-glycerophosphocholine **1** in chloroform was allowed to react with fivefold molar excess of FMOC-12-aminododecanoic acid/DCC/DMAP, in the presence of glass beads (about 1.0 g/mmol lysophospholipid) sonicated at 25 °C for 5 h. On completion of the reaction the mixture was treated with Bio-Rad AG 50-X (H^+) to remove excess DMAP, followed by chromatography of the product on silica gel ($CHCl_3$/MeOH/H_2O, 65:25:4) to give the target phospholipid as an analytically pure white solid (90.7%). Enzymatic hydrolysis of the product **2** by bee venom phospholipase A_2[13] yielded a strongly fluorescent spot on thin layer chromatography corresponding to FMOC-aminododecanoic acid, with only traces of fluorescence appearing at the phosphate-positive product of **1**.[14] Essentially the same experimental method was used to prepare compounds **3** and **4** carrying a highly fluorescent 7-mercapto-4-methylcoumarin substituted decanoic acid and a fluorescence quenching ω-bromodecanoic acid at the *sn*-2-positions, respectively. These compounds were obtained in 77% and 68% yields.

With the availability of compound **2** we were able to extend the scope of reporter groups to include a new series of phosphatidylcholines **5**–**8** shown in Scheme 2. Specifically, we discovered, that base catalyzed cleavage of the FMOC-function of these compounds with DBU, followed by N-acylation of the amino group provided a facile two-step/one-pot method for introduction of functional groups in case preparation of the substituted fatty acid is cumbersome or inconvenient. Furthermore, since the paramagnetic nitroxide spin label in compound **6** is acid labile,[15] we replaced the acidic ion exchange treatment with an alternative step on silica gel chromatography when purifying the phospholipid product.[16]

In conclusion, we developed an efficient method for acylation of lysophosphatidylcholines. The reactions occur at least ten times faster than under the conditions previously reported, the products are obtained in good yield, and we observed no evidence for acyl migration in the course of the reaction. The strength of the synthesis is in its flexibility with respect to the substituents that can be introduced, and its applicability to the development of new phospholipid analogs with desired target structures for physicochemical and enzymological studies.



Scheme 1. Reagents and conditions: (a) 5 equiv FMOC-NH$(CH_2)_{11}$COOH/DCC/DMAP, $CHCl_3$, glass beads, 25 °C, 5 h; (b) 5 equiv Br$(CH_2)_9$COOH/DCC/DMAP, $CHCl_3$, glass beads, 25 °C, 5 h; (c) 5 equiv 10-(4′-methyl-7′-mercaptocoumarin)-decanoic acid/DCC/DMAP, $CHCl_3$, glass beads 25 °C, 5 h.



Scheme 2. Reagents and conditions: (d) (i) 3 equiv DBU, $CHCl_3$, rt, 30 min, (ii) 1.5 equiv 2-naphthylacetyl chloride/DMAP, $CHCl_3$, 10 min; (e) 2 equiv DBU, $CHCl_3$, 30 min, (ii) 2 equiv 3-carboxy-PROXYL *p*-nitrophenyl ester/DMAP, $CHCl_3$, rt, 16 h; (f) (i) 2 equiv DBU, $CHCl_3$, rt, 30 min, (ii) 1 equiv 1-ferrocenecarboxylic acid *N*-hydroxysuccinimide ester/DMAP, $CHCl_3$, rt, 48 h; (g) (i) 3 equiv DBU, $CHCl_3$, rt, 30 min, (ii) 1 equiv tris-(*tert*-butyl-DOTA) *N*-hydroxysuccinimide ester, rt,16 h.

Typical experimental procedure for acylation: to a suspension of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine **1** (0.387 g, 0.8 mmol) in 25 mL ethanol-free $CHCl_3$ were added FMOC-aminolauric acid (1.605 g, 4 mmol), DCC (0.805 g, 4 mmol), DMAP, (0.477 g, 4 mmol), and 1.0 g of glass beads. The mixture was sonicated for 5 h at 25 °C. After 5 h ultrasound to this mixture were added 10 mL Bio-Rad AG 50-X (H^+) ion exchange resin and the suspension stirred for 10 min at room temperature. The mixture was then filtered, and the solid washed with 30 mL of $CHCl_3$/MeOH (1:1). The solvents collected were evaporated under reduced pressure and the residue was chromatographed on silica gel using $CHCl_3$/MeOH/H_2O (65:25:4) as eluant. The fractions containing the product were combined, evaporated, redissolved in benzene and freeze-dried to give **2** as a white solid (0.664 g, 90.7%).

Derivatization of sn-2-chain-terminal: to a stirred solution of **2** (0.275 g, 0.3 mmol) in 10 mL ethanol-free $CHCl_3$ was added DBU (0.092 g, 0.6 mmol) in 1 mL $CHCl_3$ at room temperature. After about 30 min stirring TLC ($CHCl_3$/MeOH/H_2O, 65:25:4) showed that no more **2** left in the reaction mixture, having been replaced by a new, ninhydrin-positive spot appearing close to the origin. To this solution were added DMAP (0.052 g, 0.42 mmol) and 3-carboxy-PROXYL *p*-nitrophenyl ester (0.184 g, 0.6 mmol) and the mixture was stirred overnight. The solution was loaded on a silica gel column packed with $CHCl_3$ and chromatographed with a gradient of $CHCl_3$/MeOH until all DMAP was eluted, followed by $CHCl_3$/MeOH/H_2O (65:25:4). The fractions containing the product were combined, evaporated, re-dissolved in benzene and freeze-dried to give **6** (0.159 g, 61.5%) as a pale yellow solid.

Acknowledgements

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16. All new compounds were characterized by IR, ^{1}H, ^{13}C NMR, HRMS, and elemental analysis. Selected data for compound **2**: IR (Nujol): 3330, 1728, 1690, 1534, 1246 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.87 (br t, 3H), 1.22 (br s, 38H), 1.50–1.60 (m, 6H), 2.20–2.30 (m, 4H), 3.20 (m, 2H), 3.35 (br s, 9H), 3.60–3.90 (m, 5H), 4.10–4.40 (m, 6H), 4.85 (m, 1H), 5.20 (m, 1H), 7.26–7.40 (m, 4H), 7.58 (d, 2H, J = 7.3 Hz), 7.75 (d, 2H, J = 7.3 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 14.26, 22.83, 25.08, 26.92, 29.50 (br), 29.86, 34.45, 41.26, 47.45, 54.52, 59.45, 63.14, 63.55, 66.62, 70.60, 120.08, 125.18, 127.15, 127.78, 141.44, 144.17, 156.57, 173.33, 173.71. Anal. Calcd for $C_{51}H_{83}N_2O_{10}P{\cdot}3H_2O$: C, 63.20; H, 9.26; N, 2.89. Found: C, 63.59; H, 8.86; N, 2.85. MS MH^+ $C_{51}H_{83}N_2O_{10}PH$ Calcd: 915.5858. Found: 915.5873. $[\alpha]_D^{25}$ +5.0 (c 1.01, $CHCl_3$/MeOH, 4:1). R_f ($CHCl_3$/MeOH/H_2O, 65:25:4) 0.52. Compound **3**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.87 (br t, 3H), 1.24 (br s, 34H), 1.50–1.60 (br m, 4H), 2.23–2.29 (m, 4H), 3.39 (s, 3H), 2.94 (t, 2H, J = 7 Hz), 3.37 (br s, 9H), 3.60–4.01 (br m, 4H), 4.10–4.18 (m, 1H), 4.22–4.40 (m, 4H), 5.20 (m, 1H), 6.20 (s, 1H), 7.12–7.43 (m, 4H). R_f ($CHCl_3$/MeOH/H_2O, 65:25:4) 0.48. Anal. Calcd for $C_{44}H_{74}NO_{10}PS{\cdot}2.5H_2O$: C, 59.71; H, 9.00; N, 1.58. Found: C, 59.85; H, 8.86; N, 1.61. FAB-MS MH^+ $C_{44}H_{74}NO_{10}PSH$ Calcd: 840.4844. Found: 840.4880. $[\alpha]_D^{25}$ +4.6 (c 0.98, $CHCl_3$/MeOH, 4:1). Fluorescence λ_{em} 413 nm, λ_{exc} 335 nm (MeOH). Compound **4**: FAB-MS MH^+ $C_{34}H_{67}BrNO_8PH$ Calcd: 728.3836. Found: 728.3820. Compound **5**: FAB-MS MH^+ $C_{45}H_{87}N_3O_{10}PH$ Calcd: 861.5758. Found: 861.5739. Compound **6**: FAB-MS MH^+ $C_{45}H_{87}N_3O_{10}PH$ Calcd: 861.5758. Found: 861.5739. Compound **7**: FAB-MS MH^+ $C_{47}H_{81}FeN_2O_9PH$ Calcd: 905.5102. Found: 905.5144. $[\alpha]_D^{25}$ +5.0 (c 1.01, $CHCl_3$/MeOH, 4:1). E_{ox} = 79 mV versus ferrocene (MeCN/$CHCl_3$, 5:0.2, 0.1 M Bu_4NPF_6). Compound **8**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.85 (br t, 3H), 1.21 (br s, 40H), 1.41 (br s, 31H), 2.23 (br m, 4H), 2.81 (br m, 16H), 3.21 (br s, 8H), 3.35 (br s, 9H), 3.60–3.90 (m, 4H), 4.05–4.15 (m, 1H), 4.20–4.40 (m, 4H), 5.15 (m, 1H). FAB-MS MH^+ $C_{64}H_{123}N_6O_{15}PH$ Calcd: 1247.8857. Found: 1247.8840. $[\alpha]_D^{25}$ +3.6 (c 0.84, $CHCl_3$/MeOH, 4:1). R_f ($CHCl_3$/MeOH/H_2O, 65:25:4) 0.48.