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Chemical Syntheses of Novel Fluorescent-Labelled Fatty Acids, Phosphatidylcholines and Cholesterol Esters

Wilhelm STOFFEL and Gabriele MICHAELIS

Institut für Physiologische Chemie der Universität Köln

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Summary: The synthesis of a novel class of fluorescent-labelled fatty acids of different chain lengths and unsaturation, phospholipids and cholesterol esters has been developed. The following ω-anthracene-labelled *cis*-unsaturated fatty acids have been synthesized: ω-(9-anthryl)-6*c*-octenoic, -7*c*-nonenoic, -10*c*-dodecenoic, -6*c*,9*c*-undecadienoic, -10*c*,13*c*-pentadecadienoic acid. They have been introduced into the 2-position of 1-stearoyl- and 1-linoleoyl-3-*sn*-glycerophosphocholine and cholesterol. Mass spectroscopy, ^{1}H-NMR, IR and fluorescence spectroscopy and different chromatographic procedures have been applied to confirm and characterize their structures. The properties of the different fluorescent-labelled phosphatidylcholines in monomolecular films have been determined by the Langmuir technique.

Chemische Synthese neuer fluoreszenzmarkierter Fettsäuren, Phosphatidylcholine und Cholesterinester

Zusammenfassung: Es wird die Synthese einer neuen Klasse von fluoreszenzmarkierten Fettsäuren, Phospholipiden und Cholesterinestern beschrieben. Die folgenden ω-Anthracen-markierten *cis*-ungesättigten Fettsäuren wurden synthetisiert: ω-(9-Anthryl)-6*c*-octen-, -7*c*-nonen-, -10*c*-dodecen-, -6*c*,9*c*-undecadien- und -10*c*,13*c*-pentadecadiensäure. Sie wurden in die 2-Position von 1-Stearoyl- bzw. 1-Linoleoyl-3-*sn*-glycerophosphocholin und Cholesterin eingeführt. Ihre Strukturen wurden durch Massenspektroskopie ^{1}H-NMR-, IR- und Fluoreszenzspektroskopie sowie verschiedene chromatographische Methoden charakterisiert. Die Eigenschaften der verschiedenen fluoreszenzmarkierten Lecithine im monomolekularen Film wurden mit der Langmuir-Technik bestimmt.

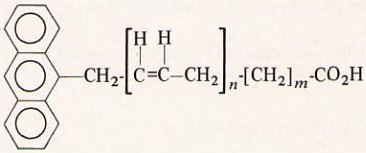
Key words: Fluorescent fatty acids, fluorescent phospholipids, spectral properties, structural proof of fluorescent probes.

Abbreviations: A, anthracene residue; PC, phosphatidylcholine; short-hand nomenclature of unsaturated fatty acids is as follows: number of carbon atoms of alkane chain, : *cis* double bond, followed by number of double bonds. The index indicates the position of double bond counted from the carboxyl group. Example: $18:2^{9,12}$/A-$8:1^{6}$-PC = 1-linoleoyl-2-[8-(9-anthryl)-6*c*-octenoyl]-3-*sn*-glycerophosphocholine.

Fluorescent probes have been widely applied for the study of the microenvironments of large biological structures such as proteins and membrane structures. The technique can either be applied to "intrinsic" fluorochromes such as tyrosine and tryptophan or to "extrinsic" probes which have been added to the system.

Fluorescent probes of polar or hydrophilic and apolar or hydrophobic structures make it possible to study regions of similar properties, provided they are located in the region from which signals are wanted. Parameters such as fluorescence polarization, lifetime and quenching measurements, and in suitable cases, the energy transfer from tryptophan to a chromophore can give valuable information about structural properties or changes in the environment of the macromolecule[1-4], phase transitions in artificial bilayers[2-19] or natural membranes[20-23], state of energy as a function of the action potential in nerve fibers [24,25], determinations of the distance between the binding site of the probe and tryptophan residues in proteins[26-28]. The most commonly used fluorochromes have been 8-anilino-1-naphthalenesulfonate or positional isomers thereof, *N*-phenyl-1-naphthylamine, 2-methylanthracene, 9-vinylanthracene and perylene. All these probes are chromophores not covalently linked to the hydrophilic or hydrocarbon region under study. Waggoner and Stryer[29] synthesized some fluorescent probes covalently linked to alkane chains, such as 12-(9-anthrylcarbonyloxy)stearic acid or dansyl derivatives linked to the ethanolamine moiety of phosphatidylethanolamine and octadecylnaphthylaminesulfonate as probes of the hydrophilic region of membranes and phospholipid bilayers. One obstacle to the use of many of the freely diffusible fluorescent probes is the difficulty of localizing them in macromolecular structures. Experiments with [12-^{3}H]12-(9-anthrylcarbonyloxy)stearic acid in this laboratory aiming at the introduction into phospholipids of eucaryotic cells (BHK 21, MDBK cells) and the fatty acid auxotroph *Escherichia coli* mutant 1059[30] failed. The ester bond between the 9-anthracenecarboxylic acid and 12-hydroxystearic acid was hydrolyzed and the chromophore resided outside the membrane structures.

We therefore developed a novel class of fluorescent-labelled fatty acids and phospholipids. The fatty acids can also be used for the acylation of other lysophospholipids and of cholesterol. We designed a synthesis of saturated and *all-cis* mono- and dienoic fatty acids with a terminal anthracene residue as fluorescent probe linked by a C-C bond to the aliphatic acid. These fatty acids of the general structure



would not only provide a non-hydrolyzable fluorescent marker, but they could also be synthesized with distinct chain lengths, thereby facilitating the exact location of the fluorescent probe in a given structure, such as artificial (liposomes) or natural membranes or reconstituted lipoprotein complexes. These tagged fatty acids were also incorporated as acyl groups into phospholipids. Furthermore, we intended to incorporate them by biosynthetic pathways into natural membranes of the aforementioned *E. coli* fatty acid auxotroph mutant and into cells in tissue culture. This paper describes

a) the synthesis of a number of anthracene-labelled fatty acids of different chain lengths and degrees of saturation,
b) their characterization and
c) studies on physical properties of these acids incorporated into phospholipids.

The subsequent paper summarizes results on their application to the study of liposomes and natural membranes.

Experimental

I) Analytical methods:

Mass spectra were recorded with a CH 5 mass spectrometer Varian MAT with a cathode emission of 300 μA, electron energy of 70 eV and 2 KV acceleration voltage. NMR spectra were registered with a 90 MHz instrument, model WH 90, Bruker Physik.

Fluorescence spectra were recorded with a Perkin Elmer Hitachi fluorescence spectrometer, model MPF III, with temperature-controlled cuvettes.

Gas-liquid chromatographic analyses were carried out on 2.5% polyethyleneglycol succinate on chromosorb columns using the Perkin Elmer model F 20 instrument.

The following solvent systems were used for thin-layer chromatography: for fatty acid derivatives, light petroleum/ether/acetic acid 60:40:1, and chloroform/methanol/water 65:25:4 for phospholipids. Spots were made visible either by their fluorescence or by charring with sulfuric acid/5% sodium dichromate; phosphorus-containing compounds with Zinzadze's reagent[31].
Radio thin-layer chromatograms were analyzed with a Packard scanner, model 7201, or a Berhtold scanner, model LB 2722. For counter-current distribution, the 500-tube instrument from H.O. Post, Scientific Instrument Company, Inc., New York, was used. Solvent system: methanol/n-heptane.

II) Synthesis of anthracene-labelled fatty acids

All reactions were carried out with light protection and in an atmosphere of purified nitrogen or argon. Melting points are uncorrected.

9-Hydroxymethylanthracene (I): 36.6 g (0.15 mol) *9-anthracenecarbaldehyde* was dissolved in 400 m*l methanol* and reduced with an aqueous solution of 7 g (0.17 mol) *$NaBH_4$* stabilized with a few drops of NaOH over a period of 40 min. The mixture was poured into 200 m*l* ice-cold saturated NaCl solution.

The product was extracted with ether. 9-Hydroxymethylanthracene crystallized on concentration of the dried ethereal solution.

Yield: 29.1 g (0.14 mol) (95% of theoret);
mp 138 - 140 °C.

9-Bromomethylanthracene (II): 29.1 g (0.14 mol) *I* dissolved in 700 m*l* ether was refluxed with 40 m*l* 48% *HBr* for 3 h. The ethereal solution was separated, washed with 5% $NaHCO_3$ and water to neutrality, dried over Na_2SO_4 and concentrated. The bromide crystallized in deep yellow needles.

Yield: 31.8 g (96% of theoret.); mp 141 - 142 °C.

6-Heptynoic and *7-octynoic* acids were prepared according to Kaluszyner[32] and Field[33] respectively, starting from *tetrahydrofuran* and *tetrahydropyran* via 1-bromo-4-chlorobutane and 1-bromo-5-chloropentane and subsequent coupling with *sodium acetylide* in liquid ammonia. Their nitriles were formed according to Smiley and Arnold[34] and the ω-acetylenic acids obtained by alkaline hydrolysis. The di-Grignard complex of these acetylenic acids was condensed with propargylbromide according to Osbond et al.[35]. 6,9-Decadiynoic and 6,9-undecadiynoic acid were purified by molecular distillation.

10-Undecynoic acid was prepared according to Khan et al.[36]. *10,13-Tetradecadiynoic* acid was synthesized following the procedure described by Kunaul[37] starting from 10-undecyn-1-ol prepared according to Couffignal et al.[38] from *10-undecen-1-ol.*

8-(9-anthryl)-6-octynoic acid: 3 g (0.12 mol) *magnesium* and 15 g (0.14 mol) *ethylbromide* were reacted in peroxide-free, dry tetrahydrofuran to the Grignard complex. 7.5 g (0.06 mol) *6-heptynoic* acid dissolved in 50 m*l* tetrahydrofuran was added over a period of 40 min at 0 °C. The mixture was stirred for 3 h at room temperature. 0.5 g CuCN was added, and after 15 min, a solution of 16 g (0.06 mol) *9-bromomethylanthracene* in

Table 1. Anthracene-labelled mono- and diynoic acids and the corresponding *cis* monoenoic and dienoic acids synthesized by condensation of 9-bromomethylanthracene and the respective ω-acetylenic acids.

ω-Acetylenic acid	Reaction product	M.P. [°C]
6-Heptynoic	8-(9-anthryl)-6-octynoic 8-(9-anthryl)-6*c*-octenoic	114 - 115 100 - 101
7-Octynoic	9-(9-anthryl)-7-nonynoic 9-(9-anthryl)-7*c*-nonenoic	110 - 112 118 - 120
6,9-Decadiynoic	11-(9-anthryl)-6,9-undecadiynoic 11-(9-anthryl)-6*c*,9*c*-undecadienoic	120 - 122 128 - 130
7,10-Undecadiynoic	12-(9-anthryl)-7,10-dodecadiynoic 12-(9-anthryl)-7*c*,10*c*-dodecadienoic	88 - 90 97 - 99
10-Undecynoic	12-(9-anthryl)-10-dodecynoic 12-(9-anthryl)-10*c*-dodecenoic	78.5 - 79.5 98.5 - 100
10,13-Tetradecadiynoic	15-(9-anthryl)-10,13-pentadecadiynoic 15-(9-anthryl)-10*c*,13*c*-pentadecadienoic	102 - 104 106 - 108

100 m*l* tetrahydrofuran. The reaction mixture was refluxed for 24 h. 50 m*l* of ice-cold 2N H_2SO_4 was added dropwise under ice cooling. The organic phase was separated and the aqueous phase extracted three times with 2N NH_4OH. The alkaline solutions were acidified under ice cooling with 6N HCl and the reaction products extracted with ether. The dried ethereal solution was concentrated and the residue chromatographed on a silicic acid column using increasing amounts of ether (5 - 10%) in light petroleum. Unreacted 6-heptynoic acid was eluted first and then 8-(9-anthryl)6-octynoic acid.

Yield: 3.7 g (20% of theoret.); mp. 114 - 115 °C, bright yellow crystals.

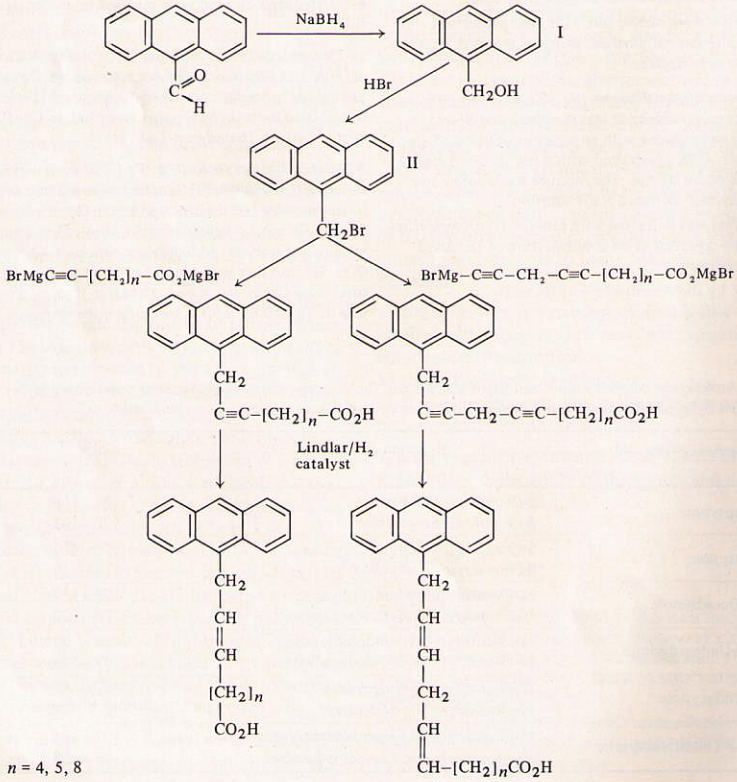
8-(9-anthryl)-6c-octenoic acid: 1.6 g (5 mmol) of the acetylenic acid was reduced with 0.5 g Lindlar catalyst in 80 m*l* ethyl acetate. The catalyst was poisoned with 0.3 m*l* 5% chinoline. The reduced product was recrystallized from light petroleum/ether.

Yield: 1.47 g (92% of theoret.); mp. 100 - 101 °C.

Counter-current distribution proved to be a more convenient and gentle method for the purification of the ω-anthryl mono- and diynoic acids. Solvent system: n-heptane/methanol, 500 partitions.

According to the coupling reaction and partial catalytic reduction described for 8-(9-anthryl)-6-octynoic acid, the acids listed in Table 1 were synthesized.

Table 2. Synthetic pathway of ω-anthracene-labelled mono- and dienoic fatty acids.

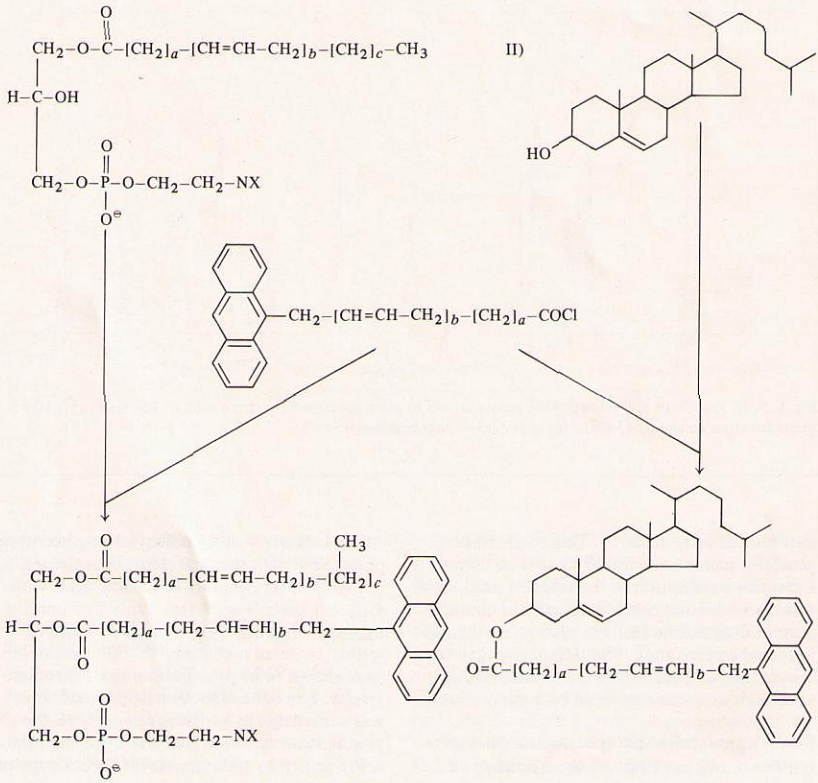


Results

Our approach to the synthesis of this new type of stable fluorescent-labelled fatty acids, phospholipids and cholesterol esters consisted essentially of the formation of an anthracene derivative suitable for the condensation with ω-acetylenic acids of the chain length and degree of unsaturation wanted. The synthetic pathway is outlined in Table 2.

9-Anthracenecarbaldehyde was reduced with sodium borohydride and the resulting 9-hydroxymethylanthracene brominated with 48% HBr in ether in almost quantitative yield. The ω-acetylenic acids were synthesized following established procedures described previously[39]. In order to obtain several fluorescent-labelled fatty acids, mono- and diacetylenic acids with chain lengths C_7 to C_{14} and one and two acetylenic bonds were synthesized. They were linked to the anthracene precursor in a Grignard reaction under the conditions used previously for the synthesis of poly-

Table 3. Scheme for the synthesis of anthracene-labelled phospholipids (I) and cholesterol esters (II).



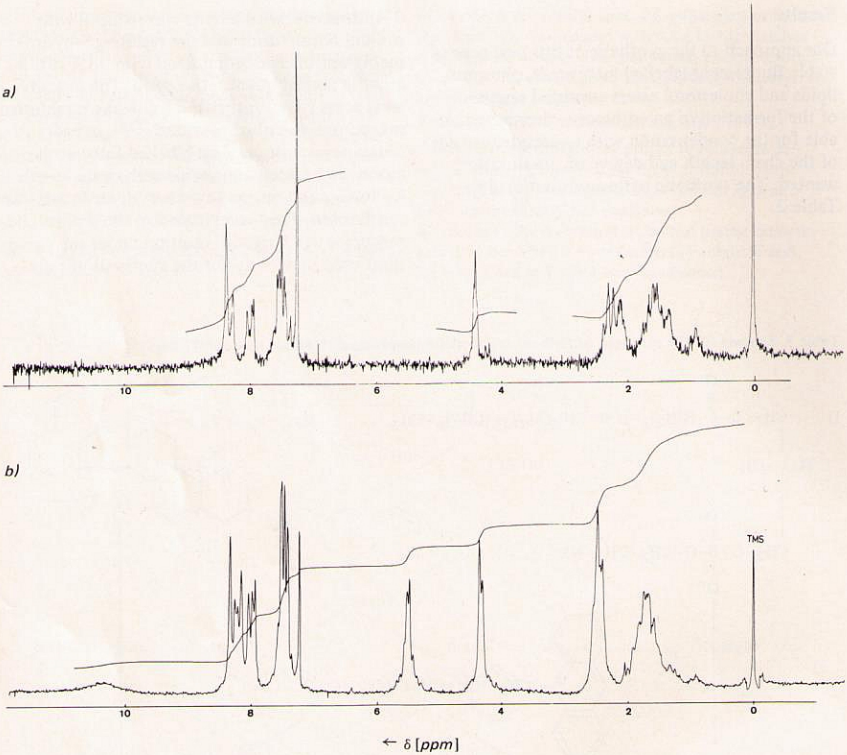
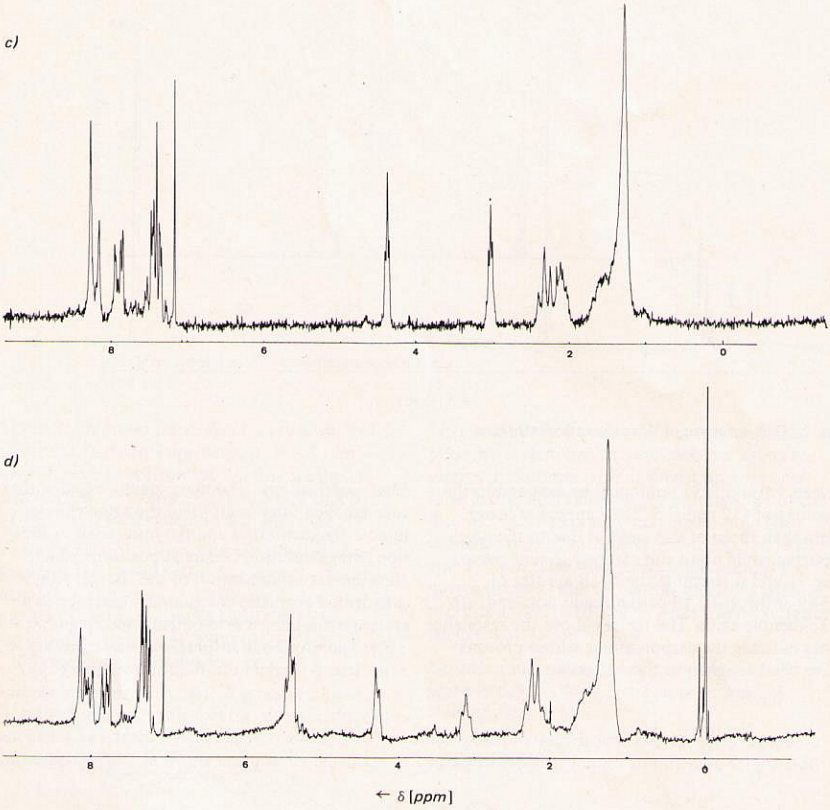


Fig. 1. NMR spectra of a) 8-(9-anthryl)-6-octynoic and b) the respective 6*c*-olefinic acid, c) 15-(9-anthryl)-10,13-pentadecadiynoic and d) 15-(9-anthryl)-10*c*,13*c*-pentadecadienoic acid.

unsaturated fatty acids[39]. This condensation generally succeeded with 20 to 30% of theory. Extensive purification of the reaction product by silicic acid column chromatography or countercurrent distribution (solvent system: methanol/heptane) was required. The latter proved to be the method of choice. The unreacted starting acetylenic acid was recovered by both methods.

Fluorescent-labelled phosphatidylcholines were synthesized by acylation of the 2-position of either 1-stearoyl- or 1-linoleoyl-3-*sn*-glycerophosphocholine with the acyl chloride of the respective ω-(9-anthryl)mono- or dienoic acids with different chain lengths (see Table 2) according to the reaction scheme given in Table 3 and described in detail previously[40]. All compounds were shown to be pure by thin-layer chromatography. Since the reduction of the triple bond was carried out in a tritium atmosphere, the phosphatidylcholines were also tested for their radioactive purity by radio thin-layer chromatography.



Cholesterol was esterified under the same reaction conditions, yielding the respective fluorescent-labelled cholesterol esters.

Characterization of fluorescent-labelled fatty acids and phosphatidylcholines

NMR-spectroscopy. ^{1}H-90 MHz NMR spectroscopy of the anthracene-labelled fatty acids with different chain lengths but the same degree of unsaturation all revealed very similar resonances. Therefore only NMR spectra of a mono- and dynoic acid, 8-(9-antryl)-6-octynoic and 15-(9-anthryl)-10,13-pentadecadiynoic acid, and their respective *cis*-olefinic ω-anthryl fatty acids are discussed (Fig. 1a, b). The signals of the aromatic protons appear between 7.4 and 8.4 ppm, those of C-8 at 4.35 to 4.4 ppm. The C-8 protons do not couple with the aromatic protons, as shown in the NMR spectrum of 9-bromomethylanthracene, Fig. 2, which is given for comparison (aromatic protons between 7.4 and 8.4 ppm, CH_2- at 5.45 ppm). The group of resonance lines be-

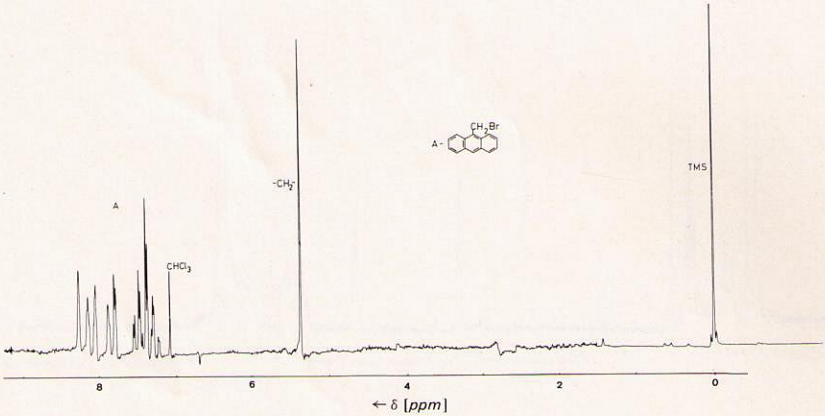
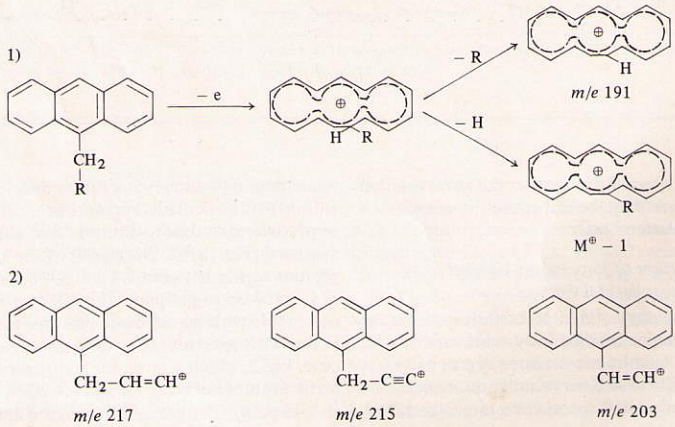


Fig. 2. NMR spectrum of 9-bromomethylanthracene.

tween 1.9 and 2.35 ppm must be assigned to the protons of C-2 and C-5. They appear at lower field than those of C-3 and C-4 due to the neighbouring triple bond and carboxylic acid group. Fig. 1c and d resemble the NMR spectra of 15-(9-anthryl)-10,13-pentadecadiynoic and -10*c*, 13*c*-dienoic acids. The figures above the resonance lines indicate the carbon atoms whose protons have been assigned to these signals.

Mass spectroscopy. The mass spectra of all anthracene-labelled fatty acids yield the same characteristic fragmentation and the mass peak in addition. Fragmentation occurs at positions which allow an easy stabilization of the charge. Alkyl-substituted aromatic compounds rearrange to a system containing a seven-membered ring (*e*/*m* = 219). Therefore our anthracene derivatives are expected to fragment in the following way:



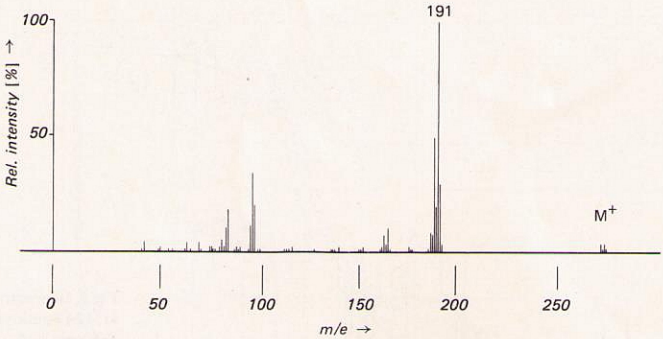


Fig. 3. Mass spectrum of 9-bromomethylanthracene.

Again two representative spectra are given which prove this fragmentation scheme. Fig. 3 represents the mass spectrum of 9-bromomethylanthracene ($M^{\oplus}$ 270 and 272) and Fig. 4 that of 8-(9-anthryl)-6-*cis*-octenoic acid ($M^{\oplus}$ 318).

IR-spectroscopy. All anthracene-labelled acids absorb at the wavelengths characteristic for the aromatic ring system at 3050 cm^{-1}, 1620 cm^{-1} and 1520 cm^{-1}, for the OH and C=O group of the carboxylic group at 3300 to 2400 cm^{-1} and 1700 cm^{-1}, respectively, Fig. 5a, b.

Fluorescence spectroscopy. Fig. 6 resembles the excitation and emission spectra of the anthracene-labelled 12-(9-anthryl)-10*c*-dodecenoic acid. The fluorescence spectra of the anthracene-labelled fatty acids as free acids and incorporated into phosphatidylcholine or as cholesterol esters are identical. Solvents with different polarity (heptane to ethanol) cause only a minute shift of the fluorescence maxima by maximal 6 nm. The aromatic system absorbs at 260, 335, 350, 370 and 390 nm. The fluorescence spectrum is the mirror image of the excitation spectrum with maxima at 395, 416 and 442 nm when excitation occurred at 370 nm.

Langmuir measurements (F/A isotherms) of phosphatidylcholines containing anthracene-labelled fatty acids.

The phosphatidylcholine species carrying anthracene-labelled mono- and dienoic fatty acids

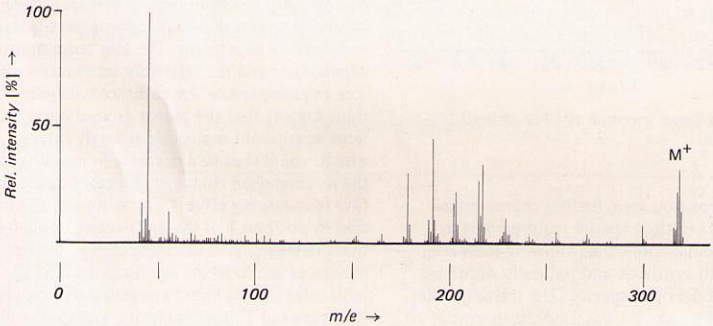


Fig. 4. Mass spectrum of 8-(9-anthryl)-6*c*-octenoic acid.

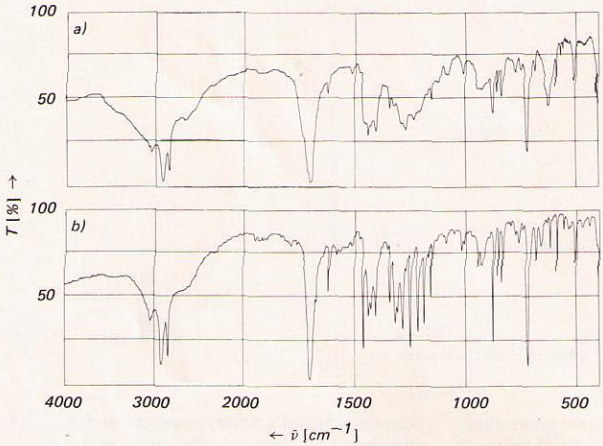


Fig. 5. IR spectrum of
a) 12-(9-anthryl)-10-dodecynoic acid,
b) 12-(9-anthryl)-10*c*-dodecenoic acid.

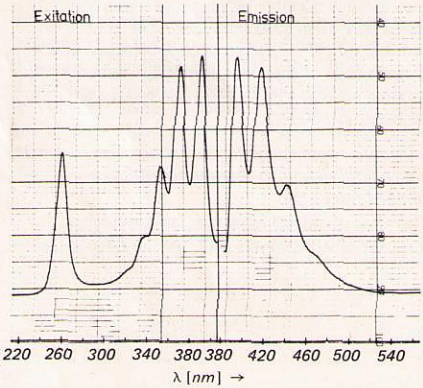


Fig. 6. Fluorescence spectrum of 12-(9-anthryl)-10*c*-dodecenoic acid.

in their 2-position were further characterized with respect to their spatial requirements in monomolecular films, thus allowing their comparison with synthetic and naturally occurring phosphatidylcholine species. The technique described previously[41] was used. Isotherms at 10°, 20°, 30° and 37.5 °C were registered, and the monomolecular film was compressed at 125 Å^2/(molecule × min) and expanded at 175 Å^2/(molecule × min).

The F/A isotherms of 18:2/A-8:1-PC are given in Fig. 7. All lecithins containing anthracene-labelled fatty acids form liquid expanded films between 110 and 130 Å^2/molecule at their collapse point. The molecular area at the collapse point increases and the pressure at the collapse point decreases with increasing temperature, as summarized in Table 4. However, the total increase in the molecular area with temperature is considerably less than that of natural phosphatidylcholines, and their collapse pressure is less temperature dependent. The low temperature dependence and the relatively small molecular area requirement of the anthracene-labelled lecithins suggest that the planar aromatic ring systems of adjacent molecules strongly interact, which would lead to a decrease in mobility of the hydrocarbon chains of the monomolecular film (condensing effect). Reduction of the linoleic acid in position 1 of the anthracene-labelled lecithins to stearic acid does not change the F/A isotherms of the lecithins significantly. The lecithin molecules form a liquid expanded monomolecular film between 130 Å^2/molecule and their collapse point. Again, the hexagonal closest packing is not

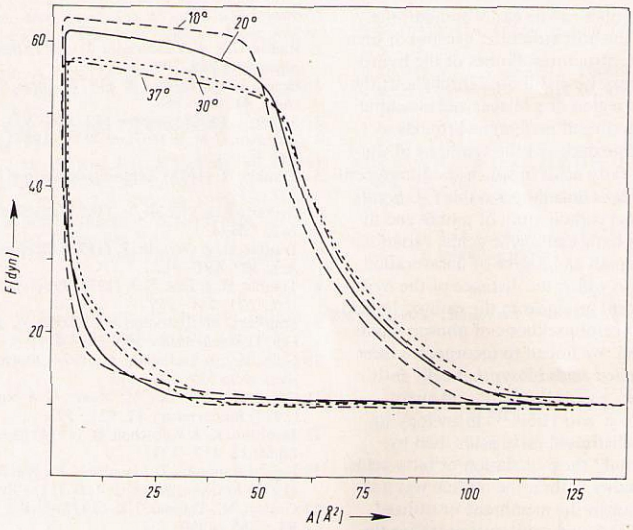


Fig. 7. F/A isotherms of 1-linoleoyl-2-[8-(9-anthryl)-6*c*-octenoyl]-3-*sn*-glycerophosphocholine.

The temperatures are indicated beside the curves.

achieved at temperatures between 10 and 37.5 °C. Also, the temperature dependence of the collapse pressure and molecular area at the collapse point is not altered significantly. The molecular area requirement of the different anthracene-labelled phosphatidylcholines at their closest possible packing ranges between 80 to 100 Å²/molecule, whereas that of natural lecithins amounts to 70 to 90 Å²/molecule.

Discussion

Fluorescent probes of hydrophilic or hydrophobic structures have been widely used to study the structure of or structural changes in regions of similar properties in macromolecules or macromolecular assemblies, such as artificial and biological membranes. Except for 12-anthrylcarbonyloxystearic acid and recently, dialkylindoles[42]

Table 4. Molecular area (A_T) of anthracene-labelled phosphatidylcholines.
The figures are given in Å²/molecule.

Temperature / Phospholipid species	10 °C	20 °C	30 °C	37.5 °C
18:2/A-8:1-PC	55.5	56.2	57.5	58.7
18:2/A-12:1-PC	51.2	52.5	50.0	54.5
18:0/A-12:1-PC	53.7	53.7	55.0	57.5
18:0/18:2-PC	50.0	56.3	58.8	60.0

none of these probes can be easily and definitely located within the host structure, because of their non-amphiphilic structures. Probes of the hydrophobic region may be distributed throughout the whole aliphatic region of a bilayer and also bind to hydrophobic sites of membrane proteins as well. We therefore designed the synthesis of fluorescent-labelled fatty acids in which the fluorescent anthracene probe is linked by a stable C-C bond to the ω-terminal carbon atom of mono- and di-unsaturated aliphatic carboxylic acids; variations in their chain length and degree of unsaturation created probes in which the distance of the hydrophobic fluorescent group from the carboxylic acid group or the glycerol backbone of phospholipids can be predicted. We hoped to incorporate these anthracene-labelled acids biosynthetically into membrane lipids, e.g. of the fatty acid auxotroph mutant of *E. coli* 1059[30] in analogy to the ω-phenyl substituted fatty acids used by Knoop[43] to study the β-oxidation of fatty acids. However, the bulky anthracene residue was not transported through the membrane or utilized by fatty acid kinase and acyltransferases for the biosynthesis of membrane phospholipids of the *E. coli* mutant.

The advantage of this type of fluorescent-labelled fatty acids is their great stability as compared to other probes, e.g. 12-(9-anthrylcarbonyloxy)-stearic acid, in which the probe is linked by an ester bond to the aliphatic chain. The acids can readily be transformed into acyl chlorides and used for the acylation of lysophospholipids such as lysophosphatidylcholines or protected lysophosphatidylethanolamines or the synthesis of cholesterol esters. No side reactions are observed when other activated esters, e.g. the *N*-hydroxysuccinimide esters, are formed. Therefore this novel type of fluorescent-labelled fatty acid probes can find wide application for the study of lipid-lipid and lipid-protein interactions.

The following publication[44] describes studies which demonstrate the versatility of these novel probes in investigations on the physical properties of artificial (liposomes) and of natural membranes.

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Prof. Dr. Dr. W. Stoffel and Dr. G. Michaelis, Institut für Physiologische Chemie der Universität, Joseph-Stelzmann-Str. 52, D-5000 Köln 41.