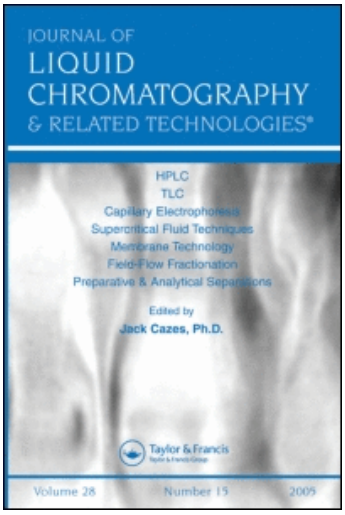

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PURIFICATION OF SYNTHETIC LIPOPEPTIDE CONJUGATES BY LIQUID CHROMATOGRAPHY

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ABSTRACT

Conventional normal phase liquid chromatography (LC) was implemented for the purification of a synthetic lipid-peptide conjugate and its electrophilic lipid precursor. N-bromoacetic distearoylphosphatidylethanolamine (DSPE-$COCH_2Br$) was extracted from the crude reaction mixture and purified in a single chromatographic step with a gradient of chloroform, methanol, and 50% aqueous acetic acid. This compound was covalently linked to Ser-Phe-Leu-Leu-Arg-Asn-$(\beta Ala)_3$-Tyr-$NHCH_2CH_2SH$, a hydrophilic decapeptide, and the conjugate was both extracted and purified in a single step by normal phase gradient LC using a hand-packed 3 mL Sep-Pak™ column. The eluent was a mixture of hexane, chloroform, 2-propanol, acetic acid, and water. Runs were monitored by thin layer chromatography and the plates developed with iodine and ninhydrin.

INTRODUCTION

Recent interest in liposome-based targeted drug delivery and membrane-mimetic approaches for advanced materials has produced a growing list of derivatized phospholipids. Recognition molecules (biotin, antigens, antibodies), drugs (methotrexate), and enzymes have all been conjugated to phospholipids (1). Purification of these compounds is often difficult, particularly as the amphiphilic

character of the molecule increases. However, successful purification has been achieved with normal phase thin layer chromatography (TLC) for phosphatidylethanolamine (PE)-biotin (2), dinitrophenylated PE (3), ω-carboxyalkyl PE (4), and methotrexate-conjugated PE (5, 6). Similarly, high-performance liquid chromatography (HPLC) and liquid-liquid extraction (LLE) have been implemented for the recovery of lipid-biotin conjugates and N-(4-(*p*-maleimidophenyl)butyryl) phosphatidylethanolamine (PE-MPB), respectively (7, 8).

Nevertheless, HPLC remains limited by its expense, and while TLC and LLE are much less costly, they are ill-suited for scaled-up preparative work. This report describes the application of micro liquid chromatography (LC) to the purification of both an electrophilic phospholipid derivative and its highly amphiphilic conjugate to a decapeptide. The advantage of this technique is its low cost and ease of implementation, since the compounds can be extracted from the crude reaction mixture and purified in a single LC step.

MATERIALS AND METHODS

Materials

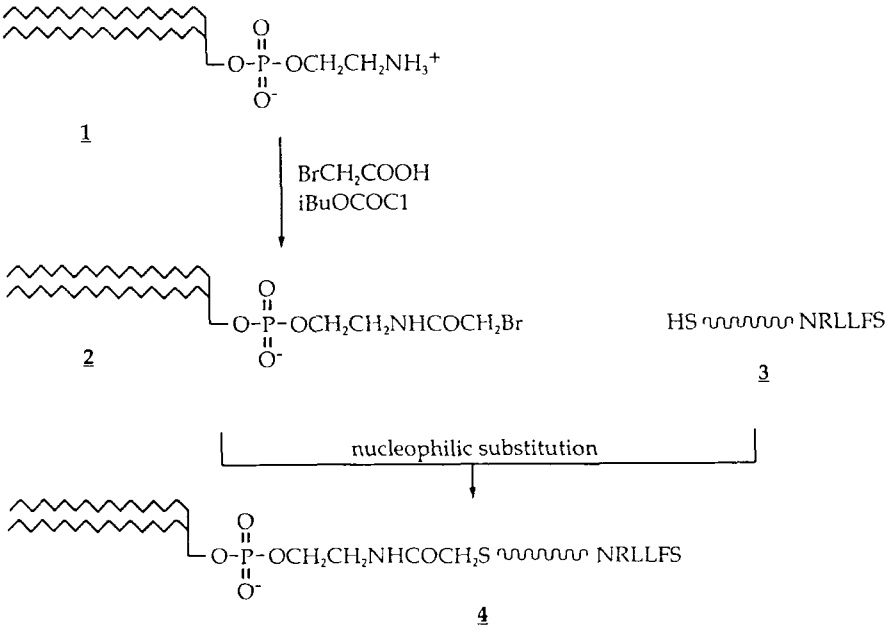
Routine TLC was performed using silica plates (20x20 cm, 250 μm) purchased from Whatman and cut to a 4x7 cm dimension. The plates were developed with iodine for the detection of all compounds. Free amines were specifically detected with a ninhydrin spray (0.2 M in absolute ethanol), and free thiols were detected by reaction with Ellman's reagent. Fine silica (silica gel Davisil™, grade 633, 200-425 mesh, 60 Å) was purchased from Aldrich. Filtration mini-columns (3-mL capacity) used for micro-LC were supplied by Baker (#7121-03). An 8-port Sep-Pak™ cartridge rack by Waters Associates was used to apply negative pressure onto the micro-LC columns. All solvents were HPLC grade from Fisher Scientific. The synthesis of N-bromoacetyl-distearoylphosphatidylethanolamine (DSPE-$COCH_2Br$) and lipopeptide conjugates has been described in greater detail elsewhere (9).

Purification of DSPE-$COCH_2Br$

Eighty-one and a half milligrams of crude DSPE-$COCH_2Br$ were dissolved in 0.6 mL $CHCl_3$/MeOH 95/5 v/v and loaded on a glass chromatography column which contained a 2x23 cm silica packing. Rinsing was performed with a total of 2x0.4 mL of the same solvent. Elution was conducted with the following gradient of $CHCl_3$/MeOH/$AcOH_{50\%aq}$ v/v/v: 10 mL, 95/5/0, fraction [1]; 10 mL, 93/7/0, [2]; 10 mL, 91/9/0, [3]; 10 mL, 90/10/0.5, [4]; 10 mL, 88.6/9.9/1.5, [5]; 30 mL, 88.7/9.8/2.5, [6-14]; 30 mL, 84.8/12.7/2.5, [15-23]; 30 mL, 82.6/14.5/2.9, [24-26]; 30 mL, 77.3/19.3/3.4, [27-28]; 30 mL, 73.3/21.9/4.8, [29-31]; 30 mL, 69.5/25.7/4.8, [32-34]; and 30 mL, 63.8/31.4/4.8, [35-40]. The fractions of interest were combined, concentrated on a rotating evaporator (40°C, 200 Torr), transferred with a minimum (2 - 4 mL) of $CHCl_3$/MeOH 2/1 v/v into two 10-mL tared glass tubes, and concentrated to 0.5 mL under a strong stream of nitrogen in a heat block at 45°C. The residue was subsequently emulsified by vortexing in 3 mL of $CHCl_3$/MeOH/H_2O 10/10/80 v/v/v and freeze-dried overnight under 0.1 mm Hg vacuum. A total of 52.6 mg (40%) of a white fluffy solid (DSPE-$COCH_2Br$) was obtained.

Synthesis and LC purification of the phospholipid-peptide conjugate

Briefly, 14.3 mg (12 μmol) of the solid phase synthesized decapeptide (Ser-Phe-Leu-Leu-Arg-Asn-$(\beta Ala)_3$-Tyr-$NHCH_2CH_2SH$ (**3**)) were weighed into a 2-dram screw-cap glass vial previously flushed with nitrogen (Scheme 1). A well-mixed clear solution of 10.4 mg (12 μmol) of DSPE-$COCH_2Br$ (**2**), 5 mL $CHCl_3$/MeOH 1/1 v/v, 300 μl water, and 32.5 μl (10 eq) of triethylamine (Et_3N) was then added upon stirring. After sparging with nitrogen, the vial was capped. Reaction was allowed for 15 hours at room temperature and was followed by TLC in E3 (Table 1). The reaction mixture was subsequently concentrated under a strong stream of nitrogen with a heat block at 45°C, vortexed in five volumes (6 mL) of $CHCl_3$/MeOH/H_2O 10/10/80 v/v/v, frozen in liquid nitrogen, and evaporated under vacuum (0.1 mm Hg) overnight (lyophilized), thus yielding 20 mg of a crude white fluffy solid. Two 10-mL Vacu-Pak filtration columns (Baker) were hand-packed with fine silica (200-425 mesh), compressed, and the packing topped with a 20 μm pre-filter (provided with the commercial polypropylene



Scheme 1. Methodology for phospholipid-peptide conjugation.

TABLE 1

Chromatography Solvent Mixtures†

	Hexane	$CHCl_3$	iPrOH	$AcOH_{glacial}$	H_2O
E0	33	9.4	52	0.9	4.7
E1	28	10	55	3	10
E2	18	10	65	3	10
E3	15	8	60	1	16
E4	15	5	60	3	17

† volumic composition

columns). Each column was loaded with 10 mg of crude dissolved in 2 mL of E0. Elution was conducted at 1 mL/min with a gradient in the following manner: the initial 2-mL load was eluted in fractions [1,2]; 2 mL, E0, [3-4]; 3 mL, E0, [5-7]; 3 mL, E0/E1 1/1 v/v, [8-10]; 6 mL, E1, [11-16]; 3 mL, E1/E2 1/1 v/v, [17-19]; and 6 mL, E2, [20-25]. Fractions [13-17] of both columns were combined and concentrated to 0.5 mL under a strong stream of nitrogen in a heat block at 45°C. The white residue was subsequently emulsified upon vortexing in 3 mL of $CHCl_3$/MeOH/H_2O 10/10/80 v/v/v and freeze-dried overnight under 0.1 mm Hg vacuum, yielding pure lipopeptide (**4**) as a white fluffy powder.

RESULTS

DSPE-$COCH_2Br$ was readily extracted from its crude reaction mixture and purified in a single step by normal phase LC. Elution was performed with a gradient of chloroform, methanol, and a 50% aqueous acetic acid solution. The fractions were analyzed by TLC in $CHCl_3$/MeOH/$AcOH_{50\%aq}$ 70/30/1 v/v/v and the compound (R_F=0.75) detected with iodine. Unlike PE (R_F=0.70), it gave a negative result upon treatment with ninhydrin. Little retention of compound on the silica column was noticed. Thus, a typical batch of 80-mg PE yielded about 50 mg of the bromoderivative after reaction and LC purification. The overall yield was 40%, conversion of DSPE into DSPE-$COCH_2Br$ being the limiting factor. ^{1}H-NMR and mass spectroscopy data have been reported (9).

Once purified, the bromoderivatized phospholipid was covalently coupled to the thiol-terminated decapeptide via a nucleophilic substitution in the presence of triethylamine. The reaction mixture was evaporated to dryness, and a single micro-scale LC sufficed to extract and purify the phospholipid-peptide conjugate. The small filtration columns were packed with fine wet silica (hexane), tightly compressed with a spatula, and the bed was topped with a pre-filter provided with the commercial plastic columns. A discrete series of eluents (Table 1) was designed by modification of a normal phase HPLC gradient of hexane, chloroform, tetrahydrofuran, 2-propanol, and water previously used for its high selectivity toward naturally occurring phospholipid classes (10). However, since the lipopeptide described in this report is markedly more hydrophilic than natural phospholipids, including phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, phosphatidylserine, and phosphatidylinositol, acetic acid was

TABLE 2

R_F Values in Given TLC Solvent Systems†

	E1	E2	E3	E4¶
DSPE-$COCH_2Br$ (**2**)	0.65	0.60	0.48	0.67
lipopeptide (**4**)	0.35	0.40	0.26	0.56
peptide (**3**)	0.15	0.15	0.12	0.51
peptide disulfide	‡	‡	‡	0.35

† for solvent systems, see Table 1
¶ triethylamine had an R_F value of 0.10 in this eluent
‡ compound not seen

added as an ion-pairing agent to minimize retention on the polar stationary phase. As noted in Table 2, the R_F values of each molecular species were distinct, indicative of the excellent selectivity of this quinternary mixture. Furthermore, the addition of acetic acid hindered spreading of the chromatographic peaks, thus improving resolution. Optimal gradient elution conditions for the given reaction mixture are as indicated in the Materials and Methods section above. A typical load of 10 mg per micro-column of dry crude reaction mixture (containing 20%-30% of lipopeptide) yielded approximately 4 mg of pure lipopeptide after lyophilization in $CHCl_3$/MeOH/H_2O 10/10/80 v/v/v. Confirmation of product chemical structure was verified by NMR.

DISCUSSION

Purification of amphiphilic lipid biomolecules is often demanding, and a variety of approaches have been described. HPLC has been used extensively for

the purification and quantitation of naturally occurring phospholipids. Silica phases and complex quinternary eluents were implemented by Redden (10) and Christie (11). Likewise, Rehman used an isocratic elution protocol with acetonitrile, methanol, and aqueous phosphoric acid (12), and Abidi eluted major lipid classes with an isocratic mixture of acetonitrile, methanol, and aqueous tetraalkylammonium phosphate on a C18 column (13). This work has been recently extended to the purification of monoalkyl- and dialkyl-surfactants, including surfactin, a cyclic heptapeptide possessing a long aliphatic moiety (14, 15), and a synthetic dialkyl peptide prepared by solid phase synthesis (16). In the latter report, two palmitoyl chains were covalently linked to the lysyl-terminus of a resin-bound peptide. Reverse phase C_{18} HPLC was feasible because of the dominant hydrophilic nature of the 23-residue peptide. Nonetheless, HPLC remains limited by its expense and complexity. Although the application of TLC is much less costly and has proven successful for both lipid separation and quantitation, it is ill-suited for scaled-up preparative work.

Conventional LC provides an alternative, inexpensive approach for amphiphile purification with preparative capabilities. LC has been used previously to successfully purify both naturally occurring phospholipids, as well as derivatives of phosphatidylethanolamine with N^{α}-iodoacetyl-(N^{ε}-dansyl)lysine and 3-(pyridyl-2-dithio) propionic acid (17-20). LC has also been applied to the purification of surfactin analogs (21). Typically, eluants have been isocratic or gradient mixtures of chloroform/methanol or chloroform/methanol/water.

Apart from its use as an isolated purification procedure, LC has also been used as a refining step in LLE procedures. Impure PE-MPB recovered after a chloroform/brine treatment required further purification by LC over silica in chloroform/methanol (20). Similarly, normal phase LC with chloroform/methanol was necessary to purify PE-COOH after initial liquid-liquid extraction (22). However, in contrast to LLE procedures, LC avoids the loss of amphiphile at the liquid-liquid interface and reduces the quantity of solvent required for product purification. For example, 25 mL was typically required to purify 10 mg of crude lipopeptide using the approach described in this report. Furthermore, expensive unreacted peptide can be recovered using an LC protocol.

In an effort to prepare receptor activating membrane-mimetic surfaces, we synthesized an amphiphilic conjugate of distearoylphosphatidylethanolamine and a

decapeptide which includes the minimal human thrombin-receptor peptide agonist Ser-Phe-Leu-Leu-Arg-Asn. Purification of this lipid-peptide conjugate and the phospholipid derivative, DSPE-$COCH_2Br$, was difficult due to the amphiphilic nature of these compounds. However, a successful result was achieved with gradient LC. In particular, a quinternary eluant in which acetic acid was added as an ion-pairing agent was effective for purification of the lipopeptide. This report provides the first description of normal phase LC for the purification of N-haloalkyl derivatives of PE and associated lipid-peptide conjugates.

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