Design, Synthesis, and Properties of a Photoactivatable Membrane-Spanning Phospholipidic Probe⊥

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Abstract: We introduce here a new photochemical probe suitable for labeling deep into the hydrophobic core of membranes: bis-phosphatidylethanolamine (trifluoromethyl)phenyldiazirine **19** (DIPETPD). This is a bipolar phospholipid provided with a covalently bonded chain designed to span the membrane and equipped with a centrally defined attachment point for the photolabeling group (trifluoromethyl)phenyldiazirine (TPD). This molecule was designed to enhance the geometrical resolution of photochemical labeling of membrane proteins by locating the photoreactive functionality in the center of the bilayer. The remarkable chemical stability of the photoreactive group TPD[1] allowed the design of a straightforward and convergent synthetic strategy. The key steps developed for molecules of this new general kind are (a) the mild and efficient coupling of two moieties of *N*-tBOC-protected lysophosphatidylethanolamine methyl ester to the photoreactive symmetric dicarboxylic fatty acid mediated by dicyclohexylcarbodiimide and (dimethylamino)pyridine and (b) the smooth deprotection of the phosphate and amino functionalities with sodium iodide and trifluoroacetic acid, respectively, to yield the final product. DIPETPD has been successfully incorporated into small and large unilamellar vesicles of different lipid composition and prepared by a variety of procedures. The bilayer location of this reagent (transmembrane vs 'U'-shaped conformations) was assayed by reaction of the amino groups at the polar heads of the bipolar phospholipid with selected membrane-impermeable reagents. Photolysis of the probe incorporated into vesicles occurs readily upon irradiation with UV light (near 360 nm). These 'loaded' vesicles show adequate stability and appear uniform and unilamellar in electron micrographs. They undergo the fusion reaction with influenza virus as efficiently as reagent-free vesicles. Evidence is presented here that DIPETPD and a reductively methylated form efficiently label the peptide ion channel form of gramicidin A (and a chemical analogue) and the influenza virus hemagglutinin. DIPETPD may help to identify transmembrane regions of integral membrane proteins and map the lipid–protein interface in a region known to be deep in the membrane. A new radioactive version of this reagent ([^{3}H]-DIPETPD)[2] has been recently used to ascertain that the HA2 subunit of influenza virus hemagglutinin inserts into the target membrane prior to fusion.[3]

Introduction

Among the increasing number of structures deposited in the Brookhaven Protein Data Bank,[4] there are so far only four integral membrane protein structures available at atomic resolution: the photosynthetic reaction centers of *Rhodopseudomonas viridis*[5] and of *Rhodobacter sphaeroides*,[6] bacteriorhodopsin from *Halobacterium halobium*,[7] and porin from *Rhodobacter capsulatus*.[8,9] The need to use detergents to solubilize these proteins compounds the difficulty in obtaining suitable crystals. The hydrophobic, membrane-associated regions of this class of proteins determine their location with respect to the lipid bilayer and mediate important functions such as signal transduction, electron transfer, and transport of ions and molecules across the membrane.

In an attempt to enhance the structural information on these membrane-associated regions, we have adopted the hydrophobic photolabeling approach.[10] Reagents designed to react within the hydrophobic milieu of the lipid bilayer have helped to identify membrane-associated peptides and map the protein–lipid interface. Such reagents may also play a role in studying dynamic phenomena, such as penetration and insertion of proteins into the lipid bilayer, translocation of proteins through the lipid bilayer, and protein-mediated membrane fusion. The only feasible chemistry involves the transient, very reactive, electron-deficient species (i.e. carbenes or nitrenes) generated by photoirradiation. For general labeling, carbenes are more suitable because they show lower selectivity than nitrenes and the half time for reaction

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⊥ Abbreviations used: DCC, dicyclohexylcarbodiimide; DIPETPD, compound **19**; DIPEP, compound **20**; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; HOBT, hydroxybenzotriazole; *N*-tBOC, *N*-(*tert*-butyloxy)carbonyl; PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; RT, room temperature (20–25 °C); TEA, triethylamine; THF, tetrahydrofuran; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP, trinitrophenyl; TPD, (trifluoromethyl)phenyldiazirine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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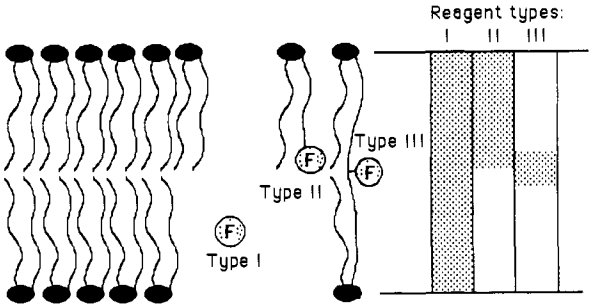

Figure 1. Regions of the lipid bilayer that are accessible to the different types of hydrophobic photolabeling reagents (I, II, and III): F indicates the photoactivatable function. Dotted areas at the right show the regions within the membrane sampled by the different reagents.

is shorter than the relaxation time for the diffusion of most molecules within the membrane. Photoadduct formation is likely to be the consequence of a direct contact between the reagent and the protein. Selected diazirines with appropriate absorption wavelengths and structures designed to minimize photoisomerization and internal rearrangements have proven useful as photochemical probes.

The types of reagents used in the past for hydrophobic photolabeling are shown schematically in Figure 1. Type I reagents are small, very hydrophobic molecules, that partition effectively into membranes, are freely diffusible, and label randomly in the lipid phase. Spiro[adamantane-2,3′-diazirine],[11] iodonaphthyl azide,[12] 3-(trifluoromethyl)-3-phenyldiazirine (TPD),[1] and its meta iodinated analogue TID[13] belong to this class. Type I reagents have been extensively used to label peptides embedded in membranes; however, identification of such structures by a positive labeling result is not unambiguous, since these small molecules can also partition favorably into the hydrophobic areas of water-soluble proteins, giving rise to spurious labeling. Type II reagents include larger, amphipathic molecules, such as phospholipids, endowed with a photoreactive functionality attached to a fatty acyl chain.[14] Type II reagents will label one leaflet of the bilayer, provided that the phospholipid is incorporated into a single monolayer of the membrane with the aid of a phospholipid transfer protein. Due to large thermal fluctuations of the fatty acyl chains at room temperature, it is unavoidable that type II reagents will also be able to sample regions close to the polar head groups at the lipid–water interface ("looping back" of fatty acyl chains). This will necessarily reduce the trans bilayer resolution of the labeling site.

With the aim of attaining better resolution of the labeled site in a direction perpendicular to the plane of the membrane, we introduce in this paper a type III reagent, named DIPETPD. This is a bipolar phospholipid molecule designed to span the membrane through a covalently linked fatty diacyl chain that contains a central attachment point for the photoreactive functionality TPD. We describe here the synthesis of DIPETPD, the conditions for the incorporation of this molecule into liposomes, the location of this probe in the lipid bilayer, and evidence for the reaction of DIPETPD with the gramicidin A channel, used as a model system of a transmembrane peptide, and with influenza virus hemagglutinin.

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Results

Design of a Photoactivatable Bipolar Phospholipid. In the design of a general labeling reagent (as opposed to an affinity label), one seeks a molecule which is as indistinguishable from the solvent as possible. For membrane proteins the relevant "solvent" is the phospholipid bilayer. Our design, based on the archaebacterial transmembrane lipids,[16] is intended to mimic the normal phospholipids as far as possible. With CPK models one can see that even the photoactivatable head group (TPD) fits quite well and is not very different in size from a comparable length of hydrocarbon chain. In addition, the TPD group is easily synthesized in high yield and is stable in the dark, but is rapidly photolyzed by irradiation with long-wave UV light, originating a highly reactive carbene that inserts readily into CH bonds.[1]

The essence of the design of DIPETPD (Figure 2) is the introduction of a symmetric covalently bonded fatty diacyl chain sufficiently long to span the membrane to constrain the motion of the photoactivatable group located at its center. The photolabeling of a membrane protein should thus be restricted only to those amino acid residues close to the center of the bilayer (see Figure 1).

The central phenyl ring is derivatized at the para position in order to position the photoreactive group outwardly, maximizing the chances for addition reactions with the environment around DIPETPD. The thioacetal junction at the middle of the molecule provides high stability under the conditions used for the synthesis and incorporation of DIPETPD into liposomes, but it is a potentially cleavable function under mild conditions by reaction with heavy metals or alkylating reagents. Such cleavage, with the consequent detachment of the phospholipid moieties of DIPETPD after the photolysis step, may facilitate the analysis of the products. Finally, the choice of phosphatidylethanolamine head groups permits analysis of the conformers present in lipid bilayers, by spectrophotometric titration of the amino groups.

A key element for the success of this whole synthetic strategy is the remarkable chemical stability of two separate groups: the 1,3-dioxanyl protecting group and the photoreactive TPD function. The TPD group is stable under a range of different chemical and physical conditions. These include strong bases (KOH), oxidizing agents (*N*-bromosuccinimide, potassium permanganate), heat (refluxing in dioxane, 80 °C), strong acids (trifluoroacetic acid 50%), acid anhydrides (acetic anhydride, di-*tert*-butyl pyrocarbonate), and mild reducing agents (sodium borohydride, thiols).[15]

The modular branched design of this synthesis permits modifications to be introduced along a certain pathway, without necessarily altering other steps. The usefulness of this feature is exemplified as follows. If need be, longer analogues of DIPETPD can be produced (e.g. to fit into thicker membranes) by following an identical synthesis but starting from the suitably long ω-halo-substituted precursor to the dicarboxylic fatty acid and the correspondingly longer chain phosphatidylethanolamine. Other chemical probes (e.g. fluorescent, spin probes, etc., adequately derivatized with a potential aldehyde) can be positioned at the junction of new dicarboxylic fatty acids, for inclusion into bipolar phospholipids. End differentiation of the final product can conceivably be performed by altering one polar head of the symmetric bipolar phospholipid by an exchange reaction catalyzed by phospholipase D.

Synthesis of the Bipolar Phospholipid. A general strategy for the synthesis of membrane-spanning bipolar phospholipids equipped with a reactive functional group probe of the membrane environment was developed in our laboratory. An early test of the synthetic methodology was provided by the synthesis of a non-photoactivatable analogue of DIPETPD (**19**), namely DIPEP (**20**), Figure 2, R = H.[16] A convergent 15-step procedure is presented here that leads to DIPETPD as the final product

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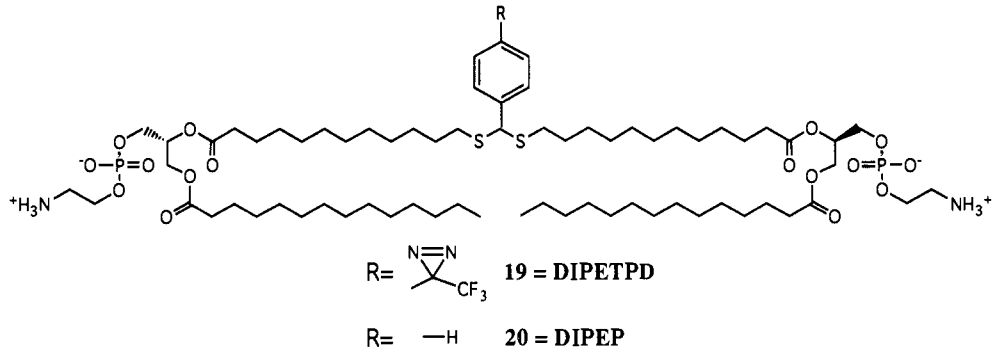

Figure 2. DIPETPD: a photoactivatable bipolar membrane-spanning phosphatidylethanolamine (type III, compound **19**). DIPEP (**20**) is a non-photoactivatable analogue of DIPETPD, used as a model compound.

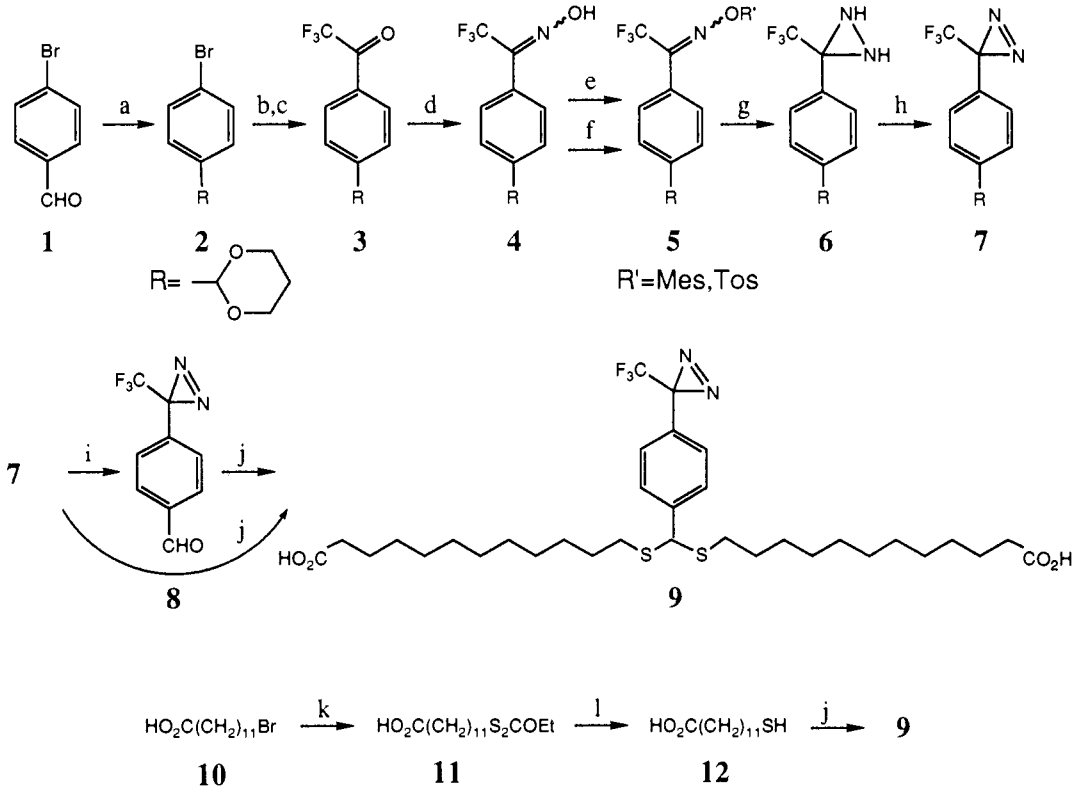

Figure 3. Synthesis of DIPETPD (part 1). Reaction conditions are as follows: (a) 1,3-propanediol and Amberlyst 15 in refluxing benzene; (b) nBuLi in diethyl ether at −30 to 0 °C; (c) (trifluoroacetyl)piperidine (prepared from piperidine and trifluoroacetic anhydride in the presence of TEA) or methyl trifluoroacetate added to the reaction mixture at −50 °C; (d) hydroxylamine in refluxing ethanol; (e) mesyl chloride, DMAP, and TEA in dichloromethane at RT; (f) tosyl chloride, DMAP, and TEA in dichloromethane at RT; (g) liquid ammonia in diethyl ether at RT (under pressure); (h) iodine and TEA in methanol at RT; (i) Amberlyst 15 suspended in acetone at RT; (j) compound **7** or **8** (0.5 equiv) mixed with compound **12** (1 equiv) with boron-trifluoride etherate in dichloromethane at 0 °C; (k) potassium ethyl xanthogenate in acetone at 4 °C; (l) ethylenediamine with a large excess of sodium borohydride in ethanol at RT.

(Figures 3, 4, and 5). The DIPETPD molecule is adequately described as a bis-phosphatidylethanolamine that is connected through a (trifluoromethyl)diazirinyl para-substituted benzylidene thioacetal of ω-hydroxy esters at the *sn*-2 position of the two phosphatidylethanolamine termini. The starting materials for this synthesis were *p*-bromobenzaldehyde (**1**; the precursor of the TPD moiety), 12-bromododecanoic acid (**10**; the precursor of the dicarboxylic fatty acid), and dimyristoylphosphatidylethanolamine (DMPE; **13**; the precursor of the polar head groups). We attempted to optimize yields at each step to avoid unnecessary purifications whenever possible, before proceeding to the next synthetic step. For analytical purposes, a small sample of each compound was routinely purified, before final characterization was made.

The 3-(trifluoromethyl)diazirinyl group was built in place of the bromo substituent in *p*-bromobenzaldehyde (**1**) by following a series of six consecutive reactions. A key to the success of this series is the efficient masking of the aldehyde as a cyclic acetal (1,3-dioxanyl compounds **2**–**7**) by reaction with 1,3-propanediol under acidic catalysis (Amberlyst 15) in the first step (to yield compound **2**). The inertness of this protective function permits the subsequent synthetic steps. This protecting group withstands (a) lithiation and reaction with either (trifluoroacetyl)piperidine or methyl trifluoroacetate to obtain **3**; (b) refluxing with hydroxylamine in ethanol to obtain **4**; (c) mesylation or tosylation[17] to obtain **5**; (d) liquid ammonia in ether to obtain **6**; and (e) oxidation with iodine in methanol to obtain **7**. Except for the rather low yield of the lithiation reaction (20–30%), all the other steps proceed almost quantitatively (ca. 90%). Building the thioacetal bridge can be performed either directly from the TPD

(17) In our hands, both mesylation and tosylation of the oxime gave high yields of products. Both derivatives react quantitatively with liquid ammonia in diethyl ether at RT (in a pressure bottle) to yield the diaziridine **6**.

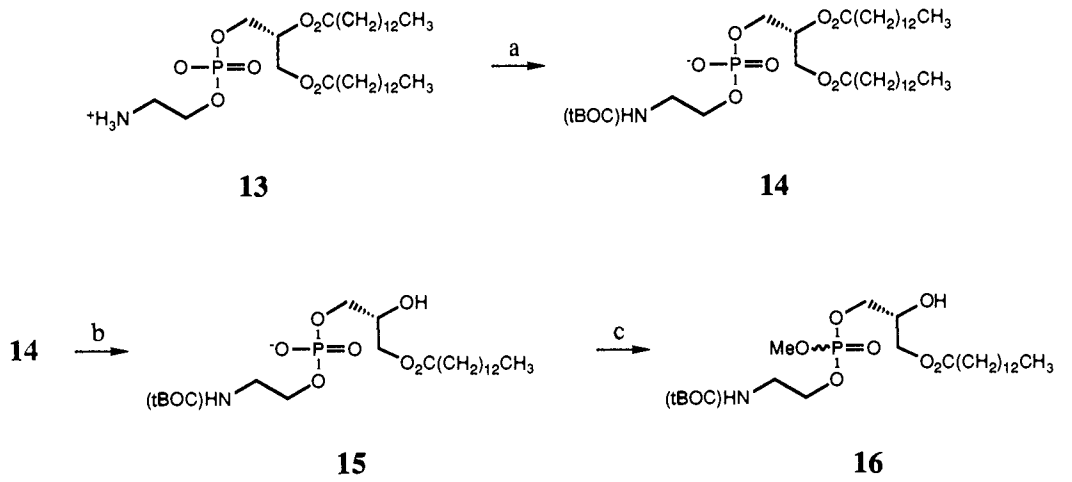

Figure 4. Synthesis of DIPETPD (part 2). Reaction conditions are as follows: (a) di-*tert*-butyl pyrocarbonate and TEA in THF at 0 °C and later at RT; (b) *Crotalus adamanteus* venom (source of phospholipase A_2) suspended in 50 mM Tris-HCl buffer, 40 mM $CaCl_2$, pH 8.4, and diethyl ether (methanol 1%) at 37 °C; (c) diazomethane in diethyl ether at RT.

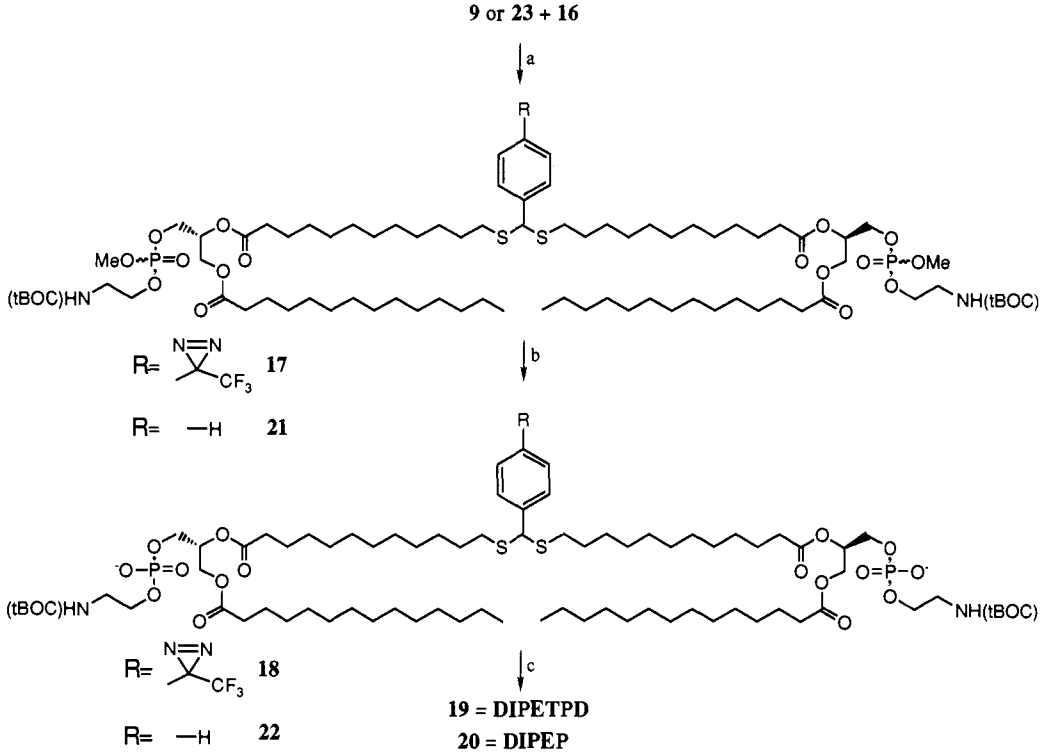

Figure 5. Synthesis of DIPETPD (part 3). Reaction conditions are as follows: (a) 0.5 equiv of **9** (or **23**) mixed with 1 equiv of **16**, DCC, and DMAP in dichloromethane at 0 °C and later at RT; (b) sodium iodide dissolved in refluxing 2-butanone (85 °C); (c) trifluoroacetic acid 50% in dichloromethane with drops of methanol at 0 °C.

1,3-dioxanyl derivative **7** or from *p*-formyl-TPD (**8**) obtained by prior deprotection of **7** back to the aldehyde with Amberlyst 15 in acetone.

12-Bromododecanoic acid (**10**) is reacted with potassium ethyl xanthogenate in acetone to yield 12-((ethoxy(thiocarbonyl))thio)-dodecanoic acid (**11**) quantitatively (98%). Conditions were sought to cleave the xanthogenate with ethylene diamine in ethanol under reducing conditions (with a large excess of sodium borohydride) to yield 12-mercaptododecanoic acid (**12**) (>90%), essentially free from the undesired disulfide (2–4% yield of byproduct) produced in the course of the reaction. This thioacid is reacted with *p*-formyl-TPD (**8**), or its 1,3-dioxanyl derivative **7**, in the presence of a Lewis acid (borontrifluoride etherate) in dichloromethane at 0 °C to yield the TPD thioacetal dicarboxylic fatty acid **9** in high yields (85%). The non-photoactivatable analogue of **9**, namely the phenyl thioacetal dicarboxylic fatty acid **23** (Figure 17, supplementary material) is obtained in a similar fashion starting from benzaldehyde **28** and 12-mercaptododecanoic acid (**12**).

Dimyristoylphosphatidylethanolamine (DMPE; **13**) serves as a building block for the two polar head groups of DIPETPD. The amino group in DMPE is first protected as the *N*-tBOC derivative by reaction with di-*tert*-butyl pyrocarbonate in tetrahydrofuran (98% yield), and the resulting phospholipid **14** is digested with crude snake venom (*Crotalus adamanteus*) in a two-phase system, diethyl ether (1% methanol)–Tris-HCl pH 8.6 buffer in the presence of Ca^{2+}, to yield *N*-tBOC-lyso-PE **15** quantitatively (100%). The phosphodiester function in this last compound reacts with diazomethane in diethyl ether solution to give the phosphotriester **16** in high yield (98%).

This synthetic approach takes advantage of an efficient and general method for the partial synthesis of mixed-chain phosphatidylethanolamines.[18] The key finding is that the reacylation of a *N*-tBOC-lysophosphatidylethanolamine methyl ester with a

carboxylic acid can be achieved in high yield (ca. 85%) at RT with DCC and DMAP dissolved in dichloromethane. We applied this procedure to couple two moieties of *N*-tBOC-lyso-PE methyl ester **16** to one moiety of the dicarboxylic fatty acid **9** (or **23**) in a single step (83% yield). This reaction produced a fully protected derivative of DIPETPD (**17**) (or of DIPEP (**21**)) that was then purified and chemically characterized extensively.

Finally, smooth deprotection of both the phosphate and amino functionalities yields the final product DIPETPD (**19**) (or DIPEP (**20**)). The phosphotriester deprotection to the original phosphodiester, i.e. the removal of the methyl group, is achieved by reaction with sodium iodide in refluxing 2-butanone (85 °C) (95% yield of **18** or **22**). Under these conditions, the reaction is complete after 30–45 min; further exposure to these high-temperature conditions causes damage to the TPD group. The amino group deprotection, i.e. the removal of the tBOC group, is complete (98% yield) after 30 min at 0 °C in the presence of trifluoroacetic acid 50% in dichloromethane. Special care should be taken to avoid strictly anhydrous conditions at this step to preserve the integrity of the thioacetal bridge. This thioacetal function resists those acidic conditions, provided that a small amount of methanol is present in the reaction mixture. An alternative order of the deprotection steps can also be followed, namely, to remove firstly the tBOC groups (to yield compound **32**, Figure 19, supplementary material) and to cleave subsequently the phospho methyl ester to yield the final product.

Chemical and Enzymatic Characterization of the Bipolar Phospholipid. ^1H and ^{13}C NMR spectra of fully protected derivatives **17** and **21** (Figure 5) were run with accurate integration. These derivatives are free from the characteristic broadening shown by phospholipids, due to the elimination of the charges at the head groups. A clear 2:1 ratio of protons belonging to the lysophospholipidic moieties versus those belonging to the dicarboxylic fatty acid is indicative of a doubly coupled product. The ^{13}C NMR spectrum shows only characteristic ester resonances, and no free carboxylate resonances, again pointing to the absence of any mono coupled product. Fast atom bombardment mass spectrometry shows no molecular ion. However, characteristic fragments, including variable portions of the polar head groups and the centrally located aromatic ring, are present. Quantitation of carbon, hydrogen, and, particularly, the heteroatoms sulfur and nitrogen (S:N, 1:1 molar ratio), by elemental analysis, points to the structure of a bis coupled product. Interestingly, heterogeneity is evident by TLC of the pure products **17** and **21**. In fact, this fully protected phospholipid is a mixture of diastereoisomers. This arises from the fixed chirality at the central secondary alcohol in both glyceryl termini and the appearance of new phosphate chiral centers at both ends resulting from the esterification to the phosphotriester. This is readily evident by ^1H NMR spectroscopy in the heterogeneity shown by the $POCH_3$ proton resonances.

The final bipolar phospholipidic products **19** and **20**, unlike the protected counterparts **17** and **21**, show a strong polar character, readily evident on their TLC behavior; i.e. the running solvent has to include very polar solvents like methanol or water. The R_f of these compounds is also strongly influenced by the acidic or basic character of the solvent (additions of acetic acid or aqueous ammonia to the running solvent), indicating the presence of ionizable groups. The staining properties are characteristic of free phospholipids (see Experimental Section), and a positive ninhydrin reaction indicates the presence of free primary amino groups. Fast atom bombardment mass spectrometry of **19** shows a peak at 1476 corresponding to the M + 2 ion. Two lines of evidence point to the bipolar nature of DIPETPD: (a) TLC analysis along the course of both deprotection reactions indicates the presence of transient, intermediate, monosubstituted species, i.e. monomethyl ester and mono tBOC protected derivatives, and (b) we synthesized mono-TNP and bis-TNP derivatives of DIPEP by controlled reaction with TNBS. Each derivative was characterized chromatographically and spectroscopically. ^1H NMR spectroscopy shows the characteristic broadening of the peaks typical of phospholipids (see Figure 20, supplementary material).

Compounds **19** and **20** are good substrates for phospholipases. This indicates that the unusual bipolar nature of these compounds does not hamper recognition by these enzymes. Phospholipase A_2 digestion (*C. adamanteus*) produces the free dicarboxylic fatty acid **9** or **23** and lysophosphatidylethanolamine. This result also shows that (a) the glyceryl C2 configuration, necessary for enzyme recognition, is conserved and (b) minimal acyl or phosphate migration occurs along the synthesis. However, we do not discard the possibility that some degree of heterogeneity present in **19** and **20** might be due to the latter. Phospholipase C digestion (*B. cereus*) yields bis-diacylglycerol **30** (Figure 17, supplementary material). Tosylation of this latter product produces **29**, a compound that is indistinguishable[19] from a product obtained by total chemical synthesis (see Figure 17, supplementary material).

Photolysis of the TPD Function. All the (trifluoromethyl)-phenyldiazirine compounds (**7–9**, **17–19**) show characteristic absorption bands at ca. 360 nm (with shoulders at around 342 and 376 nm) and 224 nm (Figure 6). Upon irradiation of an ethanolic solution of **9** with filtered UV light ($\lambda > 320$ nm), the diazirine bands decay with a half-life of around 60 s in our photolysis setup (inset to Figure 6, see Experimental Section). New, transient bands corresponding to the diazo isomer produced by photoisomerization appear at 460 and 274 nm. Characteristic isosbestic (or quasi isosbestic) points are evident at around 404, 319, 247, and 215 nm. Upon further irradiation, the diazo bands also disappear following the slower photolysis of the diazo isomer. A maximum concentration of diazo isomer (approximately 15–17% of the original diazirine) is reached between 5 and 7 min of photolysis.

Figure 7 shows the corresponding changes in the ^1H NMR spectra upon photolysis. We interpret these changes as follows: Spectrum a is the parent diazirine compound. The singlet at 4.82 ppm is characteristic of the thioacetalic methinic proton. The AA'BB' system at 7.12 and 7.45 ppm corresponds to the aromatic para-substituted phenyl ring. These are sensitive regions in which to detect photochemical changes. Proof of this is the spectrum below (b) of the sample photolyzed in ethanolic solution for 10 min. Both regions suffer changes, with complete disappearance of resonances corresponding to the diazirine (this fully agrees with the disappearance of the UV band at ca. 360 nm). In turn, new signals appear that can be interpreted as arising from the diazo, keto, and ether products. Figure 8 is a schematic diagram that describes this photochemistry. Integration of the signals and chemical shift comparison allow us to tentatively assign the new peaks as follows: diazo isomer (21% yield) δ 4.84 (s) and 7.03, ca. 7.50 (AA'BB'); ethyl ether[20] (major product, 43% yield) δ 4.86 (s) and ca. 7.34, ca. 7.50 (AA'BB'); and ketone (14%) δ 4.88 (s) and 7.60, 8.03 (AA'BB'). Notice that the yield of diazo compound estimated from the integration of signals is in good agreement with those estimated from the UV spectra. After photolysis of the dicarboxylic fatty acid **9**, new spots appear on silica gel TLC plates (solvent = chloroform/methanol, 10:1), and no sign of starting material remains: by comparison, the diazo isomer ($R_f = 0.45$) runs slightly ahead of the parent diazirine ($R_f = 0.44$), and the ketone (and ether?) trails behind ($R_f = 0.31$).

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(19) In fact, while **29** obtained from **30** should be a single isomer (with defined chirality at the glyceryl termini), **29** obtained by total synthesis starting from solketal is a mixture of LL, DD, and meso isomers.

(20) Peaks at δ 1.24 (3 H, under the multiplet), 3.56 (q, 2 H), and 4.57 (q, 1 H) were assigned to the ethyl group protons and methine proton of the ether product.

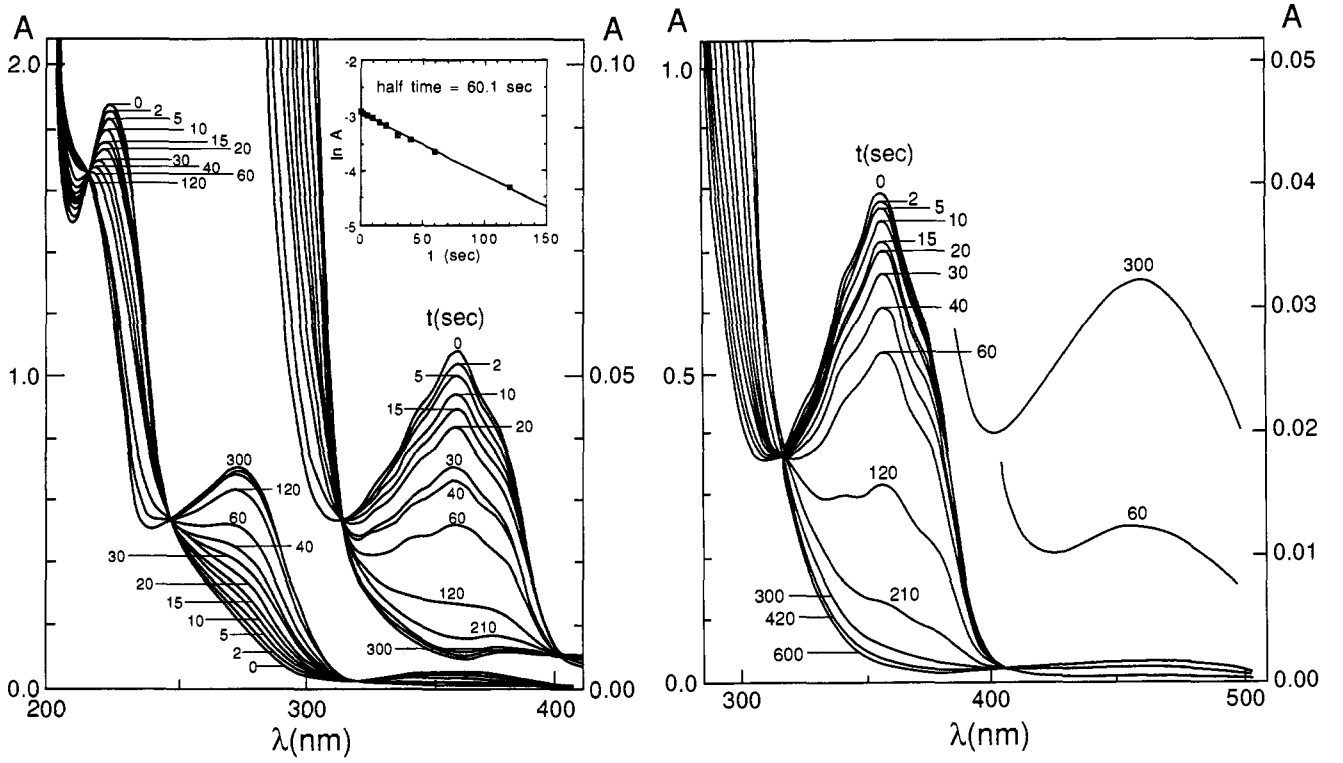

Figure 6. Photolysis of the (trifluoromethyl)phenyldiazirine (UV/vis spectra). UV/vis spectra of compound **9** (t = 0 s), and of the photolysis reaction mixture at the times (in seconds) indicated with numbers. The sample is irradiated with filtered UV light ($\lambda > 320$ nm) in ethanolic solution. On the left, spectra of a dilute solution of **9** being photolyzed are shown at two different sensitivities: on the right, spectra of a concentrated solution of **9** being photolyzed (two different sensitivities) are used to follow the changes at long wavelengths (460 nm). The inset shows the decay of the absorbance at 360 nm as a function of time of photolysis in a semilogarithmic representation. For more details, see main text and Experimental Section.

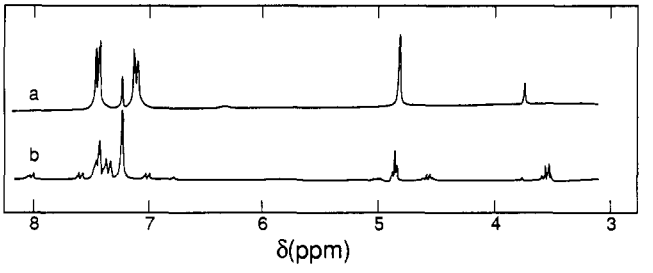

Figure 7. Photolysis of the (trifluoromethyl)phenyldiazirine (NMR spectra). ^{1}H NMR spectra (250 MHz, $CDCl_3$) of compound **9**, before (a) and after (b) photolysis for 10 min with filtered UV light ($\lambda > 320$ nm) in ethanolic solution. The peak at δ = 7.24 ppm is $CHCl_3$. For details, see main text and Experimental Section.

Finally, the photoisomerization reaction to the diazo product is very evident from the appearance of a sharp absorption peak in the IR spectrum at 2085 cm^{-1}, in a region where there is no absorption in the parent diazirine. This is in agreement with values reported in the literature.[10b]

Incorporation of the Bipolar Phospholipid into Liposomes. Experiments have been performed on the incorporation of the bipolar phospholipids DIPETPD and DIPEP into vesicles. Small unilamellar vesicles (SUVs) have been prepared by bath sonication;[21a] and large unilamellar vesicles (LUVs) were obtained either by ether injection[21b] or by high-pressure extrusion through polycarbonate filters[21c,d] (see Experimental Section for details). Both bipolar lipids DIPEP and DIPETPD incorporate readily into lipid vesicles when these compounds are mixed with the other membrane constituent lipids at the beginning of the preparation.

(21) (a) Huang, C.; Thompson, T. E. *Methods Enzymol.* **1974**, *32*, 485–489. (b) Deamer, D.; Bangham, A. D. *Biochim. Biophys. Acta* **1976**, *443*, 629–634. (c) Mayer, L. D.; Hope, M. J.; Cullis, P. R. *Biochim. Biophys. Acta* **1986**, *858*, 161–168. For a review see: (d) Szoka, F., Jr.; Papahadjopoulos, D. *Annu. Rev. Biophys. Bioeng.* **1980**, *9*, 467–508.

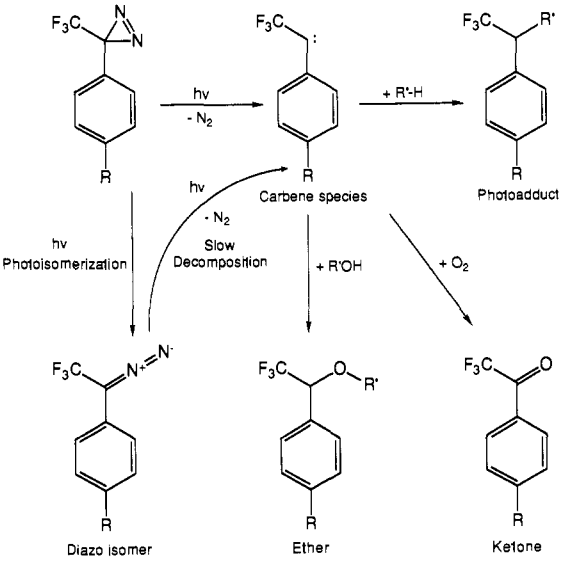

Figure 8. Photolysis of the (trifluoromethyl)phenyldiazirine (schematic diagram). The diazirine generates a highly reactive carbene by irradiation with $\lambda > 320$ nm. A major productive reaction in a hydrophobic (lipidic) environment is the insertion to C–H bonds (top right). However, carbenes show some selectivity for the reaction with nucleophiles. In polar protic solvents (alcohol), the insertion of the carbene to O–H bonds is the major reaction (yielding the ether), and some amount of quenching of the carbene by oxygen can be expected if no special precautions are taken to degas the solution (yielding the ketone). Under our experimental conditions, the maximum amount (15–20%) of the diazo isomer is reached after 5–10 min of irradiation. Further photolysis destroys this isomer, albeit more slowly (half-time of several minutes) than the diazirine (half-time ca. 60 s).

For most studies, DIPEP was used as a paradigmatic model compound of a bipolar phospholipid.

Once incorporation into vesicles is found, the question arises

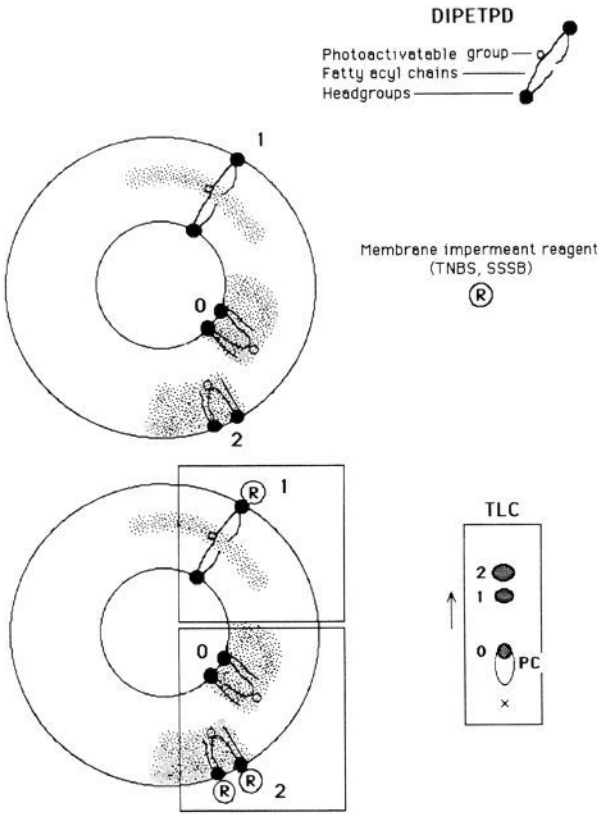

Figure 9. Conformations of DIPETPD in liposomes. DIPETPD (or DIPEP) is represented in the possible conformations that these lipids can adopt in membranes: 'U' shaped facing the interior (0), transmembranic (1), and 'U' shaped facing the external milieu (2). The dotted area indicates the likely regions sampled by the photoactivatable group in each conformation. Once the reaction with a membrane-impermeable amino group reagent (R) is complete, a certain proportion of the lipid molecules remain unreacted (0), some become labeled at one end (1), and some others become labeled at both ends (2). After extraction of the lipids, silica gel 60 F_{254} TLC analysis in solvent 2 (see Experimental Section) separates the three lipid species (notice that unreacted DIPEP moves very close to PC). Shaded spots on the plate correspond to the above mentioned products. For more details see main text and Experimental Section.

as to what conformation the bipolar phospholipid adopts (Figure 9). The flexibility inherent in the bifunctional fatty acyl chain allows the molecule to adopt either a transmembrane conformation or a bent 'U'-shaped conformation. A consequence of the former is that each polar head group faces a different compartment, the interior of the vesicle or its external milieu. In contrast, the 'U' shape positions both polar head groups on the same side, either outwardly or inwardly. We designed an assay based on the chemical reactivity of the amino groups at the polar heads with selected membrane-impermeable amino reagents. A trans bilayer orientation of the reagent will yield only monosubstituted products when the reaction is pushed to completion. On the other hand, molecules in the 'U'-shaped conformation would be expected not to react at all, if inwardly located, or to react at both ends, if outwardly located.

Reagents such as TNBS and fluorescamine have been used to label amino groups of PE in natural or artificial membranes.[22] TNBS is particularly advantageous, since the course of the reaction can be followed spectrophotometrically and the products of the reaction with DIPEP, namely, mono- and di-TNP derivatives,

(22) (a) Litman, B. J. *Biochemistry* **1974**, *13*, 2844–2848. (b) Litman, B. J. *Biochim. Biophys. Acta* **1975**, *413*, 157–162. (c) Rothman, J. E.; Kennedy, E. P. *J. Mol. Biol.* **1977**, *110*, 603–618. (d) Lee, H. C.; Forte, J. G. *Biochim. Biophys. Acta* **1979**, *554*, 375–387.

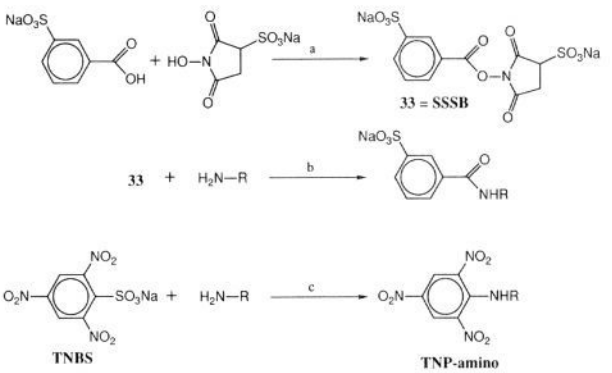

Figure 10. Membrane-impermeable amino group reagents. (a) The synthesis of SSSB (**33**) is achieved in one step by coupling the monosodium salt of *m*-sulfobenzoic acid with *N*-hydroxysulfosuccinimide with the aid of DCC and DMAP in DMF. (b) SSSB (**33**) reacts with amino groups present in vesicles containing dimyristoylphosphatidylethanolamine (DMPE) or DIPEP suspended in phosphates buffer 50 mM, pH 7.1, to yield sulfobenzamides. Hydrolysis of the reagent is the major competing reaction. (c) TNBS reacts with amino groups in phosphates buffer 50 mM, pH 8.4, to yield colored trinitrophenyl (TNP) amino derivatives.

can be readily separated by TLC (Figures 9 and 10, see also Figure 20, supplementary material).

Bilayers are generally considered to be impermeable to TNBS, but permeation rates depend on lipid composition. To avoid ambiguity, we must prove that TNBS does *not* permeate into the vesicles under our particular experimental conditions. To have two reagents for comparison, we synthesized a position isomer of the reagent described by Anjaneyulu and Staros.[23a] Our reagent is *N*-((*m*-sulfobenzoyl)oxy)sulfosuccinimide (SSSB = **33**, Figure 10), following the original impermeable crosslinker reported by Staros.[23b] This reagent converts amino groups into sulfobenzamides (Figure 10). This compound has two sulfonic groups, each located in a different half of the molecule, almost certainly preventing passage through a bilayer. The intrinsic hydrolysis rate of SSSB is higher than that of TNBS, but the reaction with amines competes effectively.

With tight, well-behaved vesicles made out of egg phospholipids, we showed that both TNBS and SSSB label the amino groups of PE to the same extent: 55–60% of the total number of amino groups present in the sample. The extent of reaction is calculated with reference to the value of a duplicate sample to which Triton X-100 has been added before the reagent in order to solubilize the lipids and, therefore, expose all the amino groups to the reagent. The comparison between SSSB and TNBS involved the reaction of an intact vesicle preparation with a suitably large excess of SSSB and letting the reaction proceed to completion. After this is over, time is allowed for the excess reagent to be quenched by the competing hydrolysis reaction. The vesicles are then solubilized with detergent (Triton X-100), and the unreacted amino groups are quantitatively estimated by reaction with TNBS. In this fashion, we modify with TNBS only those groups unreactive toward SSSB, i.e. those groups facing the interior of the vesicles. The difference between this number and 100% reveals the extent of the initial reaction with SSSB. The expected 40–45% of the amino groups were accounted for in this fashion.

With the validation of TNBS, we undertook a systematic study of the lipid composition of the vesicles, so as to optimize their tightness. The results are summarized in Table I. Moderately short-chain saturated lecithins, such as DLPC and DMPC, produce leaky preparations, even if cholesterol is added to the lipid mixture. The presence of either DMPE or DIPEP does not modify this behavior. Some tightening effect of cholesterol is observed, however, for vesicles made from DTPC. Vesicles made

(23) (a) Anjaneyulu, P. S. R.; Staros, J. V. *Int. J. Pept. Protein Res.* **1987**, *30*, 117–124. (b) Staros, J. V. *Biochemistry* **1982**, *21*, 3950–3955.

Table I. Reaction of Amino Phospholipids (DMPE and DIPEP) Incorporated into Lipid Vesicles with TNBS

membrane composition, type of vesicles[a]	% reaction of amino groups at 30 min,[b] average ± σ	permeability of TNBS[c]
	SUVs	
DLPC/DMPE/DMPA[d]	81	high
DLPC/DMPE/DMPA/ cholesterol	80	high
DLPC/DIPEP/DMPA/ cholesterol	82	high
DTPC/DMPE/DMPA/ cholesterol	50 ± 2	low
DMPC/DMPE/DMPA	60 ± 10	high
DMocPC/DMPE/DMPA	52 ± 11	moderate
DMotPC/DMPE/DMPA	67	moderate
MOPC/DMPE(18% w/w)/ DMPA	58 ± 3	low
MOPC/DIPEP(22% w/w)/ DMPA	59 ± 4[e]	low
MOPC/DIPEP(30% w/w)/ DMPA	56 ± 4	moderate
egg PC/DIPEP/DMPA	58 ± 3	low
	LUVs	
MOPC/DIPEP(30% w/w)/ DMPA	55 ± 3	moderate

[a] SUVs (small unilamellar vesicles) and LUVs (large unilamellar vesicles) were prepared according to the techniques described in the Experimental Section. The different PCs are the main components of the vesicles. Unless otherwise indicated, DMPE or DIPEP are present in the lipid mixture at a 20% (w/w) level. DMPA is added (3–5% w/w) to confer a net negative charge to the lipid vesicles. In some cases, cholesterol is added (10–20% w/w) to the lipid mixture. The extent of the reaction with TNBS does not change significantly between samples reacted immediately after being prepared or aged for 1 or 2 days. All liposomes were used within 3 days of being prepared; generally, older samples become "leaky". For mixtures which lead to vesicles that were not clearly "leaky", the numbers in the table were derived from three to eight separate preparations. [b] 100% reaction is estimated from the extent of labeling after dissolving the vesicles in the presence of Triton X-100 and letting the reaction proceed for 30 min at RT. [c] The degree of "leakiness" of the membrane is assessed from the value of the slope of the reaction kinetics curve at 30 min. Please note that here vesicle "leakiness" does not necessarily imply that TNBS actually penetrates into the interior of the vesicle prior to reaction with aminophospholipids located in the inner leaflet. Alternatively, in some cases, i.e. for phospholipids with shorter hydrocarbon chains, this effect may arise from an enhanced lipid exchange between leaflets. [d] Abbreviations are as follows: DLPC, dilauroylphosphatidylcholine (12:0, 12:0); DMPC, dimyristoylphosphatidylcholine (14:0, 14:0); DMocPC, dimyristoleoylphosphatidylcholine (14:1 cis, 14:1 cis); DMotPC, dimyristelaidoylphosphatidylcholine (14:1 trans, 14:1 trans); DTPC, ditridecanoylphosphatidylcholine (13:0, 13:0); MOPC, 1-myristoyl-2-oleoylphosphatidylcholine (14:0, 18:1 cis); DIPEP, phenyl bis-phosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine (14:0, 14:0); DMPA, dimyristoylphosphatidic acid (14:0, 14:0); TNBS, 2,4,6-trinitrobenzenesulfonic acid. [e] For this sample, after 6 and 48 h, the extent of reaction is 65 and 66%, respectively.

from PCs containing only unsaturated fatty acids (DMocPC and DMotPC) are marginally permeable. On the other hand, a complex mixture of lipids such as egg lecithin or a pure lipid species with only one unsaturated chain (such as 1-myristoyl-2-oleoyl-PC = MOPC) produces vesicles that appear to be relatively impermeable to the reagent. The addition of up to 30% by weight of DIPEP to the lipid mixture did not influence the permeability of the membranes toward TNBS. The effect of the addition of DIPEP to the vesicles is equivalent to that of DMPE; the kinetics and the final extent of reaction (around 60%) are similar for both lipids. This result indicates that the distribution of the head groups between the inner and outer layers is the same as that for DMPE, suggesting that head group packing, rather than the nature of the fatty acids of the phospholipid, is the major factor determining the conformation of the bipolar phospholipid. In all cases, a plateau level of modification at ca. 55–60% is usually reached after 30 min of reaction (Figure 11).

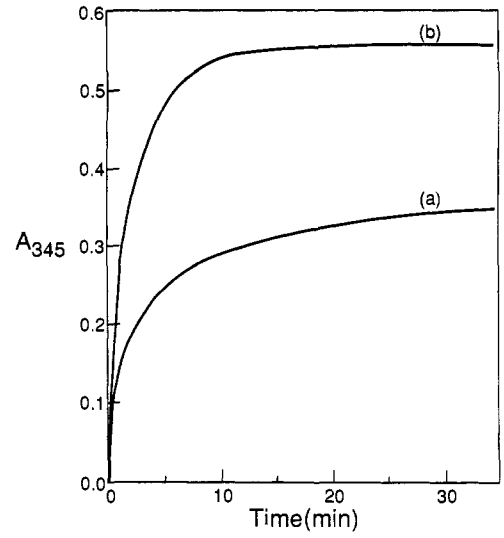

Figure 11. Reaction kinetics of the amino groups in DIPEP versus TNBS. Typical kinetics of reaction of amino groups with TNBS in the bipolar phosphatidylethanolamine DIPEP incorporated into SUVs prepared with 1-myristoyl-2-oleoylphosphatidylcholine (MOPC) as the main lipid component. The progress of the reaction is monitored continuously by measuring the A_{345} versus a blank of reagent. Curve (a) is the time course of the reaction with vesicles. Curve (b) is the corresponding kinetics when Triton X-100 is present in the medium to solubilize the vesicles. For more details see Experimental Section.

No significant increase is evident after this time. This result is expected from the outer/inner surface ratio of SUVs; the corresponding value with LUVs is somewhat lower, 55%, as expected from geometrical considerations. For most of the studies, MOPC or egg PC was used as the main lipid component. Small amounts of PA (phosphatidic acid) were added to give the vesicles a net negative charge.

After the TNBS reaction of vesicles loaded with DIPEP has proceeded for 30 min, the lipids are extracted and analyzed by TLC (Figure 9). Visual inspection of the plates always reveals spots of roughly equal yellow intensity corresponding to mono-TNP (**34**) and di-TNP (**35**) derivatives of DIPEP.[24] Considering that the extinction coefficient of the latter is about double the value of the former, this result reveals a molar ratio of 2:1 for these compounds in the reaction mixture. This is confirmed by determination of phospholipid phosphate in some selected samples (results not shown). Unfortunately, the unreacted DIPEP migrates very close to the main spot of PC, preventing an accurate direct quantitation of nonmodified phospholipid. However, the combination of the relative proportions of mono- and di-TNP derivatives with the overall extent of reaction permits us to conclude that the experimental distribution of the populations of DIPEP in the lipid bilayer is close to the statistical proportion 1:2:1, for outwardly 'U'-shaped/transmembrane/inwardly 'U'-shaped conformations. The implications of this finding for labeling are discussed later.

In joint experiments with Dr. Toon Stegmann (Department of Cell Biology, Yale), vesicles loaded with the bipolar phospholipid appear quite uniform and unilamellar by electron microscopy (negative stain). Fusion of liposomes with influenza virus has been investigated using fusion assays based on either the presence of a self-quenching fluorescent probe, octadecylrhodamine, in the membrane of the virus or a pair of fluorescent phospholipids in the membrane of the liposomes that exhibit resonance energy transfer.[25] Vesicles loaded with the bipolar phospholipid appear

(24) These derivatives were independently synthesized, isolated, and characterized by TLC and NMR and UV and IR spectroscopy, to serve as standards for the assay (Figure 20, supplementary material).

(25) Stegmann, T.; Hoekstra, D.; Scherphof, G.; Wilschut, J. *J. Biol. Chem.* **1986**, *261*, 10966–10969.

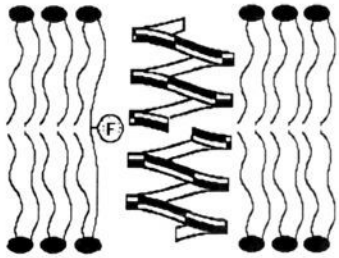

Figure 12. Ion channel conformation of gramicidin A in a lipid bilayer loaded with DIPETPD. A schematic drawing of a section of the lipid bilayer containing DIPETPD in the transmembrane conformation next to the ion channel form of gramicidin A, shown in a ribbon diagram representation. The N-termini of the peptide dimer are located close to the center of the membrane.

to undergo fusion reactions as easily as the reagent-free vesicles, with no evidence for inhibition of fusion. It is thus possible that other vesicles or cells may be loaded with DIPETPD through a fusion process. This approach was followed recently in an analysis of the kinetics of fusion of influenza virus to vesicles loaded with DIPETPD mediated by hemagglutinin.[3] In these studies, the DIPETPD probe is incorporated in complex mixtures of lipids extracted from biological membranes. Since these lipids must have to accommodate molecules of many different shapes and sizes in nature, it is expected that they can more easily accommodate the probe in the appropiate conformation, while at the same time provide an ideal target for membrane fusion.

Labeling of Gramicidin A and an Analogue by DIPETPD. Gramicidin A (gA) is a hydrophobic pentadecapeptide with the sequence HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-[L-Trp-D-Leu]$_3$-L-Trp-NHCH$_2$CH$_2$OH. In 5–20% of the population, L-Ile replaces the N-terminal L-Val.[26] The N- and C-termini are blocked, and there are no charged or polar residues. This peptide is believed to form symmetrical $\beta^{6.3}$ head-to-head dimers, the so-called "ion channel" form, that span the membrane with the N-terminus of each monomer located close to the center of the lipid bilayer.[27] The altenating L- and D- sequence results in all side chains projecting outward facing the lipid environment. A schematic ribbon diagram roughly to scale is shown in Figure 12 along with the DIPETPD molecule.

The results of photolabeling gA with DIPETPD in lipid vesicles (SUVs) are shown in Figure 13. [^{14}C-formyl]gA was used as the target peptide. TLC analysis of the products demonstrates the presence of a photoadduct (lane 4: low-R_f radioactive band, labeled **pa**), which appears only if DIPETPD is present and the sample is irradiated. The chromatographic behavior is consistent with the addition of polar groups to the hydrophobic peptide.

A peptide analogue of gA in which L-Trp replaces the natural L-Val (or L-Ile) in position 1 was synthesized and used in parallel experiments with gA. The results are summarized in Figure 14. Electron micrographs show normal, uniform SUVs with each peptide. In both cases, new radioactive components (indicated with arrows) that migrate with the same R_f appear upon irradiation of the samples. Integration of the area under the new peaks indicates that the yield of these products is different: ca. 2% for natural gA and ca. 4% for the 1-Trp analogue. The implications of this result are discussed later.

Studies Toward a Radioactive Version of DIPETPD. With the original design of DIPETPD, the intention was to insert a radioactive label for analysis after the photolysis reaction was complete ("indirect" labeling protocol). This would be accomplished by cleaving the thioacetal (e.g. with Ag^+) to regenerate the aldehyde on the now-attached photolabeling group. The aldehyde would be reduced "a posteriori" with sodium borotritide

(26) (a) Sarges, R.; Witkop, B. *J. Am. Chem. Soc.* **1965**, *87*, 2011–2020. (b) Sarges, R.; Witkop, B. *J. Am. Chem. Soc.* **1965**, *87*, 2020–2027.
(27) Urry, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 672–676.

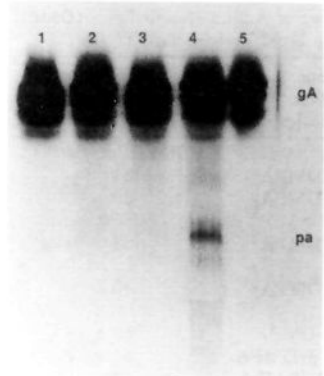

Figure 13. Photoreaction of DIPETPD with [^{14}C-formyl]gramicidin A (gA) in lipid vesicles: analysis of the products by TLC and autoradiography. Samples are prepared and processed as described in the Experimental Section. Lanes 1 and 2 do not contain DIPETPD; lanes 3 and 4 contain the reagent. Samples 1 and 3 were not irradiated; samples 2 and 4 were irradiated with filtered UV light for 30 min. Lane 5 is a standard sample of [^{14}C-formyl]gA. Analysis of the products is performed on silica gel 60 F_{254} TLC plates developed with solvent 2 (see Experimental Section) and autoradiography. Similar results were obtained with [^{14}C-formyl]1-Trp-gA (not shown). pa indicates the presence of a photoadduct.

of high specific activity to yield an alcohol, or derivatized further by reaction with an amine (Schiff's base formation) and selective reduction with sodium cyanoborotritide.[28] Prereduction of the sample with cold borohydride before cleavage was expected to reduce the background radioactive labeling. This procedure would avoid the necessity of synthesizing a complete reagent with high specific activity. This protocol does work and has been used in experiments on synthetic transmembrane fragments of bacteriorhodopsin and glycophorin. In both cases the above-described "indirect" procedure generates tritiated products only when DIPETPD is present in the vesicles and photolysis takes place (J. Hunt and J. M. Delfino, unpublished), but it is unlikely to be easily and generally applicable because of the technical difficulties associated with each particular membrane protein system intended for study.

A radioactively labeled derivative at high specific activity will be extremely useful to follow the labeled products through structural analysis. This is especially important in the case of a photoreagent, given the intrinsic low yields of the reaction. Reductive methylation of the PE head groups of DIPETPD with formaldehyde and sodium borotritide or cyanoborotritide[29] (see Figure 21, supplementary material for this reaction on PE) produced a labeled reagent with minimum effort. This approach is possible because the mild reaction conditions employed preserve the integrity of the photoreactive group TPD and the bridging thioacetal. These methylated forms of DIPETPD behave as does the parent reagent in terms of incorporation into liposomes and ability to label transmembrane peptides. A nonradioactive preparation of methylated DIPETPD was used to label [^{14}C-formyl]gA and [^{14}C-formyl]1-Trp-gA in experiments similar to those reported above, with comparable results (Figure 15). The methylated derivative of DIPETPD produces at least two distinctly different photoadducts (bands shown by arrows in lanes 1 and 4), which, in all likelihood, result from the intrinsic heterogeneity of the methylated reagent (mono- and dimethyl amino groups are possible at each end; for details see Figure 21, supplementary material). Labeling experiments of gA with the radioactive reagent were complicated by difficulties in the analysis of the products, mainly due to the extreme hydrophobic character of

(28) For the reduction of aldehyde groups present in some proteins with sodium cyanoborotritide see: van Poelje, P. D.; Snell, E. E. *Anal. Biochem.* **1987**, *161*, 420–424.
(29) (a) Borch, R. F.; Bernstein, M. D.; Durst, H. D. *J. Am. Chem. Soc.* **1971**, *93*, 2897–2904. (b) Jentoft, N.; Dearborn, D. G. *J. Biol. Chem.* **1979**, *254*, 4359–4365.

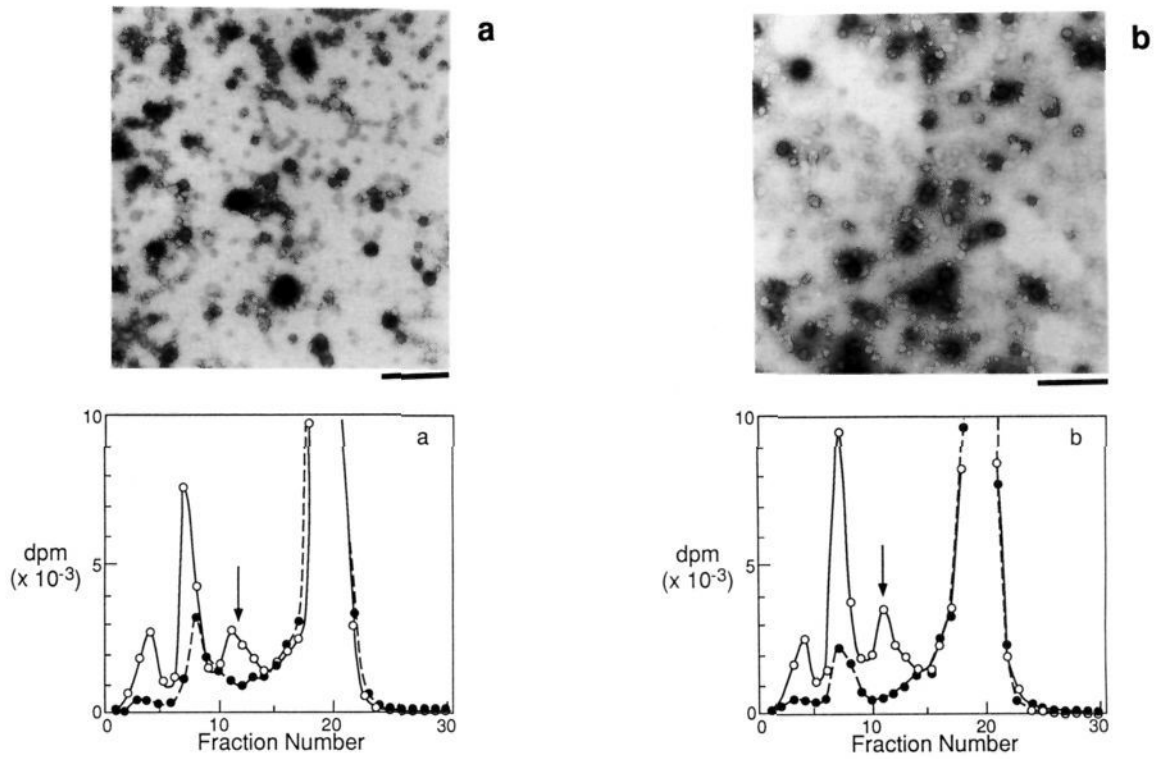

Figure 14. Photoreaction of DIPETPD with [^{14}C-formyl]gA and [^{14}C-formyl]1-Trp-gA in lipid vesicles: electron micrographs of vesicle preparations and TLC analysis of the photoadducts. Electron micrographs (negative stain technique) of lipid vesicles (SUVs) prepared with dimyristoylphosphatidylcholine (DMPC), DIPETPD, and either (a) [^{14}C-formyl]gA or (b) [^{14}C-formyl]1-Trp-gA are shown in the top portion of the figure. The length of the bar equals 1000 Å. A quantitative TLC analysis of the photoproducts is shown in the bottom portion of the figure for the same samples. After photolysis, the peptides and lipids are extracted and analyzed on silica gel 60 F_{254} TLC plates developed with solvent 2 (see Experimental Section). Three-millimeter slices of silica are scraped off the plate, and their radioactivity is measured by liquid scintillation counting. Open circles represent the measurements on a photolyzed sample. Control samples (without photolysis) are shown in black circles. The arrows indicate the position of the photoadducts.

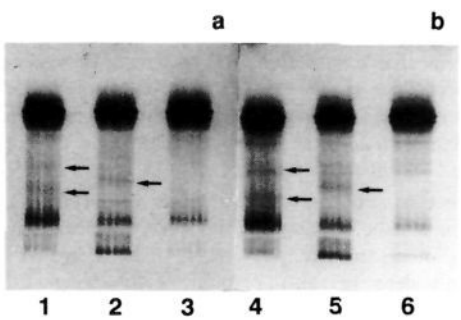

Figure 15. Photoreaction of DIPETPD and a methylated derivative with [^{14}C-formyl]gA and [^{14}C-formyl]1-Trp-gA in lipid vesicles: analysis of the products by TLC and autoradiography. The experimental procedure and analysis is similar to that described before in the legend to Figure 13. (a) [^{14}C-Formyl]gA or (b) [^{14}C-formyl]1-Trp-gA is the target peptide. Methylated DIPETPD (lanes 1 and 4), DIPETPD (lanes 2 and 5), or no photoactivatable lipid reagent (lanes 3 and 6) is included in the lipid mixture. The position of the photoadducts is indicated with arrows.

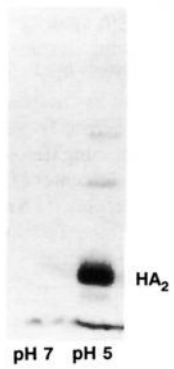

Figure 16. Labeling of influenza virus hemagglutinin (HA) with a tritium methylated derivative of DIPETPD. Autoradiograph of products separated by SDS-PAGE after incubation of liposomes containing a tritium methylated derivative of DIPETPD with influenza virus at 37 °C. The procedure is described in the Experimental Section. Fusion of virus with liposomes occurs under acidic conditions (right lane, pH 5). A control sample incubated at pH 7 is also shown (left lane). After the incubation, both samples are photolyzed. Only HA_2, the polypeptide chain of HA including the segments known to interact with the lipid bilayer, namely the "anchor" peptide and the "fusion" peptide, is labeled with the photoactivatable reagent under fusion conditions.

this peptide. In addition, further analysis at the level of individual amino acids in this case is hampered by the poor repetitive yields of the Edman degradation of gA (75–80%), again, a consequence of the hydrophobic nature of gA.

This tritium methylated version of DIPETPD was used in early experiments on the influenza virus hemagglutinin (HA) system in collaboration with Dr. Toon Stegmann. HA is an integral membrane protein responsible for the fusion between this virus and target cells. Here DIPETPD is loaded into vesicles that serve as the target for fusion with the virus. Figure 16 shows the experimental results. At neutral pH no fusion reaction occurs, and consequently, no photolabeled protein is found. In contrast, at pH 5, fusion between virus and vesicles occurs readily and hemagglutinin becomes labeled. Only polypeptide chains of hemagglutinin interacting with the lipids become labeled (HA_2), while extramembrane portions (HA_1) do not. The fact that DIPETPD does not exchange between membranes prior to fusion and is then a suitable reagent to study this vectorial process was

established in this early work. The more detailed studies of that system have been reported elsewhere.[3]

In agreement with this result, Dr. James Clifton[30] demonstrated that water-soluble proteins such as carbonic anhydrase and staphylococcal nuclease, when added to a reconstituted preparation of the vitamin B_{12} receptor in asolectin vesicles loaded with DIPETPD, do not become labeled while the receptor does. This points out again the high specificity for the labeling of membrane proteins shown by DIPETPD (results not shown).

The further use of the methylated version of the DIPETPD reagent has been abandoned because it would not be useful in detailed analytical studies. For sequence analysis of photolabeled peptides, one will want to remove the bulk of the bipolar reagent following attachment by cleavage of the thioacetal. The tritium label would be lost at this point. Clearly, the most desirable compound would have the label stably attached to the photochemical group in the primary reagent. This has been accomplished and will be reported elsewhere.[2] The synthesis follows the same path described in this paper, except for the initial insertion of the tritium in the aldehyde function.

Discussion

In this paper we introduce a new photoactivatable reagent DIPETPD designed to label regions deeply embedded within the hydrophobic core of membranes. This compound appears to lack the drawbacks of reagents such as TID (Type I, Figure 1), namely, the occurrence of spurious labeling of protein domains located outside the membrane. On the other hand, DIPETPD, being a bipolar phospholipid, was designed to fix the position of the photoactivatable group by anchoring it in the middle of a dicarboxylic fatty acid. This should prevent problems associated with Type II reagents (Figure 1), namely, the expected "blurring" of the labeling site due to the large excursions of the ends of fatty acyl chains of phospholipids to which the photoactivatable group is attached.

DIPETPD can be loosely classified within a heterogeneous class of molecules designed to span the lipid bilayer. Among these one can count the following: a spin-labeled transmembrane reagent based on bipolar fatty acids,[31] transmembrane bispyridinium polyenes that operate as "molecular wires",[32] "bolaamphiphilic" molecules that generate "covalent monolayer" membranes,[33] and a membrane-spanning steroid metalloporphyrin that immobilizes a heme group in the center of a membrane to perform regioselective oxidation of steroids.[34] An independent synthetic effort toward bifunctional photoactivatable phospholipids based on benzophenone was also published.[35]

The demonstrated conformational heterogeneity of DIPEP (a model compound for DIPETPD) in membranes merits further discussion. We found an essentially identical distribution of head groups between the inner and outer layers for DMPE and DIPEP in vesicles of varying lipid composition. This result is not surprising in view of the fact that no large rotational barriers exist along the aliphatic chain connecting the head groups in DIPETPD, therefore allowing head group packing at the interfacial region to drive this distribution.

The expected flexibility at the central hinge region in DIPETPD would then allow roughly equal partitioning between the two different conformers of this large molecule (a 1:2:1 distribution among outwardly 'U', 'trans', and inwardly 'U' conformations was indeed found for DIPEP incorporated into lipid vesicles). Circumstantial evidence for the flexibility of the thioacetalic junction was gathered from the isolation of a 26-member ring macrocycle (compound **31**, Figure 18, supplementary material) obtained through an intramolecular Dieckmann condensation of the dimethyl ester of the phenyl thioacetal dicarboxylic fatty acid **23**.

In the 'trans' conformation, adequate packing interactions between the dicarboxylic fatty acid and both myristoyl chains allow DIPETPD to be accommodated in membranes. In addition, in the 'U'-shaped conformation, modeling of DIPETPD shows that the thioacetalic junction introduces no strain that could hamper van der Waals contacts along the two arms of the bent dicarboxylic acid. Both 'trans' and hairpin ('U') conformations locate the TPD group in the vicinity of the middle plane of the lipid bilayer.

Molecules in the 'trans' conformation should unambiguously label regions located in the center of the bilayer, since motion of the TPD group attached to a stretched chain should be very small. On the other hand, molecules in the 'U-shaped' conformation do not necessarily implicate a departure from this behavior. Movement of the TPD group in this case is conceivable, but it requires the concerted bending of two fatty acyl chains attached to the probe. This less probable event makes the bent covalent chain much stiffer than a normal fatty acyl chain of the type found in reagents of Type II. We expect that, on average, DIPETPD would sample regions close to the geometric center plane of the lipid bilayer with a higher probability than any of the previously available hydrophobic reagents.

A direct consequence of the design of DIPETPD is the possibility of labeling almost exclusively membrane-spanning regions of integral membrane proteins. In this regard, we recently presented evidence that an amphiphilic peptide thought to interact laterally with the lipid bilayer and, therefore, shallowly embedded in the membrane, the so-called "fusion peptide" of influenza virus hemagglutinin, is only marginally labeled with DIPETPD,[3] whereas PTPC/11[14e] (a Type II reagent) labels it clearly. Our current belief is that positive labeling with DIPETPD may provide diagnostic evidence for transmembrane domains of integral membrane proteins. The evidence gathered so far on the different membrane protein systems studied supports this hypothesis. The use of DIPETPD to strictly identify membrane-spanning stretches of amino acids may prove to be a useful experimental counterpart to predictive methods for transmembrane regions in proteins based on amino acid sequences.[36]

Apart from gramicidin A (and the 1-Trp analogue), DIPETPD, when co-reconstituted into vesicles containing integral proteins or peptides, labeled the following systems: the vitamin B_{12} receptor from *Escherichia coli*,[30] hemagglutinin of influenza virus,[3] synthetic peptides corresponding to the transmembrane domain of glycophorin and bacteriorhodopsin,[37] the nicotinic receptor

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of *Discopyge tschudii*,[38] and the calcium pump of human red blood cells.[39] Peptides containing putative membrane-spanning segments incorporate most of the label, while extramembranous peptides show insignificant reaction with DIPETPD.

For statistical reasons, the nonspecific photolabeling reaction of DIPETPD occurs mainly with the surrounding lipid environment. A low yield is always expected for reaction with the peptide target. However, analysis of these yields is useful in understanding the characteristics of the labeling by this reagent. With gramicidin A, the dominant reaction with carbenes occurs with the indole rings of tryptophan.[14c] Phospholipidic reagents of type II preferentially label these residues in gA, in spite of their location in the outer portion of the bilayer in the C-terminal half of this peptide. In the 1-Trp analogue, a fifth Trp is substituted for the less reactive Val in position 1.[40] We observed that this substitution doubles the yield of the photoadduct (from 2 to 4%). The rate increment would have been substantially less if the competition were with reaction at the Trp residues located in the C-terminal half. This result supports the geometrical regioselectivity of the DIPETPD reaction. Further refinement of the precise locale of labeling at amino acid resolution is required to adequately address this point.

We envision that the general structure of the bipolar phospholipid could provide an adequate framework to locate other probes of membrane environment such as spin label or fluorescent groups deep into the hydrophobic core of the lipid bilayer. In the transmembrane conformation, the thioacetal bridge at the center of the molecule is restricted in its movement (entirely losing one translational degree of freedom). This situation allows one to position groups attached to the chain, not only at a precise depth within the membrane but also potentially with a defined orientation with respect to the plane of the membrane. This may be particularly advantageous, for example, in studies of polarization of fluorescence or in spectroscopic studies on oriented membrane samples (spin labels, NMR, IR, CD, etc.).

Conceivably, head group differentiation of DIPETPD might produce asymmetric reagents that could orient themselves in a unique fashion across the membrane. A hypothetical example would be modification of DIPETPD with phospholipase D to create a hybrid molecule with head groups of PE and PC. This molecule could likely orient itself in SUVs following the known tendency of PE to reside with preference in the inner leaflet and PC in the outer leaflet. In principle, if this situation is achieved, one can bias the natural distribution of conformations of these bipolar molecules ('trans' vs 'U' shaped), favoring a single defined transmembrane orientation. Another possibility would be to take advantage of a pH gradient across the membrane as the driving force for the orientation of amphiphilic molecules in vesicles.[41] A unique orientation of an asymmetric transmembrane reagent opens the possibility of locating the probe groups at variable depths across the membrane.

DIPETPD does not appear to exchange readily between membranes. This physical characteristic of DIPETPD may find a structural correlate in the "double anchor" nature of the molecule: four fatty acyl chains (two normal fatty acids and a "double" fatty acid linked to the probe group) insert into the lipid bilayer, as opposed to only two fatty acyl chains present in natural phospholipids. As pointed out by others,[14c] the presence of polar heteroatoms within the photoactivatable fatty acyl chain increases the transfer of the phospholipid between membranes. In this regard, the low polar character of the thioacetal bridge of DIPETPD may effectively mimic a purely aliphatic chain.

A separate feature of the design of DIPETPD is the potential cleavability of the thioacetal bridge. With the twofold purpose of (a) eliminating the phospholipid attached to the photoadduct to facilitate analysis of products and (b) radioactively tagging the photolabeled peptides, we also pursued the "indirect labeling" procedure describe in the Results section. Here, we cleave first the thioacetal bond and reduce the nascent aldehyde with selective tritiated reagents. We do not expect this protocol to be generally applicable in all cases; therefore, further elaboration along these lines led us to the synthesis of a new radioactive version of DIPETPD, in which the central methinic proton is replaced by tritium and which is therefore capable of effectively transferring a tritium label to the photoadducts. These studies are the matter of a separate publication.[2] This last labeled version of DIPETPD proved to be useful to assess the role of hemagglutinin in the mechanism of influenza virus fusion.[3]

Experimental Section

General Procedures. ^1H and ^{13}C NMR spectra were recorded on a Bruker WM 250 NMR spectrometer, FTIR spectra on a Nicolet 5SX/5DX FTIR spectrometer equipped with a CDC Control Data 9427H Cartridge Disk Drive, mass spectra on a Hewlett-Packard 5985 GC/MS system mass spectrometer equipped with a HP 5840A gas chromatograph, and FAB spectra on a Carlo Erba/Kratos HR GC/MS MS80RFA mass spectrometer at the Yale University Chemistry Instrument Facility. UV spectra were recorded on a Varian Cary 219 UV spectrophotometer, and fluorescence spectra on a Perkin Elmer MPF-3 spectrofluorometer.

Normally, column chromatography is performed on silica gel 60 (E. Merck) or Sephadex LH-20 (Pharmacia). Thin-layer chromatography (TLC) is routinely performed on 5.0 × 1.6 cm plates of silica gel 60 F_{254} (E. Merck). The sample is applied at about 0.7 cm from the bottom and 0.5 cm from the sides, three to four 1-μL samples per plate. Depending on the solvent, the chromatography takes 2–5 min to develop. R_f values reported here are only approximate and should be considered in comparison with standard compounds. Different solvent systems are employed; among the most commonly used are the following: solvent 1, chloroform/methanol (20:1, v/v); solvent 2, chloroform/methanol/water/acetic acid (65:25:4:1); solvent 3, chloroform/methanol/ammonium hydroxide (20:5:1); solvent 4, chloroform/methanol/water (65:25:4); solvent 5, chloroform/methanol/ammonium hydroxide (75:25:4). Spots on the TLC plates were visualized by fluorescence quenching under illumination with a 254-nm UV light source and/or staining with various reagents: ammonium molybdate/ceric sulfate (general stain; 40 g of ammonium molybdate tetrahydrate and 0.4 g of cerium(IV) sulfate in 400 mL of 10% aqueous sulfuric acid), molybdenum blue (stain for phospholipids; Sigma), ninhydrin (stain for amino groups; 0.2% acetone solution). Occasionally, charring of the plate by immersion in 15% aqueous sulfuric acid and heating (>350 °C) on a hot plate was used to detect fatty acids and derivatives.

All reagents employed were of analytical grade and were purchased from Aldrich Chemical Co., Sigma, or Fluka. Benzene, THF, diethyl ether, dichloromethane, and chloroform were distilled before use from appropriate desiccants. DMF is dried over activated molecular sieves. All other solvents were reagent grade and used without further purification. Radiochemicals were purchased from Amersham (Arlington Heights, Illinois). Phospholipids were purchased from Avanti Polar Lipids (Birmingham, Alabama).

Photolysis. Photolysis experiments are routinely carried out employing a cylindrical 450-W Hanovia medium-pressure mercury lamp as the light source. The lamp is cooled down by a circulating cold water jacket, and the light is filtered through a 1.5 cm thick saturated copper sulfate sleeve. This filter cuts off essentially all the UV emission below 320 nm. In order to control the exposure time, we located a shutter in between the light source and the sample holder. Samples to be photolyzed with volumes under 2 mL are placed in quartz cuvettes that fit into thermostated cuvette holders. Larger samples are placed into thin (<1 cm) cross section round-shaped quartz vessels that can hold up to 30 mL; they are thermostated by air flowing from a heat gun. Samples to be photolyzed are routinely first degassed and then bubbled, layered with argon, and capped tightly.

Radioactivity Measurements. TLC plates containing ^3H or ^{14}C products are sprayed with En^{3}Hance (New England Nuclear); the surface is then

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covered with preflashed Kodak XAR-5 film for autoradiography, and the film is exposed at −70 °C in the dark. Direct quantitation of radioactivity present on TLC plates (10 cm long runs) is achieved by cutting 2–3 mm wide slices along the run, scraping the silica off the glass plate, and counting the samples by liquid scintillation.

Synthesis and Characterization of Compounds: 4-(2-(1,3-Dioxanyl))-bromobenzene (2). 4-Bromobenzaldehyde (**1**, Aldrich, 15.3 g, 82.5 mmol) is dissolved in 100 mL of dry benzene; 1.5 g (10% by weight) of activated Amberlyst 15 (strongly acidic resin catalyst) and 7.5 mL (104 mmol, 1.25 molar excess) of 1,3-propanediol are added. After addition of some Boileezers and flushing the vessel with nitrogen gas, the mixture is refluxed with stirring for 2.5–3 h. Water is extracted in the process by azeotropic removal and collected in a Dean–Stark trap.

The reaction mixture is filtered, the filtrate is washed twice with water, and the organic phase is dried with $MgSO_4$. Finally the solvent is eliminated under reduced pressure to yield a pale-yellowish oil that solidifies to a pale-yellow powder. This material appears to be pure by TLC (solvent = hexanes/ethyl acetate, 2:1). No trace of the starting material (upper spot) is detected. The NMR spectrum shows essentially pure product. Yields are quantitative. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.46 (complex doublet, J_{gem} = 13.5 Hz, 1 H), 2.22 (m, J_{diax} = 12.4 Hz, 1 H), 3.98 (complex triplet, 2 H), 4.27 (complex double doublet, 2 H), 5.47 (s, 1 H), 7.36, 7.50 (AA'BB', J_{AB} = 8.5 Hz, 4 aromatic H). FTIR (film): wavenumber cm^{-1} 3442 (br, m), 2969 (s), 2953 (s), 2864 (s), 1490 (m), 1470 (m), 1462 (m), 1399 (s), 1384 (s), 1237 (s), 1211 (m), 1108 (s), 1012 (s). MS (EI, 20 eV): *m/e* (%) 244.1 (62.1, M + 1), 243.2 (95.2, M), 242.2 (58.5, M − 1), 241.1 (100.0, M − 2), 186.1 (28.2), 185.1 (60.1), 184.1 (29.7), 183.1 (55.7), 163.2 (24.3), 158.0 (5.4), 157.0 (6.3), 156.0 (5.6), 155.0 (4.7).

1-(4-(2-(1,3-Dioxanyl))phenyl)-2,2,2-trifluoro-1-ethanone (3). 4-(2-(1,3-Dioxanyl))bromobenzene (**2**, 9.05 g, 37.2 mmol) is dissolved in 200 mL of dry diethyl ether. The round-bottom flask is gassed with dry argon and capped tightly with a rubber stopper. The solution is cooled to −30 °C. *n*-Butyl lithium (18 mL of a 2.5 M solution in hexanes, 45 mmol) is added slowly with a syringe to the stirred solution. After the addition is completed, the reaction mixture is allowed to warm up to 0 °C for a period of 2 h. At the end, the solution is cooled down to −50 °C and 8.76 g (48.4 mmol) of (trifluoroacetyl)piperidine (see below) dissolved in 40 mL of diethyl ether is slowly added with a syringe. The reaction was allowed to proceed for an additional 3 h at −50 °C and then quenched with a concentrated ammonium chloride aqueous solution.

The ether phase was washed five times with the ammonium chloride solution and three times with water, then dried with $MgSO_4$, and filtered, and the solvent was evaporated under reduced pressure. The crude product (light-yellowish oil) is purified by silica gel column chromatography employing chloroform/petroleum ether (4:1) as the eluting solvent. The product shows long spots on a TLC plate (solvent = chloroform/ethyl acetate, 9:1), probably due to the polar character of the trifluoro ketone, which exists normally as the hydrated form. The nonoptimized overall yield after purification typically ranges between 20 and 30%. An alternative procedure that yields equivalent results employs methyl trifluoroacetate (Aldrich) instead of (trifluoroacetyl)piperidine. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.50 (complex doublet, J_{gem} = 13.5 Hz, 1 H), 2.25 (m, J_{diax} = 12.4 Hz, 1 H), 4.03 (complex triplet, 2 H), 4.31 (complex double doublet, 2 H), 5.57 (s, 1 H), 7.68, 8.08 (AA'BB', J_{AB} = 8.2 Hz, 4 aromatic H). ^{13}C NMR ($CDCl_3$, 63 MHz): δ 25.60, 67.35, 100.08, 114.22, 118.92, 126.80, 127.18, 127.56, 127.81, 129.99, 130.41, 145.91. FTIR (film): wavenumber cm^{-1} 3400 (br), 2959 (w), 2867 (w), 1717 (s), 1382 (m), 1195 (s), 1173 (s), 1152 (m), 1137 (s), 1104 (s), 1026 (s). MS (EI, 20 eV): *m/e* (%) 277.1 (0.8, M + OH), 260.1 (26.2, M), 259.1 (17.8, M − 1), 243.2 (1.0), 201.1 (28.8), 191.2 (6.1), 173.1 (1.6), 163.2 (4.5), 149.1 (2.2), 133.1 (59.2), 105.1 (23.1), 87.1 (23.0), 86.1 (24.0), 49.1 (12.1), 42.2 (100.0).

(Trifluoroacetyl)piperidine. Triethylamine (14 mL, 100 mmol) is dissolved in 100 mL of diethyl ether; to this solution is added 12 mL of piperidine (121 mmol) at RT. Trifluoroacetic anhydride is added in portions during a span of 30 min up to a total volume of 14.2 mL (100 mmol). The mixture is then refluxed with stirring for an additional 3 h.

The reaction mixture is washed first three times with 0.1 N HCl and then once with water; the ether phase is dried with $MgSO_4$, and the solvent is evaporated. The oil that remains is distilled under reduced pressure (bp 110 °C) to give 8.8 g of product, a transparent oil (unoptimized yield is approximately 60%). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.52–1.68 (m, 6 H), 3.43–3.60 (m, 4 H). FTIR (film): wavenumber cm^{-1} 2942 (m), 2863 (w), 1694 (s), 1511 (w), 1466 (m), 1447 (m), 1373 (w), 1307 (m), 1286 (w), 1243 (m), 1192 (s), 1139 (s), 1126 (s), 1024 (w), 1006 (m).

1-(4-(2-(1,3-Dioxanyl))phenyl)-2,2,2-trifluoro-1-ethanone Oxime (4). **3** (100 mg, 0.385 mmol) dissolved in 0.2 mL of ethanol is added dropwise to a refluxing solution of neutralized hydroxylamine hydrochloride, i.e. 83.5 mg (1.15 mmol) and 46.2 mg of NaOH (1.15 mmol), dissolved in 2.7 mL of ethanol. A white precipitate of sodium chloride is present in the reaction mixture. The reaction is allowed to proceed for 14–16 h. At the end, the reaction appears complete as judged by TLC (solvent = chloroform/ethyl acetate, 9:1). Only two spots corresponding to the syn and anti isomers of product **4** are evident.

The solvent is evaporated from the reaction mixture, and the solid residue is partitioned between diethyl ether and an aqueous solution of 0.01 N HCl. The organic phase is washed four times with this acidic solution and three times with water, then dried with $MgSO_4$, and filtered, and the solvent is removed by rotary evaporation. The crude unoptimized yield of product **4** is 95%. The purity of this material, ascertained by NMR and TLC, is sufficient to continue with the next synthetic step. For analytical purposes a sample was purified further by silica gel column chromatography using a mixture of chloroform/ethyl acetate (30:1) as the solvent. A separation of the isomers of **4** is achieved by this procedure. Yield of product is 95%, and usually better than 76% after chromatography. ^{1}H NMR ($CDCl_3$, 250 MHz): mixture of syn and anti isomers, δ 1.46 (complex doublet, J_{gem} = 13.5 Hz, 1 H), 2.22 (m, J_{diax} = 12.4 Hz, 1 H), 3.99 (complex triplet, 2 H), 4.28 (complex double doublet, 2 H), 5.53 (s, 1 H), 7.47, 7.57 (overlapping AA'BB' systems, J_{AB} = 8.3 Hz, 4 aromatic H), 8.74, 8.97 (broad singlets, 1 H). FTIR (film): wavenumber cm^{-1} 3303 (br, m), 2866 (m), 1448 (m), 1379 (m), 1343 (m), 1240 (m), 1190 (s), 1153 (m), 1128 (s), 1105 (s), 1010 (s). MS (EI, 20 eV): *m/e* (%) 275.1 (21.7, M), 274.1 (39.8, M − 1), 258.1 (44.1, M − OH), 216.1 (34.1), 200.0 (8.4), 87.2 (17.6), 57.2 (7.2), 43.2 (100.0).

1-(4-(2-(1,3-Dioxanyl))phenyl)-2,2,2-trifluoro-1-ethanone *O*-(Methylsulfonyl)oxime (5, R′ = Mes). **4** (40 mg, 0.145 mmol) is dissolved in 0.5 mL of dichloromethane. To this solution are added mesyl chloride (0.115 mL, 1.46 mmol), TEA (0.405 mL, 2.91 mmol), and DMAP (18 mg, 0.147 mmol) dissolved in 2 mL of the same solvent. The reaction is allowed to proceed overnight at RT. The reaction appears complete at the end of this period, as judged by TLC (solvent = chloroform/ethyl acetate, 9:1).

Purification of product **5** is achieved by silica gel column chromatography employing straight chloroform as the solvent. Yields are better than 95%. ^{1}H NMR ($CDCl_3$, 250 MHz): mixture of syn and anti isomers, δ 1.45 (complex doublet, J_{gem} = 13.5 Hz, 1 H), 2.20 (m, J_{diax} = 12.4 Hz, 1 H), 3.09 (s, 3 H), 3.98 (complex triplet, 2 H), 4.26 (complex double doublet, 2 H), 5.52 (s, 1 H), 7.46, 7.62 (AA'BB', J_{AB} = 8.3 Hz, 4 aromatic H).

1-(4-(2-(1,3-Dioxanyl))phenyl)-2,2,2-trifluoro-1-ethanone *O*-(*p*-Tolylsulfonyl)oxime (5, R′ = Tos). **4** (40 mg, 0.145 mmol) is dissolved in 0.5 mL of dichloromethane. To this solution are added tosyl chloride (278 mg, 1.46 mmol), TEA (0.405 mL, 2.91 mmol), and DMAP (18 mg, 0.147 mmol) dissolved in 2 mL of the same solvent. The reaction is allowed to proceed overnight at RT. The reaction appears complete at the end of this period, as judged by TLC (solvent = chloroform/ethyl acetate, 9:1).

Purification of product **5** is achieved by silica gel column chromatography employing a two-step procedure with (a) chloroform/hexanes (1:1) and (b) straight chloroform as the solvents. Yields are typically 98%. ^{1}H NMR ($CDCl_3$, 250 MHz): mixture of syn and anti isomers, δ 1.44 (complex doublet, J_{gem} = 13.5 Hz, 1 H), 2.19 (m, J_{diax} = 12.4 Hz, 1 H), 2.43, 2.45 (s, 3 H), 3.97 (overlapping complex triplets, 2 H), 5.49, 5.50 (s, 1 H), 7.30–7.57 (AA'BB', J_{AB} = 8.4 Hz, 4 aromatic H), 7.32, 7.85 (AA'BB', J_{AB} = 8.4 Hz, 4 aromatic H). FTIR (film): wavenumber cm^{-1} 2967 (m), 2928 (w), 2857 (m), 1597 (m), 1389 (s), 1339 (m), 1278 (m), 1237 (m), 1195 (very strong), 1180 (s), 1151 (s), 1104 (s), 1019 (m), 1004 (m). MS (EI, 20 eV): *m/e* (%) 429.2 (8.7, M), 370.2 (0.3), 273.1 (4.0), 258.1 (15.1, M − TosO), 213.1 (100.0), 155.1 (7.3), 91.1 (24.7).

3-(4-(2-(1,3-Dioxanyl))phenyl)-3-(trifluoromethyl)diaziridine (6). **5** (R′ = Mes or Tos; 0.145 mmol) dissolved in 0.3 mL of a mixture of cold diethyl ether/chloroform/liquid ammonia (1:1:1) in a pressure bottle; the vessel is tightly closed, and the reaction mixture is then allowed to warm up to RT and stirred for 12 h under dim light. A precipitate appears in the tosylate reaction mixture. Caution should be taken to cool down the reaction mixture before opening the pressure vessel. Gaseous ammonia is then evaporated spontaneously at RT. At the end of this period, complete conversion of the starting material into product **6** (of

lower R_f) is achieved under these conditions, as judged by TLC (solvent = chloroform/ethyl acetate, 9:1).

Purification of **6** is achieved by silica gel column chromatography employing chloroform/ethyl acetate (20:1) as the solvent. Yields are better than 92% of crude product, and better than 65–70% after chromatography. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.44 (complex doublet, J_{gem} = 13.5 Hz, 1 H), 2.16 (broad doublet, 1 H), 2.20 (m, J_{diax} = 12.4 Hz, 1 H), 2.77 (broad doublet, J = 8.1 Hz, 1 H), 3.98 (complex triplet, 2 H), 4.26 (complex double doublet, 2 H), 5.50 (s, 1 H), 7.53, 7.61 (AA'BB', J_{AB} = 8.3 Hz, 4 aromatic H). FTIR (film): wavenumber cm^{-1} 3240 (m), 2966 (m), 2929 (w), 2857 (m), 1470 (w), 1461 (w), 1429 (w), 1379 (m), 1320 (w), 1306 (w), 1279 (m), 1237 (m), 1217 (m), 1148 (s), 1102 (s), 1051 (w), 1021 (m). MS (EI, 20 eV): *m/e* (%) 275.2 (2.2, M + 1), 274.2 (20.2, M), 273.1 (100.0, M – 1), 253.2 (5.1), 216.2 (19.0), 215.2 (70.9), 200.1 (7.8), 195.1 (16.4), 187.1 (21.0), 167.1 (17.6), 147.2 (7.3), 132.1 (2.5), 119.2 (11.4), 101.1 (6.5), 87.1 (73.8), 59.2 (10.7), 42.1 (7.0).

3-(4-(2-(1,3-Dioxanyl))phenyl)-3-(trifluoromethyl)diazirine (7). **6** (10 mg, 0.036 mmol) is dissolved in 0.2 mL of methanol where 33 μL of TEA (0.237 mmol) has been added. Iodine (30 mg, 0.118 mmol) dissolved in the same solvent is added dropwise to the stirred solution. The immediate disappearance of the yellow color of iodine is evident upon addition to the diaziridine solution. The solution is stirred in the dark for 0.5–1 h after this addition is completed. The complete disappearance of the starting material and the appearance of the product (of higher R_f) is followed by TLC (solvents = chloroform/ethyl acetate, 9:1, and straight chloroform).

After evaporation of the solvent, the reaction mixture is partitioned between dichloromethane and dilute aqueous HCl. The organic phase is then washed successively with water (until neutrality), with an aqueous sodium thiosulfate solution (to reduce the remaining iodine), and again with water. After drying the organic phase with $MgSO_4$ and filtration, the solvent is evaporated to yield essentially pure product **7** as a light-yellowish oil. Yields of crude product are quantitative and better than 73% after purification by silica gel column chromatography.

While handling these compounds (**7–9**, **17–19**), care should be taken not to expose them to direct sunlight. However, they stand dim artificial illumination (fluorescent light). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.44 (complex doublet, J_{gem} = 13.5 Hz, 1 H), 2.19 (m, J_{diax} = 12.4 Hz, 1 H), 3.96 (complex triplet, 2 H), 4.24 (complex double doublet, 2 H), 5.48 (s, 1 H), 7.18, 7.50 (AA'BB', J_{AB} = 8.3 Hz, 4 aromatic H). FTIR (film): wavenumber cm^{-1} 2962 (m), 2945 (m), 2863 (m), 2844 (w), 1613 (w), 1471 (w), 1460 (w), 1419 (w), 1390 (w), 1379 (w), 1349 (m), 1341 (m), 1324 (w), 1238 (s), 1229 (s), 1213 (m), 1195 (m), 1177 (s), 1148 (s), 1138 (s), 1113 (m), 1107 (m), 1051 (m), 1015 (s). MS (EI, 20 eV): *m/e* (%) 272.1 (0.2, M), 271.1 (1.0, M – 1), 245.2 (13.4, M – N_2 + 1), 244.2 (100.0, M – N_2), 225.1 (2.6), 203.1 (1.6), 187.1 (8.3), 186.1 (78.8), 185.1 (11.6), 172.1 (2.4), 159.1 (9.2), 158.1 (75.9), 157.1 (12.0), 138.1 (5.7). UV (ethanol): λ (nm) 372 (sh), 356 (max), 341 (sh), 324 (sh), 296 (min), 271 (sh), 265 (max).

3-(4-Formylphenyl)-3-(trifluoromethyl)diazirine (*p*-Formyl-TPD, 8). There is generally no need to obtain this compound, since the protected form (**7**, see above) can react directly with the thioacid (**12**) to yield the thioacetal **9**. However, in some instances, the free aldehyde **8** was obtained from **7** by deprotection catalyzed by Amberlyst 15 in acetone solution. Filtration to remove the resin beads and evaporation of the solvent yield product **8** in quantitative yield. Care should be exercised at this step not to evaporate under high vacuum, since compound **8** is relatively volatile and, consequently, losses may occur.

12-((Ethoxy(thiocarbonyl))thio)dodecanoic Acid (Xanthogenate 11). 12-Bromododecanoic acid (**10**, Aldrich) (3.0 g, 10.42 mmol) is dissolved in pure acetone. To this solution is added 2.0 g of potassium ethyl xanthogenate (Fluka) dissolved in the same solvent. The final volume of the reaction mixture is about 300 mL. The mixture is stirred at 4 °C in the dark during 24 h. After the reaction is over, a white precipitate is observed (KBr).

The workup procedure is the following: the acetone is evaporated completely, and the residue is taken in diethyl ether/water. Drops of 1 N HCl (until strong acidic reaction on pH paper) and NaCl solution (to break the emulsion) are added to the aqueous phase. Ether extractions of this aqueous phase are repeated four times, the extracts are pooled together, dried with $MgSO_4$, and filtered, and the solution is concentrated to dryness with the aid of a rotary evaporator and a vacuum pump. NMR analysis of the product shows essentially pure xanthogenate of the fatty acid (**11**), and only traces of the starting material remain. This material is perfectly suitable for continuing the synthesis; no purification is required at this stage. The crude yield is 3.26 g (98% of the theoretical value). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.25 (m, 14 H), 1.40 (t, J = 7.1 Hz, 3 H), 1.63 (m, 4 H), 2.33 (t, J = 7.5 Hz, 2 H), 3.09 (t, J = 7.4 Hz, 2 H), 4.63 (q, J = 7.1 Hz, 2 H). FTIR (film): wavenumber cm^{-1} 3022 (br, m), 2985 (m), 2978 (m), 2950 (m), 2924 (s), 2852 (s), 2650 (br, m), 1702 (s), 1470 (s), 1450 (m), 1432 (m), 1409 (m), 1396 (m), 1355 (m), 1326 (m), 1303 (w), 1278 (s), 1234 (s), 1222 (s), 1204 (s), 1148 (w), 1114 (s), 1088 (w), 1050 (s). MS (EI, 20 eV): *m/e* (%) 320.0 (2.3, M), 287.1 (11.3), 241.1 (1.4), 231.3 (1.1), 213.1 (7.9), 199.2 (9.1), 181.1 (7.7), 163.2 (7.9), 139.1 (3.3), 122.0 (100.0).

12-Mercaptododecanoic Acid (12). The xanthogenate of the fatty acid (**11**, 0.48 g, 1.5 mmol) is dissolved in a freshly prepared solution of 3.8 g of sodium borohydride in 100 mL of 100% ethylenediamine in pure ethanol. Ethylenediamine had been freshly distilled from CaH_2 (bp: 115–116 °C). The solution was stirred vigorously overnight at RT under a nitrogen atmosphere.

The workup procedure is the following: the solvent is evaporated in a rotary evaporator, and further removal is achieved under high vacuum to leave a white paste. Care is taken to avoid any contact with air, i.e. venting of the evacuated flask is performed using a balloon filled with argon. The residue is dissolved in ethyl ether/water and cooled down in an ice bath. Concentrated sulfuric acid is added dropwise to destroy the excess amount of sodium borohydride. Addition of acid is continued under constant stirring, keeping the temperature at around 0 °C and avoiding excessive foam production, until a strongly acidic reaction on pH paper is observed. After evolution of hydrogen gas ceases, the aqueous phase is extracted five times with diethyl ether, and the extracts are pooled together and dried with $MgSO_4$. After filtration, the solvent is evaporated to yield a white powdery residue. This residue weighs about 1.4 g and contains essentially pure product (**12**), presumably together with an ether-extractable byproduct of sodium borohydride. As such, this material was perfectly suitable to continue the synthesis. No further purification is attempted at this stage to avoid the risk of oxidation of **12** to its disulfide form. It is advisable to go on with the next synthetic step without prolonged delay. NMR analysis of this sample (in $CDCl_3$ solution, after filtering out the insoluble residue) shows essentially pure (>90–95%) thiol acid **12**, traces of its disulfide byproduct, and no starting material. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.25 (m, 14 H), 1.59 (m, 4 H), 2.33 (t, J = 7.5 Hz, 2 H), 2.50 (q, J = 7.3 Hz, 2 H). ^{1}H NMR ($CDCl_3$, with drops of D_2O): 1.25 (m, 14 H), 1.58 (m, 4 H), 2.32 (t, J = 7.5 Hz, 2 H), 2.49 (t, J = 6.8 Hz, 2 H). FTIR (film): wavenumber cm^{-1} 3080 (br, m), 2920 (s), 2847 (s), 1690 (s), 1520 (w), 1498 (w), 1465 (m), 1435 (w), 1408 (m), 1325 (w), 1304 (w), 1280 (m), 1227 (w), 1204 (w), 1188 (m). MS (EI, 20 eV): *m/e* (%) 236.1 (4.4), 232.2 (6.0, M), 216.2 (5.2), 215.2 (11.5), 214.2 (73.4, M – H_2O + H), 196.1 (8.8), 181.2 (16.4), 163.2 (6.3), 154.2 (6.3), 138.2 (7.4), 129.1 (12.3), 112.1 (35.3), 98.2 (100.0).

12,12'-Dithiobis(dodecanoic Acid) (Byproduct). Following the same workup procedure described above for compound **12**, isolation of the dithio (oxidized) form of compound **12** occurs when a smaller excess or no sodium borohydride is present in the reaction mixture. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.25 (m, 28 H), 1.59 (m, 8 H), 2.33 (t, J = 7.5 Hz, 4 H), 2.67 (q, J = 7.5 Hz, 4 H). MS (EI, 20 eV): *m/e* (%) 462.2 (22.5, M).

[((Trifluoromethyl)diazirinyl)phenyl] Thioacetal Dicarboxylic Fatty Acid 9. **7** (or **8**) (0.36 mmol) is dissolved in dichloromethane. A 2.5-fold molar excess (0.9 mmol) of freshly prepared thiol acid **12** is added to this solution, and the mixture is stirred under nitrogen in an ice bath under dim light. Care should be taken to avoid direct sunlight; dim room light however is nondeleterious to the compound. The reaction is initiated by the dropwise addition of about 100 μL of boron trifluoride etherate solution (Fluka). The progress of the reaction is followed by TLC (solvent = dichloromethane/methanol, 10:1). After 45–60 min, (a) the complete disappearance of the UV positive spot corresponding to the starting material (aldehyde) running near the solvent front and (b) the appearance of a main UV positive product (thioacetal **9**) as a tailing spot with R_f 0.4–0.5 that appears deep blue after staining with ammonium molybdate/ceric sulfate solution are evident.

The workup procedure is as follows: Saturated $NaHCO_3$ solution is added to the reaction mixture to neutralize the Lewis acid (until final pH 8–8.3). Then the aqueous phase is reacidified with diluted sulfuric acid (until pH 1), and the product is extracted four times with diethyl ether. The extracts are pooled together, dried with $MgSO_4$, and filtered, and the solvent is evaporated under reduced pressure. The main product in this crude material is thioacetal **9**. Purification is achieved by column silica gel chromatography employing successively dichloromethane/

methanol/acetic acid (320:10:1 and 150:10:1). Isolated yields are usually better than 80%. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.24 (m, 28 H), 1.43–1.63 (m, 8 H), 2.33 (t, J = 7.4 Hz, 4 H), 2.40–2.60 (m, 4 H), 4.82 (s, 1 H), 7.12, 7.45 (AA'BB', J_{AB} = 8.3 Hz, 4 H). After photolysis of **9** in ethanolic solution, the characteristic signals corresponding to the diazirine at 4.82, 7.12, and 7.45 ppm disappear, and the following new signals appear: 1.24 (3 H, under the multiplet), 3.56 (q, 2 H), 4.57 (q, 1 H), 4.84, 4.86, 4.88 (singlets) and 7.03, 7.34–7.50, 7.60, 8.03 ppm (AA'BB' systems), corresponding to the photolysis products, diazo isomer and ethyl ether and ketone (see Figure 7 and main text for details). FTIR (film): wavenumber cm^{-1} 3037 (br, m), 2926 (s), 2853 (s), 2675 (m), 1709 (s), 1613 (m), 1516 (m), 1464 (m), 1457 (m), 1436 (m), 1412 (m), 1365 (sh), 1344 (m), 1288 (m), 1229 (s), 1186 (s), 1157 (s), 1125 (sh), 1052 (m). After photolysis in ethanolic solution, a new band at 2085 cm^{-1} appears (diazo isomer). MS (EI, 20 eV): m/e (%) no M ion, 462.3 (24.1), 403.2 (58.6), 402.1 (93.1), 401.1 (37.9), 246.2 (62.1), 236.1 (44.8), 215.2 (31.0), 214.2 (44.8), 213.2 (62.1), 204.0 (34.5), 203.0 (41.4), 185.1 (51.7), 173.1 (41.4), 129.0 (31.0), 112.1 (31.0), 101.1 (55.2), 98.2 (89.7), 97.2 (65.5), 96.1 (31.0), 95.2 (31.0), 87.1 (100.0). MS (CI): m/e no M ion, 459, 447, 433, 429, 415, 403, 371, 337, 317, 289, 257, 233, 231, 215, 201, 103. MS (FAB) (thioglycerol): m/e no M ion, 435, 429, 359, 329, 326, 303, 205, 201, 163, 153, 149, 137. UV (ethanol): λ (nm) 380 (sh), 360 (max, approximate ϵ = 300), 342 (sh), 313 (min), 274 (sh), 224 (max), 211 (min). After complete photolysis in ethanolic solution, the band at 360 nm corresponding to the diazirine disappears, and new bands corresponding to the diazo isomer appear at 460 (max, approximate ϵ = 68) and 274 (max, approximate ϵ = 22 000) nm. Upon further irradiation these bands also disappear, following the slower photolysis of the diazo isomer. Quasi isosbestic points are 404, 319, 247, and 215 nm (see Figure 6 and main text for details).

***N*-tBOC-dimyristoylphosphatidylethanolamine (14).** In a 100-mL flask, 1 g of dimyristoylphosphatidylethanolamine (DMPE, **13**, Sigma) is dissolved in 40 mL of anhydrous THF; 2 mL of TEA are added, and the reaction is started at 0 °C by the addition of 4 g of di-*tert*-butyl dicarbonate. After 5 min, the stirred reaction mixture is allowed to warm up to RT. The reaction is continued for 16 h.

At the end, the solvent is evaporated under reduced pressure, the residue is dissolved in chloroform and washed with water, and the organic phase is dried with $MgSO_4$. Product **14** is purified by silica gel column chromatography, firstly eluting with chloroform and then with solvent 2. Yields are quantitative (98%). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 0.86 (t, J = 6.6 Hz, 6 H), 1.23 (m, 40 H), 1.42 (s, 9 H), 1.56 (m, 4 H), 2.25 (m, 4 H), 3.37 (broad m, 2 H), 3.75–4.45 (complex m, 6 H), 5.10 (broad m, 1 H), 5.21 (m, 1 H). FTIR (film): wavenumber cm^{-1} 3386 (br, m), 2956 (sh, m), 2923 (s), 2870 (sh, m), 2853 (s), 1742 (s), 1732 (sh, s), 1716 (s), 1699 (sh, s), 1515 (m), 1466 (m), 1456 (m), 1365 (m), 1247 (s), 1174 (s), 1113 (s), 1073 (m), 1045 (m), 1012 (sh, m).

Lyso-*N*-tBOC-myristoylphosphatidylethanolamine (15). This compound is obtained from **14** following the reaction conditions described below for phospholipase A_2 hydrolysis. Silica gel TLC analysis (solvent 2) indicates complete transformation of **14** into **15** after 8–10 h at 37 °C.

Purification of **15** by silica gel column chromatography is performed only for analytical purposes. In order to avoid acyl or phosphate migration, for bulk synthesis, we bypassed the purification step. After the hydrolysis is complete, the chloroformic extract, containing **15** and free myristic acid in equimolar amounts, was directly subjected to the next step of methylation with diazomethane (see below). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 0.86 (t, J = 6.6 Hz, 3 H), 1.23 (m, 20 H), 1.42 (s, 9 H), 1.61 (m, 2 H), 2.33 (t, J = 7.9 Hz, 2 H), 3.33 (broad m, 2 H), 3.79–4.23 (complex m, 7 H), 5.75 (broad m, 1 H).

Lyso-*N*-tBOC-myristoylphosphatidylethanolamine Methyl Ester (16). Compound **15** is dissolved in diethyl ether, and an excess amount of freshly prepared diazomethane is added (until the yellow color persists in the reaction mixture). Then the solvent and the excess reagent are evaporated under reduced pressure, and the residue (**16**) is washed with ether. Silica gel TLC analysis (solvent 2) indicates complete transformation of **15** into **16**.

Purification of **16** by silica gel column chromatography is performed only for analytical purposes. In order to avoid acyl or phosphate migration, for bulk synthesis, the extract obtained after digestion of **14** with phospholipase A_2 (containing **15** and free myristic acid in equimolar amounts) is reacted directly with diazomethane. After methylation is complete, this equimolar mixture of **16** and myristoyl methyl ester becomes a suitable starting material for the following coupling step of **16** to **9** (or **23**). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 0.85 (t, J = 6.6 Hz, 3 H), 1.22 (m, 20 H), 1.42 (s, 9 H), 1.59 (m, 2 H), 2.31 (t, J = 7.5 Hz, 2 H), 3.41 (broad m, 2 H), 3.77 (overlapping doublets, J = 11.2 Hz, 3 H), 4.04–4.25 (m, 7 H), 5.08 (broad m, 1 H). ^{13}C NMR ($CDCl_3$, 63 MHz): δ 14.28, 22.86, 25.07, 28.57, 29.33, 29.45, 29.54, 29.65, 29.78, 29.84, 32.10, 34.27, 41.04, 54.82, 64.50, 64.55, 67.47, 67.56, 68.97, 69.05, 69.11, 69.18, 69.26, 83.38, 83.43, 173.98. FTIR (film): wavenumber cm^{-1} 3370 (br, m), 2974 (sh, m), 2957 (m), 2923 (s), 2869 (sh,m), 2854 (m), 1742 (m), 1738 (m), 1732 (m), 1721 (s), 1716 (s), 1713 (s), 1698 (m), 1694 (m), 1682 (sh,m), 1536 (m), 1530 (m), 1524 (m), 1519 (m), 1515 (m), 1504 (m), 1467 (m), 1461 (m), 1455 (m), 1391 (w), 1367 (m), 1269 (s), 1254 (s), 1173 (s), 1041 (s). MS (EI, 20 eV): m/e (%) 448.2 (10.3), 434.2 (14.4), 407.2 (13.4), 379.3 (44.3), 365.2 (37.1), 335.1 (40.2), 285.2 (74.2), 279.1 (49.5), 223.1 (88.7), 169.1 (100.0). MS (FAB) (thioglycerol): m/e (%) 562 (0.3, M + Na), 540 (0.3, M + H), 538, 440 (19.4), 419, 379 (6.6), 285 (100.0). MS (FAB) (thioglycerol, NaCl): 562 (54.1, M + Na), 440 (15.0), 419 (17.7), 379 (6.3), 285 (100.0).

DIPETPD, Bis-*N*-tBOC-, Bis(methyl ester) (17). In order to azeotropically remove traces of water or alcohol from the sample, compound **16**[42] is washed three times with anhydrous benzene and the solvent removed under high vacuum. **16** (0.530 g, 0.98 mmol) is dissolved in dry dichloromethane. To this solution are then added 0.246 g of **9** (0.45 mmol) and 0.120 g of DMAP (0.98 mmol). The mixture is cooled down in an ice bath before 0.567 g of DCC (2.75 mmol) dissolved in the same solvent is added dropwise with stirring. The reaction mixture is kept for 5 min at 0 °C, then allowed to warm up to RT, and left overnight at this temperature. The appearance of a white precipitate of DCU is evident after a few minutes. The progress of the reaction is followed by TLC using chloroform/methanol (20:1) as developing solvent.

At the end, the reaction mixture is filtered to remove most of the DCU. The organic phase is quickly washed with 0.2 N HCl to remove DMAP, dried with $MgSO_4$, and filtered, and the solvent is evaporated under reduced pressure to yield a slightly yellowish oil. This residue is dissolved in less than 5 mL of chloroform/methanol (1:1) and the sample applied to a column of Sephadex LH-20 (4.5 × 20 cm) equilibrated in this same solvent. Elution is performed by gravity, and 5 mL fractions are collected. The chromatographic separation is followed by TLC as described above. Ammonium molybdate/ceric sulfate staining of the plates reveals a good separation by sizing between the desired product **17** (blue spot on TLC eluting first) and the remaining DCU (grayish spot on TLC with similar R_f). Fractions containing compound **17** are pooled together, and the solvent is evaporated under reduced pressure. Care should be taken to completely eliminate methanol before the next chromatographic step. The residue is dissolved in chloroform, applied to a column of silica gel 60 (2.2 × 20 cm), and eluted with a mixture of chloroform/methanol (50:1). The chromatographic separation is followed by TLC as described above. This last procedure separates the desired product (**17**) from remaining **16** present in the reaction mixture. Fractions containing the product are concentrated, and the resultant transparent, slightly yellowish oil residue is put under high vacuum until constant weight is attained. Usual yields of pure product **17** range between 75 and 83%. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 0.85 (t, J = 6.6 Hz, 6 H), 1.23 (m, 68 H), 1.42 (s, 18 H), 1.45–1.65 (m, 12 H), 2.29, 2.31 (overlapping triplets, 8 H), 2.45 (m, 4 H), 3.37 (broad q, 4 H), 3.72, 3.77 (overlapping doublets, J = 11.0 Hz, 6 H), 4.00–4.37 (m, 12 H), 4.81 (s, 1 H), 5.08 (broad m, 2 H), 5.22 (m, 2 H), 7.11, 7.44 (AA'BB', J_{AB} = 8.3 Hz, 4 H). After photolysis in ethanolic solution, the aromatic region changes as follows: the characteristic signals of the diazirine at 7.11 and 7.44 ppm disappear, and the following new signals appear: 7.03, 7.20, 7.34–7.50 ppm (new AA'BB' systems), corresponding to the photolysis products, diazo isomer and ethyl ether and ketone. FTIR (film): wavenumber cm^{-1} 3340 (br, m), 2925 (s), 2853 (s), 1743 (s), 1715 (s), 1517 (m), 1465 (m), 1457 (m), 1390 (w), 1365 (m), 1344 (w), 1272 (m), 1253 (m), 1175 (s), 1159 (s), 1105 (w), 1045 (s). After photolysis in ethanolic solution, a new band at 2084 cm^{-1} appears (diazo isomer). MS (FAB) (thioglycerol): m/e 1605, 850, 499, 399, 285, 169. UV (chloroform/methanol, 1:1): λ (nm) 376 (sh), 358 (max, approximate ϵ = 300), 342 (sh), 313 (min). After photolysis in solution, the band at 358 nm corresponding to the diazirine disappears, and a new, transient band corresponding to the diazo isomer appears at 460 (max) nm. Quasi isosbestic points are 404 and 318 nm. Anal. Theory % (Exptl. %): C, 58.52 (58.38); H, 8.64 (8.71); N, 3.29 (3.23).

(42) For the synthesis of **17** we routinely employed the equimolar mixture of **16** and myristoyl methyl ester, resulting from the hydrolysis of **14** with phospholipase A_2 and further reaction with diazomethane. In this way, we avoid the exposure of lysophospholipid derivatives **15** and **16** to silica gel, thus minimizing the chances of acyl or phosphate migration. Removal of myristoyl methyl ester is achieved in the course of the purification of **17**.

DIPETPD, Bis-*N*-tBOC- (18). **17** (150 mg, 95 μmol) is dissolved in 8 mL of a refluxing (85 °C) solution of 120 mg of NaI (753 μmol) in 2-butanone, and the solution is refluxed for 30–45 min.

At the end, the solvent is evaporated under reduced pressure, and the residue is suspended in chloroform and washed with water. The organic phase is dried with $MgSO_4$, filtered, and concentrated. Yields of **18** are usually better than 95%. ^1H NMR ($CDCl_3$, 250 MHz): The spectrum of **18** shows characteristic broadening, typical of phospholipids. δ 0.85 (t, J = 6.6 Hz, 6 H), 1.24 (m, 68 H), 1.42 (s, 18 H), 1.57 (m, 12 H), 2.27, 2.30 (overlapping triplets, 8 H), 2.46 (m, 4 H), 3.37 (broad, 4 H), 3.90–4.40 (complex m, 12 H), 4.82 (s, 1 H), 5.21 (m, 2 H), 7.12, 7.45 (AA′BB′, J_{AB} = 8.1 Hz, 4 H). FTIR (film): wavenumber cm^{-1} 3380 (br, w), 2924 (s), 2853 (s), 1742 (s), 1717 (s), 1515 (m), 1466 (m), 1457 (m), 1366 (m), 1344 (w), 1241 (s), 1176 (s), 1159 (s), 1104 (m), 1057 (m), 1037 (m).

DIPETPD (19). Compound **18** (10–30 mg) is dissolved in 1–3 mL of an ice-cold solution of trifluoroacetic acid 50% in dichloromethane, containing drops of methanol (this is an important detail, since, in the absence of methanol, cleavage of the thioacetal bridge may occur). The reaction is allowed to proceed for 30–45 min at 0 °C.

At the end, the solvent is evaporated under high vacuum at 0 °C. The residue is washed with a chloroform/methanol (1:1) solution and the solvent evaporated again. The pale-yellowish transparent oil that remains is essentially pure **19**. Yields are quantitative (98%). This compound quenches the fluorescent indicator of a TLC plate and stains characteristically with molybdenum blue (phospholipid), ammonium molybdate/ceric sulfate, and ninhydrin (free amino groups). ^1H NMR ($CDCl_3$, 250 MHz): The spectrum of **19** shows characteristic broadening, typical of phospholipids. δ 0.85 (t, J = 6.6 Hz, 6 H), 1.24 (m, 68 H), 1.57 (m, 12 H), 2.26, 2.29 (overlapping triplets, 8 H), 2.45 (m, 4 H), 3.12 (broad m, 4 H), 3.85–4.40 (complex m, 12 H), 4.82 (s, 1 H), 5.20 (m, 2 H), 7.12, 7.45 (AA′BB′, J_{AB} = 8.0 Hz, 4 H). FTIR (film): wavenumber cm^{-1} 3130 (sh, w), 2924 (s), 2851 (s), 1734 (s), 1684 (m), 1616 (sh, m), 1550 (m), 1465 (m), 1449 (m), 1377 (w), 1344 (w), 1230 (s), 1205 (s), 1185 (s), 1175 (s), 1159 (s), 1100 (sh, m), 1080 (s), 1029 (m). MS (FAB) (thioglycerol): m/e 1476, 902, 747, 641, 500, 426, 285, 217. MS (FAB) (w/MAGIC): 1254, 1240, 1102, 1088, 888, 793, 641, 500, 441. UV (chloroform/methanol, 1:1): λ (nm) 376 (sh), 358 (max), 342 (sh), 313 (min). After photolysis in solution, the band at 358 nm, corresponding to the diazirine, disappears, and new, transient bands corresponding to the diazo isomer appear at 460 (max) and 274 (max) nm. Quasi isosbestic points are 408, 320, and 248 nm. Anal. Theory % (Exptl. %, corrected for the presence of silica in the sample): C, 57.80 (58.14); H, 8.62 (8.79); N, 3.80 (3.28).

***m*-Sulfobenzoic Acid, Sulfosuccinimidyl Ester (SSSB, 33).** *m*-Sulfobenzoic acid, monosodium salt (0.235 g, 1.05 mmol), dissolved in 20 mL of dry DMF, is mixed with 0.250 g (1.15 mmol) of *N*-hydroxysulfosuccinimide, sodium salt, and 13 mg (0.10 mmol) of DMAP. The reaction is started by the addition of 0.432 g (2.10 mmol) of DCC. Cloudiness develops after a few minutes. The solution is stirred overnight at RT.

Workup procedure: The suspension is filtered to remove the precipitate of DCU, and 10 mL of anhydrous diethyl ether is added. Immediately, a whitish precipitate appears. This material is washed five times with dry ether, dried under high vacuum, and kept in a desiccator until use. The yield of SSSB (**33**) is 98% (dry powder). This compound was tested by reaction with ammonia and DMPE. No further purification is recommended to avoid hydrolysis of the active ester. The UV spectrum of **33** in DMF solution shows a peak at 270 nm.

Reductive Methylation of DIPETPD (19) with BT_4Na. The whole procedure is performed in a hood authorized for handling large amounts of tritium. The source of tritium most generally used is BT_4Na (89 Ci/mmol); in some experiments we use instead $(CN)BT_3Na$ (12 Ci/mmol). The procedure is as follows: DIPETPD (**19**, 1–5 mg) is dissolved in the minimum volume (ca. 0.2 mL) of chloroform/isopropanol (2:1). An excess amount of concentrated aqueous formaldehyde is next added to the mixture, and finally the solid tritiated reagent (0.1–1 Ci) is added. The reaction mixture is stirred continuously for 2–3 h.

At the end, aqueous HCl is added to destroy any remaining borotritide. The tritium gas release is trapped in a Pd/C filter connected to the reaction vessel. After the experiment, the trap is soaked in an ethanolic solution of oleic acid to yield solid radioactive waste (stearic acid). The solvents are evaporated under reduced pressure from the reaction mixture to yield an oily residue. After several cycles of methanol washings and repeated evaporations, the residue is extracted with chloroform/methanol (2:1). The product (methylated DIPETPD) is purified by silica gel column chromatography and gel filtration or Sephadex LH-20. Characterization of the radioactive product is achieved by normal-phase HPTLC and autoradiography. The compound runs as a single spot on TLC. Slices from a chromatogram are scraped off the plate and counted by liquid scintillation. This radioactivity measurement, together with the UV absorption at 356 nm (diazirine) allowed us to calculate the specific activity of the product (26.5 Ci/mmol, when starting from BT_4Na).

Liposome Preparation (Small Unilamellar Vesicles, SUVs). A thin dry film of phospholipids is obtained by evaporation of a chloroform/methanol (2:1) solution of the lipid components in a round-bottom flask under a stream of argon or nitrogen, with continuous shaking in a mildly hot water bath. Traces of solvent are eliminated under high vacuum (<10 mTorr) overnight. All the following operations are performed at 65 °C and under an Ar atmosphere. The film is hydrated with the appropriate buffer (usually phosphates 50 mM, pH 7.0 or 7.5) for 1 h. Glass beads are added to help suspend the lipid film while intermittently vortexing during 15–30 min. The resultant milky suspension of multilamellar vesicles (MLVs) is then sonicated during 1 h in a thermostated (65 °C) bath sonicator (Branson 2200, Branson Ultrasonics). At the end, the suspension shows an opalescent bluish appearance.

After being cooled down to RT, the suspension is firstly centrifuged for 5–10 min in an Eppendorf bench centrifuge to remove remaining large aggregates (usually, a very small pellet or no pellet at all is observed) and then filtered through a 0.45 μm HA Millipore filter mounted on a syringe (most often, practically no material is left in the filter). These vesicles are generally used immediately afterward, or stored at RT and used always within 3 days after being prepared.

Liposome Preparation (Large Unilamellar Vesicles, LUVs). These vesicles are prepared from different mixtures of phospholipids by high-pressure extrusion through polycarbonate filters.[21c] A dry thin film of phospholipids is obtained as described above by evaporation of a chloroform solution. The milky suspension of MLVs (see above) is subjected to five freeze–thaw cycles between liquid nitrogen and warm water, and then passed through three stacked polycarbonate filters (Nucleopore) of decreasing pore size (0.4, 0.2, and 0.1 μm) mounted on the extruder, assembled with the 10-mL barrel (Lipex Biomembranes, Vancouver). The extrusion is usually repeated three to four times to obtain vesicles of defined size. The temperature is kept at 65 °C during the extrusion procedure. The preparation is stored at RT and used within 3 days.

Early experiments were performed employing LUVs prepared by ether injection.[21b] In this method, an ethereal solution containing the mixture of lipids, typically 6.0 mg of 1-myristoyl-2-oleoylphosphatidylcholine (MOPC), 0.5 mg of dimyristoylphosphatidic acid (DMPA), and 2 mg of DIPEP dissolved in 5 mL of diethyl ether, ca. 2.5 μmol of lipid/mL, is slowly injected (0.25 mL/min) through the bottom of an open chamber holding 5 mL of phosphates buffer 50 mM, pH 7.1, thermostated at 78 °C. Immediate evaporation of the solvent occurs, leaving a suspension of LUVs. The preparation is allowed to stand at the same temperature for 10–15 min after injection is finished to help remove traces of organic solvents. After being cooled down to RT, the suspension is passed through a 1.2-μm Millipore filter to remove large lipid aggregates. The filtrate is flushed with nitrogen and stored at RT. This preparation is used within 3 days.

Reaction of SSSB (33) with DIPEP or PE Incorporated into Lipid Vesicles. A freshly prepared and concentrated solution (100–200 mg/mL) of *m*-sulfobenzoic acid, sulfosuccinimidyl ester (SSSB, **33**) is added to a sample of liposomes containing DIPEP or PE. The reaction proceeds in phosphates buffer 50 mM, pH 7.1. Hydrolysis of the reagent is the major competing reaction; this accounts for the quenching of the excess amount of reagent after a few minutes. After the reaction is complete, the extent of reaction of the amino groups with SSSB is estimated from the difference in the extent of reaction with TNBS between (a) this SSSB-reacted sample, after Triton X-100 is added to solubilize the lipids, and (b) an identical sample reacted only with TNBS in the presence of Triton X-100 (100%). See below for more details.

Reaction of TNBS with DIPEP or PE Incorporated into Lipid Vesicles. A sample of liposomes (250 μL, 4 mg/mL of total phospholipids) prepared with phosphatidyl choline (PC), phosphatidic acid (PA), and either DIPEP (**20**) or phosphatidylethanolamine (PE) (see Table I) is diluted in 1.9 mL of phosphates buffer 50 mM, pH 8.4. The reaction is initiated by the addition of TNBS to this suspension: 40 μL of a freshly prepared solution of reagent (20 mg/mL) are added to the liposomes with rapid mixing (0.4 mg of reagent/mL, final concentration) at 25 °C. The absorbance at 345 nm is continuously recorded immediately after the mixing (t = 0). The blank is a solution of reagent with no lipid vesicles present. A correction value for the scattering of each vesicle sample is evaluated

from the reading at 345 nm of the vesicle suspension versus phosphates buffer, before the addition of TNBS takes place. A parallel sample of vesicles to which 30 μL of 10% Triton X-100 are added (1.5 mg of detergent/mL, final concentration), and which is incubated briefly in a hot water bath, serves to evaluate the maximum extent of the reaction (100%). Under these conditions, the reaction is complete after 30 min of incubation with TNBS. The absorbance of a sample of "nonleaky" vesicles should also reach a plateau after 30 min of reaction. The usual level of this plateau is 55–65% of the value observed in the presence of Triton X-100. However, if a significant absorbance increase after this time is recorded, this indicates "leakiness" of the vesicles toward the reagent.

The reaction is stopped by addition of 0.2 mL of lysine (100 mg/mL). The lipids are then extracted with chloroform/methanol (2:1), and the organic phase is dried with $MgSO_4$, filtered, and concentrated under reduced pressure. TLC analysis on silica gel is performed using solvent 2 as the developing solvent. This procedure allows the separation of unlabeled DIPEP (**20**), mono-TNP-DIPEP (**34**), and bis-TNP-DIPEP (**35**). Estimation of the relative amounts of TNP derivatives is usually achieved by visual inspection of the TLC plates under room and UV light. A quantitative estimation was performed in some cases by elution of the separated lipid derivatives from the TLC plate and determination of phospholipid phosphates.[43] Under the chromatographic conditions employed, DIPEP comigrates with PC.

Photoreaction of DIPETPD with [^{14}C-Formyl]gA and [^{14}C-Formyl]-1-Trp-gA in Lipid Vesicles. In experiments involving gramicidin A (gA) and its analogue 1-Trp-gA the reconstitution of the peptides into the membrane is straightforward, i.e. a methanolic solution of the peptide is added to the lipid mixture dissolved in chloroform/methanol (2:1). A typical mixture is the following: 4 mg of dimyristoylphosphatidylcholine (DMPC), 0.5 mg of [^{14}C-formyl]gA (or [^{14}C-formyl]1-Trp-gA), and 0.5 mg of DIPETPD (approximately 20:1:1, on a molar basis). This mixture is processed as above to obtain SUVs. Unlike pure lipid vesicles made only with DMPC (14:0, 14:0), these peptide loaded vesicles exhibit an enhanced fluidity at RT. The characteristic UV spectrum of DIPETPD (λ_{max} ca. 360 nm) is observed superimposed over the scattering background of the vesicle preparation. Negatively stained electron micrographs (tungstate stain) of these samples are taken to characterize the size and shape of these peptide-loaded vesicles (see Figure 14). These vesicles are irradiated with filtered UV light ($\lambda > 320$ nm) for 1 h at 40–45 °C to label gramicidin A in its membrane channel form. After photolysis for 20–30 min, the complete disappearance of the UV absorption band at 360 nm is observed. The aqueous suspension is then lyophilized and the lipids are solubilized in chloroform/methanol (2:1). Alternatively, the vesicle suspension is acidified with concentrated HCl and repeatedly extracted (5×) with chloroform/methanol (2:1). The combined extracts are dried with $MgSO_4$ and filtered. Concentration of the extracts is performed under a stream of nitrogen or argon. The labeled peptides are analyzed by HPTLC on silica gel 60 plates. Visualization of the spots is achieved by (a) UV quenching of the fluorescent indicator on the plate and (b) staining with ammonium molybdate/ceric sulfate solution or *p*-aminobenzaldehyde solution. Detection and quantitation of the radioactivity of ^{14}C are achieved by autoradiography and liquid scintillation counting of scraped-off silica fractions, respectively.

Labeling of Influenza Virus Hemagglutinin (HA) with a Tritium Methylated Derivative of DIPETPD. The procedure is essentially that described by Stegmann et al.[3] To 50 μL of liposomes prepared with egg PC, egg PE, bovine brain gangliosides (Type III) (6:3:1, approximately 20 nmol of total P_i), and trace amounts of tritium methylated DIPETPD in 1 mL of degassed 135 mM NaCl, 10 mM MES, 5 mM HEPES, 15 mM citrates, pH 5 (fusion takes place) or pH 7 (no fusion takes place), incubated at 37 °C, is added 80 μL of influenza virus (X-31 recombinant strain, approximately 20 nmol of P_i, 80 μg of protein). After 30 min, 1 mM HEPES, pH 8, is added to neutralize the acidic mixture (pH 5), and both samples are subsequently photolyzed during 15 min at RT. Gel electrophoresis and autoradiography are carried out as described before.[3]

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Supplementary Material Available: Additional experimental procedures, five figures, and bibliographic references (22 pages). Ordering information is given on any current masthead page.

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