papers and notes on methodology

Synthesis and characterization of a highly fluorescent peptidyl-phosphatidylethanolamine

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Abstract The synthesis of a fluorescent lipid for use in studies of immune recognition of model membranes is described. The molecule has the basic structure HAPTEN-SPACER-LIPID, where fluorescein is the hapten, an oligopeptide (triglycine) is the spacer, and dipalmitoylphosphatidylethanolamine (DPPE) is the lipid. The spacer, which is necessary for immunological reactivity, is first linked via a peptide bond to DPPE. The free N-terminus of the peptidyl-DPPE is then reacted with 5-dichlorotriazinylaminofluorescein (DCTAF) to yield fluorescein-chlorotriazinyltriglycyl-DPPE (FG_3P). The structure is confirmed by mass spectrometry and Fourier transform NMR. When FG_3P is incorporated into phospholipid vesicles it retains the brilliant fluorescence and high-affinity immunological reactivity of fluorescein. The general synthesis scheme may prove useful in other membrane and lipoprotein applications. —**Petrossian, A., A. B. Kantor, and J. C. Owicki**. Synthesis and characterization of a highly fluorescent peptidyl-phosphatidylethanolamine. *J. Lipid Res.* 1985. **26:** 767–773.

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Spectroscopic techniques are among the most important tools for analyzing membranes and lipoproteins. A successful experimental strategy has been to attach a spectroscopic probe covalently to a phospholipid, incorporate this as a minor component of the system being studied, and obtain structural and/or dynamic data from the spectroscopic behavior of the probe. Such molecules are important in the analysis of ligand–receptor-mediated recognition processes. McConnell and co-workers (for reviews see references 1 and 2) have studied a variety of immunological phenomena with model membranes using spin-label haptens that have been covalently attached to lipids. We describe here the detailed synthesis of a fluorescent hapten linked to a phospholipid, a molecule that has several advantages over spin-labeled lipids for antibody–membrane recognition. Luedtke and Karush (3), Sinha and Karush (4), and Weltzien et al. (5) have also recognized the value of a fluorescent membrane-bound hapten. We have reported the interactions of antibodies with liposomes derivatized with our hapten elsewhere (6, 7).

The rationale for the design of the molecule follows. Our molecule has the overall structure HAPTEN-SPACER-LIPID: the hapten is linked to the phospholipid head group by a spacer chain that projects the hapten far enough out from the membrane so that an antibody can bind it (8). This requisite spacer makes our synthesis more complex than the direct attachment of a fluorophore such as NBD (9) or dansyl (10) to a phospholipid.

Fluorescein was chosen as the hapten partly because of its brilliant fluorescence, which is almost quantitatively quenched upon antibody binding (11). It is highly immunogenic and one of the best-studied haptens in immunology (12). The molecule is also an effective reporter group, yielding important information about its environment and mobility (7).

The spacer chosen was an oligopeptide. In addition to creating analogies to membrane proteins, this choice accessed the rich variety of molecular properties and synthetic techniques that are obtainable for peptides (13). For the synthesis reported here, we used glycylglycylglycine, attaching the phospholipid to the carboxyl group and then the hapten to the α-amino group. Triglycine is conformationally flexible, and the polar amide group should inhibit interactions between the spacer and the nonpolar region of the membrane.

The phospholipid chosen was DPPE. The amino group

Abbreviations: CBZ, benzyloxycarbonyl group; CBZ-Gly_3, benzyloxycarbonyltriglycine; CBZ-Gly_3DPPE, benzyloxycarbonyltriglycine-DPPE; DCTAF, 5-dichlorotriazinylaminofluorescein; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DMSO, dimethylsulfoxide; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; Et_3N, triethylamine; FG_3P, fluorescein-chlorotriazinyl-triglycyl-DPPE; FITC, fluoresceinisothiocyanate; Gly_3DPPE, triglycyl-DPPE; NBD, nitrobenzoxydiazol; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; TLC, thin-layer chromatography.

is a convenient attachment site for the spacer. Natural PE's with mixed alkyl chains would have produced a heterogeneous product, which we wanted to avoid. We chose a saturated acyl chain rather than an unsaturated one for stability against oxidation. Phospholipids with acyl chains shorter than sixteen carbons are more readily soluble in reaction solvents than is DPPE and have been used to make hapten-substituted derivatives (14). However, phospholipids with shorter chains have been shown to transfer between membranes fairly readily (15), an undesirable property for our work. Furthermore, shorter-chain phospholipids are frequently associated with bilayer instability; for example, high rates of probe flip-flop (7) and ionic permeation (W. Hubbell, personal communication) have been observed in DMPC vesicles. We therefore preferred to use DPPE rather than DMPE even though it complicated the synthesis somewhat.

MATERIALS AND METHODS

Chemicals

Chloroform and methanol were obtained from Mallinckrodt, Inc. (Paris, KY). When used as a reaction solvent, the chloroform was distilled from Linde Type 3A Molecular Sieves (Union Carbide, South Plainfield, NJ) and stored over the sieve drying agent at 4°C. Palladium (10%) on activated carbon and Et_3N were obtained from Aldrich Chemicals (Milwaukee, WI). The primary and secondary amine contaminants of Et_3N were reduced by fractional distillation with a 40-cm insulated column filled with Tamer Tabs (Northwest Products, Succasunna, NJ). Isobutylchloroformate was obtained from Eastman Kodak (Rochester, NY) and CBZ-Gly_3 was from Bachem (Torrance, CA). Both were used without further purification. Other chemicals were obtained from Sigma (St. Louis, MO).

Thin-Layer chromatography

Analytical and preparative silica gel 60 plates, without fluorescent indicator, were manufactured by E. Merck (Darmstadt, Germany; catalog numbers 5762 and 5764, respectively). The preparative plates were 2-mm thick and used a polymer binder. The following developing solvents were used (ratios by volume): (A) chloroform–methanol–water 65:25:4, (B) chloroform–methanol–acetic acid–water 65:25:4:2, (C) chloroform–acetone–methanol–acetic acid–water 6:8:2:2:1, (D) chloroform–methanol–28% ammonia 65:25:5.

Identification of the bands from continuously developed preparative plates was confirmed by analytical TLC. The R_f values (±0.05) are given as a reference; spots were identified by comparison with standards. Visualization was by ninhydrin (0.2% in acetone) for amino groups, I_2 vapor for other organic compounds, and direct fluorescence. When developed with an acidic solvent, fluorescence was enhanced with NH_3 vapor, since fluorescein derivatives are most luminescent in the dianion form.

Lipid and hapten

The DPPE (Avanti Polar Lipids, Birmingham, AL) and fluorescein (disodium salt; Sigma) were both one spot on TLC with developing solvents (A) and (B) and were used without further purification. DCTAF · 2 HCl was obtained from either Research Organics (Cleveland, OH) or Molecular Probes (Junction City, OR).

Analysis

Gravimetric methods can be unreliable for estimating lipids separated by TLC (16). Although we need not worry about calcium sulfate or indicator dye, the silica gel and perhaps the polymer binder may be eluted and weighed. We minimized this difficulty in determining our yields in two ways. First, the product (or intermediate) was filtered through Whatman 43 paper and then 1.0 μm Nucleopore (Pleasanton, CA) polyester membranes. Second, the final fluorescent product was assayed for phosphorus content (17), and the extinction coefficient of the fluorophore (per phosphorus) was determined.

Proton NMR was done on the U.C.B. Chemistry Dept. Fourier-transform machine in *a*) deuterated chloroform–methanol 2:1 and *b*) deuterated DMSO (Merck, Montreal, Canada). Negative-ion liquid secondary-ion mass spectrometry (SIMS) using a Cs^+ gun was performed on Gly_3DPPE and FG_3P dissolved in a glycerol–thioglycerol 1:1 matrix (18).

Optical spectroscopy

Fluorescence spectra were obtained on a Spex Fluorolog 2 spectrofluorometer; absorbance measurements were made on a Varian Superscan spectrophotometer. For some measurements the FG_3P was incorporated as a minor component into phospholipid vesicles made by the ethanol-injection method (7, 19).

EXPERIMENTAL

The synthetic scheme is presented in **Fig. 1**. Roman numerals refer to the structures shown there.

Activation of CBZ-Gly_3 (I)

Well-pulverized CBZ-Gly_3 (113 mg, 350 μmol) was dissolved in 15 ml of chloroform with Et_3N (145 μl, 1 mmol). A mole equivalent of isobutylchloroformate (II) (45.2 μl, 350 μmol) was diluted into 1 ml of chloroform and then slowly added to the mixture. The stirred reaction continued for 30 min in the dark at room temperature.

Preparation of Gly_3DPPE (VI)

DPPE (IV) (200 mg, 290 μmol) dispersed in 20 ml of chloroform was added to the above mixture and reacted

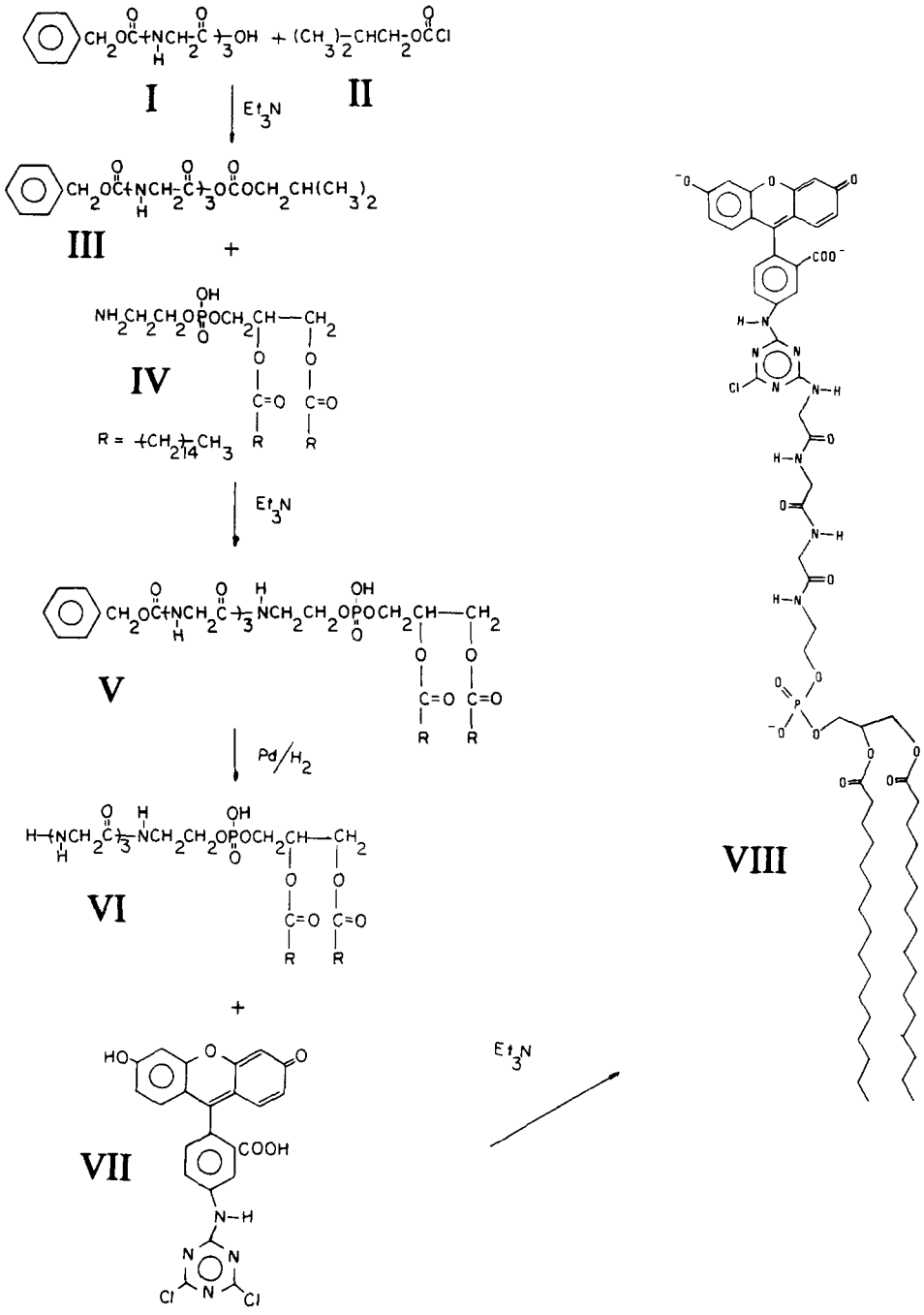

Fig. 1. Synthesis scheme. The compounds are (I) CBZ-Gly_3, (II) isobutylchloroformate, (III) mixed anhydride intermediate, (IV) DPPE, (V) CBZ-Gly_3DPPE, (VI) Gly_3DPPE, (VII) DCTAF, and (VIII) FG_3P.

for 4 hr at room temperature with continuous stirring. The solvent and base were removed by rotary evaporation and the dried residue containing CBZ-Gly_3DPPE (V) was dispersed in 15 ml of methanol. Acetic acid (1 mmol) was added and the amine protecting group (CBZ) was removed by catalytic hydrogenation. Hydrogen gas was bubbled through the reaction mixture at 1 atm in the presence of 600 mg Pd-carbon; the reaction was monitored by analytical TLC and was terminated after 2 hr. The Gly_3DPPE product (VI) and unreacted DPPE adsorbed to the carbon, which was transferred as a slurry to a 1-cm-wide groove on a preparative TLC plate and developed continuously with solvent (A) for 8 to 10 hr. Ninhydrin staining revealed two bands: one, which was

the greyish-yellow color often characteristic of glycine N-terminal peptides (20), migrated one-third up the plate from the initial spot and was identified as Gly_3DPPE. A separate purple DPPE band migrated half-way up the plate. CBZ-Gly_3DPPE and CBZ-Gly_3, visualized with I_2, migrated poorly. The Gly_3DPPE band was excised, extracted with solvent (A), filtered, and dried by rotary evaporation, with T < 30°C. The yield was estimated gravimetrically.

Preparation of FG_3P (VIII)

The purified Gly_3DPPE (70–80 μmol, 60–70 mg) was dissolved in 3.3 ml of chloroform–methanol 10:1. The fluorophore, DCTAF (VII) (450 μmol, 255 mg), was dissolved in 6 ml of chloroform–methanol 1:1 with Et_3N (140 μl, 1 mmol) and stirred in the dark with Gly_3DPPE for 4 hr. FG_3P was purified by continuous development preparative TLC with solvent (A) for 12 hr. The product migrated three-fourths up the plate and was distinguished from the spots immediately above and below by intensity. The excess DCTAF migrated to the top of the plate. The product was extracted with methanol–chloroform 4:1 (R_f = 0.8), and its identity was confirmed. The yield was determined spectrophotometrically. The compound was purified further by single-development preparative TLC with solvent (C). This final product was assayed for phosphorus and studied spectroscopically.

RESULTS AND DISCUSSION

Peptidyl-phosphatidylethanolamine

Analytical TLC of the isolated Gly_3DPPE showed little DPPE or other contaminants visible with ninhydrin or I_2. Mass spectrometry (**Fig. 2A**) yielded a nominal mass to charge ratio (m/e) of 861 for the $(M-H)^-$ parent ion (i.e., after proton abstraction); the molecular weight of Gly_3DPPE (most probable isotopic composition) is 862.5.

The Gly_3DPPE yield was typically 25–30%, based on DPPE. Chloroform is usually a solvent of choice for PE's, but it is generally not the most suitable solvent for peptide synthesis via the mixed-anhydride method. Low yields and impure product are often obtained (21). Furthermore, our DPPE solution was not clear, but formed a turbid, well-dispersed suspension. We failed to attain better yields at lower temperatures or with other solvents and tertiary amines commonly used in peptide synthesis.

Gly_3DPPE should be valuable for attaching a wide variety of molecules to liposomes and cell membranes. Many functional groups can be linked directly or indirectly to its α-amino group (22). For linkage of soluble proteins to preformed liposomes containing a lipid-based cross-linking agent, Gly_3DPPE should be a more reactive cross-linker than phospholipids without spacers (e.g., DPPE) or with more hydrophobic spacers (e.g., alkyl chains). Such derivatized vesicles have practical immunological and clinical applications (8, 23–25).

FG_3P

DCTAF (VII) is more stable and more easily purified than FITC and has been a successful alternative for labeling proteins (26–28). Moreover, when bound to the N-terminal amino group of peptides, FITC can participate in an Edman degradation (29). Analytical TLC of the DCTAF revealed 95% of the fluorescence in the major spot, which compares satisfactorily with the 98% reported elsewhere (26). We compensated for DCTAF depletion in side reactions by adding a sixfold excess.

After the purification of the product by continuous-development preparative TLC, analytical TLC revealed a major spot with 90% of the total fluorescence in solvents (A) R_f = 0.25 and (C) R_f = 0.68. Several other fluorescent spots were observed, but no ninhydrin-positive spots were present. Solvent (D), which is especially good for separating the free N-terminus species (FG_3P, R_f = 0.05; free N-terminus species, R_f > 0.2), confirmed the absence of DPPE and Gly_3DPPE. A yield estimate, corrected for contaminants and determined spectrofluorometrically, was 90% based on the upper Gly_3DPPE estimate and 25% based on DPPE. The hapten reaction was essentially complete.

NMR in deuterated (a) methanol–chloroform 2:1 and (b) DMSO revealed the proton shifts of the alkyl chain (a: 0.8–1.8 ppm, b: 0.7–1.4 ppm), the fluorescein ring (a: 6.5–7.2 ppm, b: 5.4–7.5 ppm) and the methylene protons of the triglycine (a: 3.8–4.1 ppm, b: not resolved) in the expected positions and with intensities in the correct ratios, within experimental error.

Attempts to obtain mass spectra by field desorption, electron impact, and fast-atom bombardment failed. Secondary-ion mass spectrometry was successful, giving the spectrum in Fig. 2B. The $(M-H)^-$ parent ion has a nominal mass/charge of 1319 and the molecular weight of FG_3P is 1320.6. In sum, the product is identified as FG_3P.

The product described above has been used successfully in our initial immunological studies (6, 7). FG_3P was further purified by single-development preparative TLC with solvent (C). Analytical TLC of this final purified product gave a major spot with at least 95% of the total fluorescence in each of the solvents [R_f = (A) 0.25 (B) 0.53 (C) 0.68 (D) 0.05]. Some minor bands appeared; slow loss of the final triazinyl Cl accounts for one of the impurities. Its characteristics when incorporated into liposomes are presumably very similar to FG_3P. Approximately 85% of the FG_3P was recovered for a corrected yield of 20% based on DPPE. This compares well with the 70% yield obtained for the direct attachment of NBD to mixed chain PE's (9), the 30% yield for a DNP-spacer-

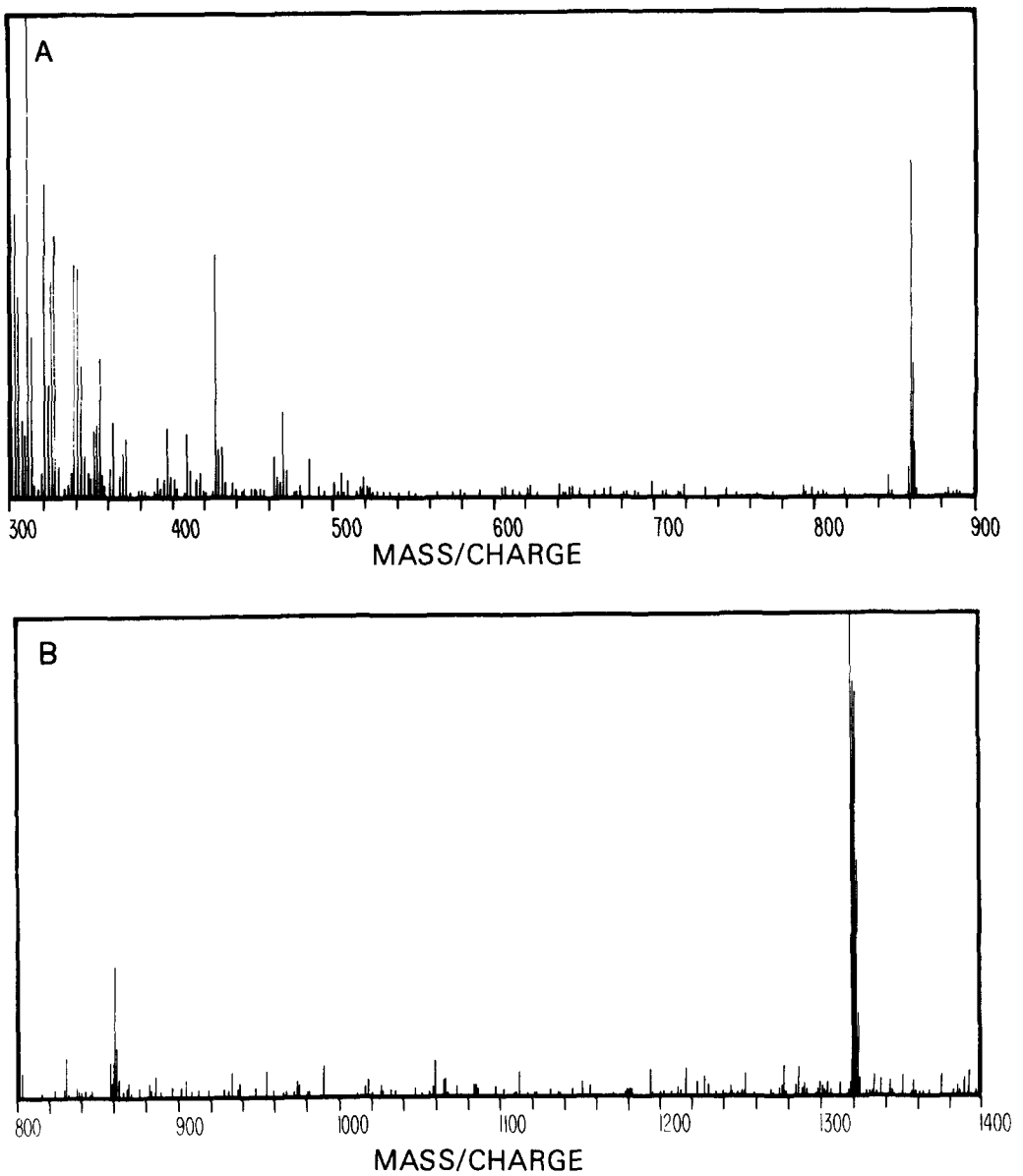

Fig. 2. Mass spectra. (A), Gly_3DPPE, compound (VI) in Fig. 1, isotopically most probable molecular weight 862.5. (B) FG_3P, compound (VIII) in Fig. 1, isotopically most probable molecular weight 1320.6.

DPPE molecule (30), and the 20% yield for a NBD-spacer-cholesterol (31). This final product was used for the spectroscopic studies.

Spectroscopy

The characteristics of fluorescein that led us to choose it as a hapten have been retained in FG_3P, as is demonstrated by absorbance and fluorescence spectroscopy. FG_3P is insoluble in aqueous solution but soluble in ethanol. We compared the spectral properties of fluorescein, DCTAF, and FG_3P in ethanol solutions made basic with NH_3 to ensure that each fluorophore was in the dianion state. The DCTAF chlorides can be substituted by both ethanol and ammonia (32, 33); TLC indicates a new product is formed. Spectra are presumably for at least a monosubstituted product, most likely by NH_2 (32). All three compounds have nearly identical excitation and emission spectral shapes. Maxima, extinction coefficients, and relative fluorescence intensity are also similar (**Table 1**). Note that there is some disagreement in the reported data for fluorescein and DCTAF; the similarity among the three is the most important result. When FG_3P is quantified by phosphorus assay, its extinction coefficient agrees with the result reported for fluorescein in basic ethanol solution (34).

In typical experimental use, FG_3P is incorporated into

TABLE 1. Summary of spectroscopic parameters

Fluorophore	Extinction Coefficient	Excitation Maximum	Emission Maximum	Relative Fluorescence
	$M^{-1}\ cm^{-1}$	*nm*	*nm*	
In ethanol (0.1 M NH_3)				
Fluorescein	92,000[a]	502	521	1.0
DCTAF	85,000	502	521	1.0
FG_3P	92,000	502	521	1.0
In borate buffer (pH 9.5)				
Fluorescein	88,000[a]	492	513	1.0
DCTAF	82,000[b]	492	513	0.50[c]
FG_3P (vesicles)	>70,000	497	520	

Our fluorescence parameters were obtained on solutions of 75 nM free fluorophore or 3 nM vesicle-bound fluorophore. Absorption measurements were made on 3 μM fluorophore solutions. In all cases the fluorophore is essentially all in the dianion state. The pK_a measurements for the aqueous solutions are discussed elsewhere (7). For the free fluorophore excitation spectra, the emission wavelength is 560 nm and the excitation and emission slits are 0.9 and 1.8 nm, respectively. For the emission spectra, the excitation wavelength is 460 nm with the same slit widths. The vesicles are DPPC–cholesterol 2:1 with 0.1 mol% FG_3P. The excitation and emission slits are, respectively, 0.9 and 7.2 nm for the vesicle measurements. Relative fluorescence is normalized by absorbance and compared to fluorescein in the same solvent.

[a] Values are from chromatographically purified and dried samples (34, 35). Values for the wavelength maxima vary somewhat among investigators.

[b] Value is from Barskii et al. (28); we obtained similar results.

[c] Blakeslee and Baines report 0.49 (26).

phospholipid vesicles that are dispersed in aqueous solution. Its spectroscopic characteristics then differ somewhat from those obtained in ethanol solution. We have incorporated FG_3P as a minor component (0.1 mol %) in DPPC–cholesterol 2:1 vesicles. Under appropriate conditions the fluorophore is essentially completely in the dianion state, and its lateral density is low enough to avoid self-association that would lead to concentration quenching (7). The FG_3P excitation and emission spectral maxima are red-shifted from those of the free fluorophores (fluorescein and DCTAF) in aqueous solution (Table 1). The amount of the shift depends on the lipid composition of the vesicles, and we attribute the effect to a lipid-dependent partition of the fluorophore between the head-group region and the aqueous phase near the vesicles (7).

Determination of the extinction coefficient of FG_3P in vesicles is complicated by turbidity and possible refractive screening of the fluorophore on the inner monolayer. In Table 1 we report a lower limit to the extinction coefficient at the absorbance maximum, assuming all FG_3P molecules behave alike. Based on the similarities of fluorescence intensities and extinction coefficients reported here, plus the similarity of fluorescence lifetimes of aqueous fluorescein and FG_3P in vesicles (7), we conclude that the quantum yields of the dianions of these fluorophores are essentially the same. The quantum yield of our precursor fluorophore, DCTAF, is about half that of fluorescein; reaction of DCTAF with NH_3 has been reported to increase the quantum yield (26), and a similiar effect occurs in FG_3P.

The retention of immunological reactivity of FG_3P in vesicles is demonstrated by fluorescence quenching observed in the presence of anti-fluorescein antibodies. Details are available elsewhere (6).

In conclusion, the synthetic scheme incorporates features of both lipid and peptide chemistry. Variations on the method may prove generally useful for applications that require the attachment of molecules to liposomal membranes while preserving access to intermolecular interactions in the aqueous phase near the membranes.

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