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SYNTHESIS OF A CHIRAL PHOSPHOLIPASE-C AND D INSENSITIVE MEMBRANE PROBE WITH EXCIMER EMISSION PROPERTIES.

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Abstract: A new cationic membrane probe bearing a trimethylammonium function in the head group and pyrenyl groups in the two acyl chains has been synthesized. The new probe will allow the measurement of membrane dynamics by excimer emission and should be more stable than similar ones containing phosphodiester linkages. These are degraded in systems where the levels of phospholipase-A, C and D activity are high. The synthesis utilizes the 4-carbon synthon (S)-3-hydroxy-γ-butyrolactone to provide the chiral diacyl substructure.

INTRODUCTION

Fluorescent spectroscopy is a tool that is routinely used in the study of membrane organization and dynamics[1]. Although many non-lipid fluorescent probes are used, those which bear a reasonable structural similarity to membrane lipids are generally the most useful. A common strategy for using phospholipid analogs as probes is to attach a pyrenyl group to each of the acyl chains[2]. In such a configuration, the pyrenyl groups interact to form a dimer complex which emits differently to single pyrenyl groups by a phenomenon known as excimer emission. This form of emission requires that the two pyrenyl groups be in close proximity allowing the formation of the excimer (dimer) complex. It is then possible to follow the hydrolysis of the acyl chains as a result of phospholipase-A activity by monitoring the decrease of this excimer emission[3]. If the probe is insensitive to phospholipase-A activity or in the absence of such activity, such probes allow the measurement of the extent of packing of the membrane hydrocarbon chains. This is because the excimer emission spectrum is a function of the average separation of the pyrenyl groups[4]. If the probe is in a tightly packed domain of a membrane, the average separation of the pyrenyl groups will be small and the resulting spectrum will reflect this. The intensity of the excimer emission decreases with a decrease in packing density. The excimer emission is also affected by the microviscosity of the medium. In the case of membrane lipids, this is determined by the lateral mobility of the lipid molecules and their average orientation. In viscous media, the pyrenyl rings in the probe are, on average, in the same relative orientation to a greater extent.

One problem in the design of membrane probes is that those which contain phosphodiester linkages are susceptible to cleavage by phospholipase-C and D. In the case of phospholipase-D, the phosphodiester linkage distal to the diacyl

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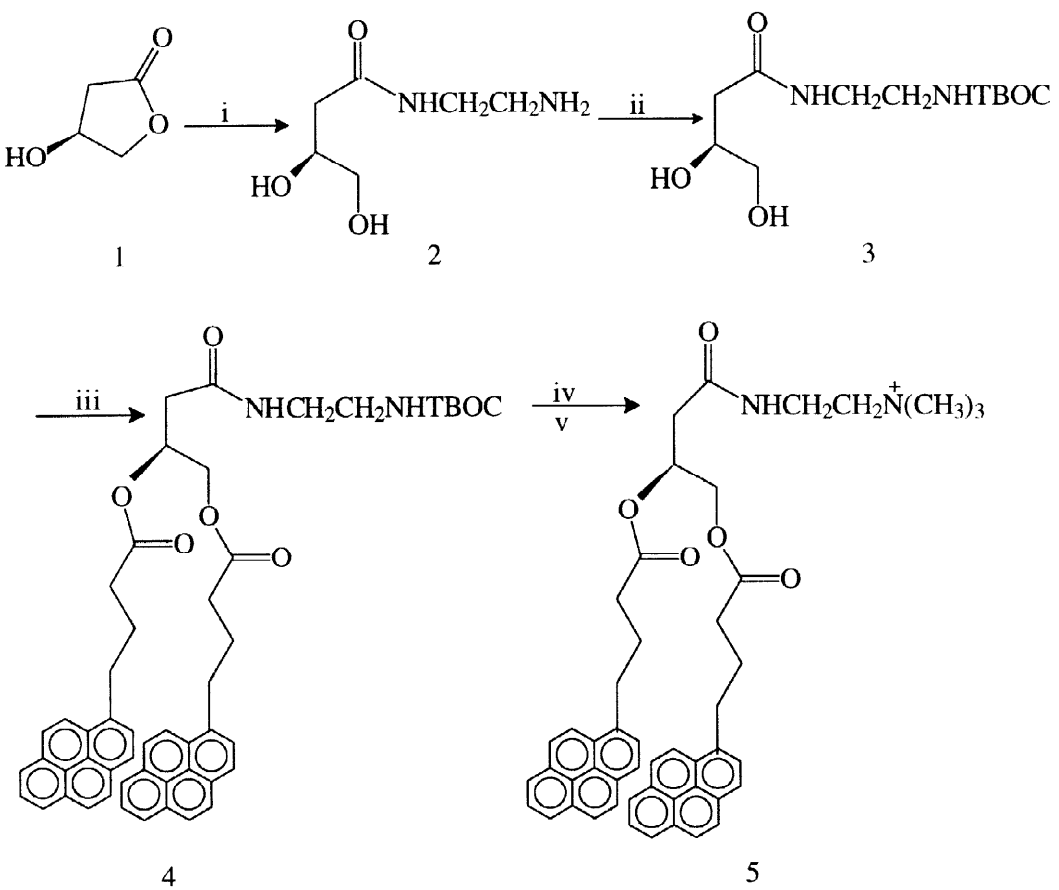
glycerol moiety is cleaved so that a phosphatidic acid remains. Phospholipase-C activity results in the cleavage of the proximal bond and a diacylglycerol is left. The design of a lipid analog that maintains the diacyl structure and the polar aspects of the head group but without the phosphodiester linkage is desirable. The preparation should be simple and the product should be optically pure. Here we describe the preparation of a phosphatidyl choline analog **5** lacking the phosphodiester group but bearing the trimethylammonium ethyl group and pyrenyl functions in each acyl chain. The molecule has a net positive charge. Phosphatidyl choline in the membrane environment also has a net positive charge since the negative charge on the phosphate group is neutralized by calcium ions at the interface between the hydrocarbon core and the solvation shell. The synthesis is based on (S)-3-hydroxy-γ-butyrolactone as the source of the chiral diol fragment to which the acyl groups are attached. The oxygen atom at the 3-position of glycerol in glycerolipids is replaced by a carbon atom in this structure. This probe was designed to be stable to phospholipase-C and D activity but is also very stable to phospholipase-A. It was designed for use in studying membrane dynamics and order by analysing excimer emission spectra using fluorescence spectroscopy and for performing studies at the single cell level using fluorescence microscopy.

RESULTS AND DISCUSSION

The chiral head group of the fluorescent probe was obtained from carbons 3-6 of D-glucose by selectively degrading it to (S)-3,4-dihydroxybutyric acid and then cyclizing it to the 3-hydroxy-γ-lactone[5]. The lactone was then ring-opened with an excess of ethylenediamine to give the 2-aminoethyl (3,4-dihydroxy)butyramide and the amino group protected with a t-butyloxycarbonyl function[6] (scheme I). The acyl groups were then installed, the t-butyloxycarbonyl group removed and the amino group was completely methylated using iodomethane to give the product. One important feature of this synthesis is the ease with which the chirality is integrated into the molecule. The starting lactone is obtained >99.8 % optically pure (by chiral gas liquid chromatography on a cyclodextrin column) and the subsequent transformations cannot disturb the chiral center. The chiral center is not β to a carbonyl function and there is not a problem with migration of a protecting group as can happen with protected glycerols in phospholipid synthesis. The ring opening to form the amide is very facile. The chemistry is quite simple and actually represents a general method to access optically pure glycero lipids if one so desires. The dihydroxybutyramide (or equivalent structure such as a hydrazide) can be protected and converted to a 3-carbon (glyceryl) derivative by reactions such as the Curtius and Hoffmann. The synthesis of optically pure phospholipids and analogs of phospholipids is a very laborious affair that usually involves the use of chiral 3-carbon synthons such as glycidols and isopropylidene glycerols. These are expensive and the installation of the phosphate group requires some effort. The chemistry involves the selective functionalization of the glyceryl moiety, the protection and activation of the phosphate group, the coupling steps, the acylation reactions and some deprotection steps . Phospholipids and related molecules such as platelet activation factors are very important molecules that have a wide variety of functions in biological systems. These range from structural (formation of membranes and compartments) to dynamic (formation of domains) to modulatory (signal transduction and activation of biochemical cascades).

The analog 5 displayed the excimer emission properties that were required. The emission spectrum at the absorption maximum of free pyrenebutyric acid (327 nm) is shown in figure 1A and the corresponding spectrum from the analog (probe) 5 is shown in figure 1B. The highly intense, broad spectrum is the excimer emission spectrum. An intense excimer emission was also obtained from the probe in phosphatidyl choline liposomes (figure 1C). The intensity of the excimer band decreases as the average separation of the rings increases. If the molecule is degraded by a phospholipase A (which removes a pyrenebutyric acid residue) the two pyrenyl rings drift apart and the excimer emission disappears completely. Treatment of the probe for several hours at pH 7.5 with a phospholipase-A from *Rhizobium trifolii* followed by extraction and thin-layer chromatography analysis indicated that no free pyrenebutyric acid was liberated and hence the probe was stable to this enzyme.

Scheme I



1 2 3 4 5

Reaction Conditions, Reagents and Yields: (i) excess $H_2NCH_2CH_2NH_2$, THF, 70 °C, 107%; (ii) tBuOH, $(tBuO_2C)_2O$, Na_2CO_3, H_2O, 76%; (iii) DMAP, DCC, CH_2Cl_2, 1-pyrenebutyric acid, 47%; (iv) CF_3COOH; (v) K_2CO_3, CH_3I, 65 °C, 61%.

Figure 1A

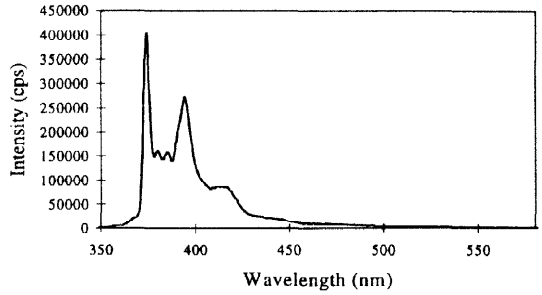


Figure 1B

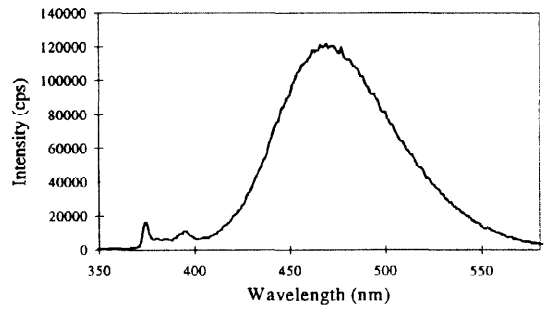


Figure 1C

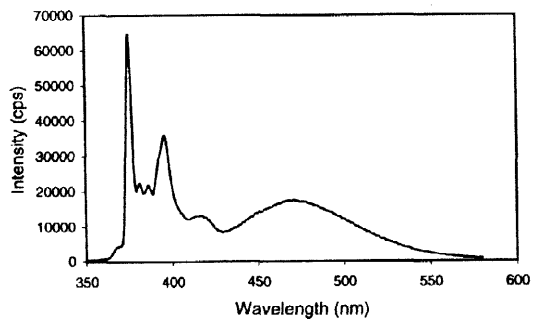


Figure 1A. Fluorescence emission spectrum of pyrene butyric acid with excitation at 327 nm. (B) Spectrum of fluorescence probe 5 showing the broad excimer emission between 400 and 550 nm. (C) Spectrum of probe in phosphatidyl choline unilamellar vesicles showing excimer emission.

EXPERIMENTAL

Preparation of 1

Maltose monohydrate (10 g, 28 mmol) was dissolved in 0.16 M sodium hydroxide (500 mL, 80 mmol) and 37 mmol of hydrogen peroxide in 500 mL of water was added. The mixture was heated at 70 ^{0}C for 10 hours, the pH adjusted

to 1 with sulfuric acid and the solution concentrated to a syrup which was stirred with sodium bicarbonate (4.2 g, 50 mmol) and 100 g of crushed ice. The slurry was extracted with ethyl acetate (500 mL) and the extract stirred with Norit (10 g), filtered and concentrated. The product was chromatographed on silica (9:1 chloroform - methanol) (Alternatively, the lactone could be purified by distillation, b.p. 98 - 100 ^{0}C, 0.3 mm Hg). The yield was 2.3 grams (85%). The optical purity was 99.9% on chiral GC using a BetaDex cyclodextrin bonded phase from Supelco (Bellefonte PA). $[\alpha]_D$: -85.6^0 (c = 3.1, CH_3CH_2OH) [Lit.[7], $[\alpha]_D$: -86.1^0 (c=3.1, CH_3CH_2OH)]; IR: (neat) 1783 (s), 1737 (s) cm^{-1}; ^{1}H NMR (300 MHz): δ 2.28 (1 H, dd, J = 18.0, 0.2 Hz), 2.74 (1 H, dd, J = 18.0, 5.8 Hz), 4.13 (1 H, dd, J = 9.8, 0.2 Hz), 4.32 (1 H, dd, J = 9.8, 4.5 Hz), 4.49 (1 H, m); ^{13}C NMR (75 MHz, CDCl3): 177.2, 76.8, 67.8, 37.7.

Preparation of 2

A solution of 1.0 g (10 mmol) (S)-3-hydroxy-γ-butyrolactone (prepared as described above or obtained from Synthon Corporation, Lansing MI) and 1.8 g (30 mmol) ethylene diamine in 1 ml THF was heated at 70 ^{0}C for 12 hours. The reaction mixture was dried on a rotary evaporator to give the product as a syrup. It was used without further purification. IR (KBr): 3357 (bs), 1644 (s), 1562 (s), 1040 (s) cm^{-1}; ^{1}H NMR (300 MHz, D_2O): δ 3.90 (1 H, m), 3.43 (1 H, dd, J = 11.3, 4.5 Hz), 3.34 (1 H, dd, J = 11.3, 6.0 Hz), 3.09 (2 H, t, J = 6.0 Hz), 2.55 (2 H, t, J = 6.0 Hz), 2.29 (1 H, dd, J = 14.5, 4.5 Hz), 2.19 (1 H, dd, J = 14.5, 8.9 Hz); ^{13}C NMR (75 MHz, D_2O): 173.9, 68.7, 64.8, 41.6, 39.6, 39.5; HRMS Exact mass: calcd for $C_6H_{15}N_2O_3$ $[M+H]^+$, 163.1084. Found 163.1078.

Preparation of 3

To a two-necked round bottom flask containing Na_2CO_3 (7.0 g, 66 mmol) freshly dissolved in water (66 mL) and equipped with a reflux condenser, a dropping funnel and a magnetic stirring bar were added compound 2 (6.5 g, 40 mmol) and tert-butyl alcohol (30 mL) with stirring.The mixture was stirred and 19.0 g di-tert-butyl dicarbonate was added dropwise through the dropping funnel. Stirring was continued for 16 hours and then 200 mL pentane and 80 mL H_2O were added and the layers separated. The aqueous layer was extracted again with 200 mL pentane and then twice with ethyl ether. It was dried and compound 3 was recovered from the salts by extraction of the solid with methanol. The methanol solution was concentrated to yield compound 3 as an amorphous solid in a highly pure state. It was used without further purification . IR ($CHCl_3$): 3351 (bs), 1779 (m), 1694 (s), 1655 (s) cm^{-1}; ^{1}H NMR (300 MHz, D_2O): δ 3.88 (1 H, m), 3.41 (1 H, dd, J = 12, 4 Hz), 3.31 (1 H, dd, J = 12, 6 Hz), 3.08 (2 H, t, J = 6 Hz), 3.02 (2 H, t, J = 6 Hz), 2.26 (1 H, dd, J = 15, 6 Hz), 2.16 (1 H, dd, J = 15, 9 Hz), 1.22 (9 H, s); ^{13}C NMR (75 MHz, D_2O): δ 173.45, 167.29, 80.72, 68.68, 64.78, 39.59, 39.09, 38.99, 27.50; HRMS Exact mass: calcd for $C_{11}H_{23}N_2O_5$ $[M+H]^+$, 263.1608. Found 263.1617.

Preparation of 4

Compound 3 (0.26 g, 1.0 mmol) was dissolved in THF and the solution was dried under vacuum to remove moisture. Anhydrous dichloromethane (10 mL), 0.63 g (2.2 mmol) of 1-pyrenebutyric acid and 0.24 g (2.0 mmol) of DMAP were added and the solution was stirred. Dicyclohexylcarbodiimide (0.45 g, 2.2 mmol) in 10 mL of anhydrous

dichloromethane was added through a dropping funnel. The solution was stirred overnight at room temperature with a drying tube. The reaction mixture was filtered and the precipitate was washed with a small amount of chloroform. The filtrate was concentrated on a rotary evaporator and was then purified by flash column chromatography on silica gel starting with pure chloroform and increasing the polarity to chloroform/methanol (1:2) in a stepwise fashion. The yield was 47%. IR ($CHCl_3$): 3455 (bs), 3029 (m), 3023 (m), 3017 (m), 3011 (m), 2980 (m), 2942 (m), 2880 (m), 1736 (s), 1705 (s), 1679 (s), 1605 (w), 1588 (w) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$):δ 7.76-8.21 (18 H, m, Ar-H), 6.19 (1 H, broad, NH), 5.44 (1 H, m), 4.82 (1 H, broad, NH), 4.38 (1 H, dd, J = 12.3, 3.0 Hz), 4.17 (1 H, dd, J = 12.3, 6.0 Hz), 3.29 (4 H, t, J = 7.5 Hz), 3.23 (2 H, t, J = 5.3 Hz), 3.13 (2 H, t, J = 5.3 Hz), 2.41 (6 H, m), 2.13 (4 H, m), 1.40 (9 H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 213.2, 173.0, 172.4, 168.9, 120-130 (many peaks), 79.6, 68.7, 64.3, 40.7, 39.9, 37.6, 33.6, 33.4, 32.5, 32.4, 28.2, 26.5, 26.4; HRMS Exact mass: calcd for $C_{51}H_{50}N_2O_7$ $[M]^-$, 802.3620. Found 802.3647.

Preparation of 5

Trifluoroacetic acid (0.5 mL) was added to 0.38 g of compound 4. The solution was allowed to stand for 15 minutes and was concentrated on a rotary evaporator. Acetone (3 mL) and 0.32 g (5.0 equivalents) of potassium carbonate were added to the solution which was then cooled in an ice bath. Iodomethane (3.0 g) was added to the solution which was refluxed at 65 ^0C overnight and cooled to room temperature. Chloroform (5.0 mL) was added and the reaction mixture was filtered to remove inorganic salts. The filtrate was concentrated and compound 5 was obtained by flash column chromatography on silica gel using a gradient elution from pure chloroform to chloroform/methanol 1:1. The yield was 61%. IR ($CHCl_3$): 2953 (s), 2880 (w), 1734 (s), 1671 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.76-8.21 (18 H, m, Ar-H), 5.46 (1 H, m), 4.32 (1 H, dd, J = 12.8, 3.9 Hz), 4.12 (1 H, dd, J = 12.8, 6.0 Hz), 3.43 (2 H, s), 3.40 (2 H, s), 3.10 (4 H, s), 2.82 (9 H, s), 2.51 (2 H, J = 6.0 Hz), 2.33 (4 H, m), 2.00 (4 H, m); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.3, 173.0, 170.2, 121.6-135.3 (many peaks), 68.3, 64.51, 64.50, 54.2, 54.0, 53.8, 37.3, 33.9, 33.6, 32.3, 32.2, 26.5, 26.4.; HRMS Exact mass: calcd for $C_{49}H_{49}N_2O_5^+$ $[M]^+$, 745.3644. Found 745.3658.

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