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Syntheses of Phosphatidylcholines, Sphingomyelins and Cholesterol Substituted with Azido Fatty Acids

Photocrosslinking with Nearest Neighbouring Lipids in Liposomes Chemical and Mass Spectroscopic Proof

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Summary: The chemical synthesis of azido-substituted saturated, monoenoic and dienoic fatty acids of high specific tritium radioactivity, together with improved methods for their introduction into phosphatidylcholine, sphingomyelin and cholesteryl ester molecules are outlined. Phospholipid vesicles with these photosensitive probes were irradiated and the main crosslinking products isolated and characterized by high resolution mass spectroscopy.

Synthesen von mit Azidofettsäuren substituierten Phosphatidylcholinen, Sphingomyelinen und Cholesterin. Photochemische Quervernetzung mit nächsten Nachbar-Lipidmolekülen in Liposomen. Chemische und massenspektroskopische Untersuchungen

Zusammenfassung: Die Synthesen von azido-substituierten gesättigten, einfach und doppelt ungesättigten Fettsäuren mit hoher spezifischer Tritium-Radioaktivität sowie verbesserte Methoden für deren Einbau in Phosphatidylcholin-, Sphingomyelin- und Cholesterinester-Moleküle werden beschrieben. Phospholipidvesikeln mit diesen photosensitiven Sonden wurden bestrahlt und die Hauptquervernetzungsprodukte isoliert und durch Massenfeinbestimmung charakterisiert.

Key words: Azido- and ^{3}H-labelled lipids, chemical synthesis, photocrosslinking in liposomes, NH-crosslinked fatty acid dimers.

One goal in membrane and lipoprotein structural studies is the understanding of the interactions of lipid and protein components in their nearest neighbour array. Physical probes (fluorescence, ESR, NMR) allow only a random interpretation of the lipid and protein interaction on the basis of altered mobilities of the aliphatic fatty acyl chains or polar head groups of the complex lipid molecules.

Another approach to get insight into the molecular architecture can be gained from chemical crosslinking neighbouring lipids and polypeptide segments. Lipid molecules tagged with a photosensitive azido group which on ultraviolet irradiation generates a highly reactive nitrene, will crosslink to their nearest neighbour due to the electron deficiency by addition to double bonds or insertion into C-H-bonds (for review see ref.[1]). The aliphatic nitrenes have an extremely short life time (10^{-11} s) as compared to aromatic nitrenes (10^{-6} s)[2]. Therefore, the lateral diffusion of components of a membrane or

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lipoprotein can be neglected in reactions of aliphatic nitrenes. The characterization of the crosslinked products should indeed give a momentary picture of adjacent structures in a supramolecular arrangement. Naphthylazide was used to label membrane proteins[3–5]. The advantage of our approach is that the photosensitive groups are in defined positions of the phospholipid bilayer. For the tracing of the crosslinked lipid molecules in biochemical studies, it was necessary to synthesize azido-labelled fatty acids of very high specific radioactivity. The azido group was placed either at the methyl terminal end or towards the center of the fatty acyl chain.

We describe in this paper the synthesis of 5-azido-[8,9-$^3H_2$]palmitic, 16-azido-[9,10-$^3H_2$]palmitic, 12-azido-[12 or 9,10-$^3H_2$]oleic and 18-azido-[9,10,12,13-$^3H_4$]linoleic acid. Furthermore the improved chemical syntheses of phosphatidylcholines and sphingomyelins, which are substituted by two or one azido fatty acid, and of photosensitive cholesteryl esters are outlined. Liposomes consisting of these phosphatidylcholines were irradiated and the crosslinking products isolated and characterized.

Materials and Methods

16-Bromo-9-hexadecenoic acid and 12-hydroxyoleic acid (ricinoleic acid) were purchased from Fluka (Switzerland), tritium gas from Amersham-Buchler (Braunschweig) and polyene phosphatidylcholine kindly provided by *Dr. Betzing*, Fa. Nattermann & Cie, Köln. 12-Hydroxy-[9,10-$^3H_2$]stearic acid resulted from the catalytic hydrogenation of 12-hydroxyoleic acid with PtO_2 in a tritium atmosphere with methanol/ethyl acetate 1:1 (v/v) as solvent and was purified by silicic acid chromatography with petroleum ether/ethyl ether mixtures with increasing ether concentrations (10 to 50%, v/v). Lysophosphatidylcholine was prepared by phospholipase A_2 (*Crotalus adamanteus*, Fa. Celo, Zweibrücken) hydrolysis of polyene phosphatidylcholine in sodium borate buffer pH 8.0.

All reactions involving intermediates with the azido group were carried out with protection from light.

25-Azido-[25-^{3}H]27-norcholesterol (specif. radioactiv. 7.7 Ci/mol) was synthesized as described before[6]. Cholesterol or 25-azido-27-norcholesterol were esterified according to the following procedure: 120 mg (0.26 mmol) cholesterol and 80 mg (0.62 mmol) 4-(dimethylamino)-pyridine[7,8] were dissolved in 5 ml dry chloroform. 100 mg (0.31 mmol) fatty acyl chloride was added and the mixture stirred at room temperature for 5 h. The solvent was evaporated, the residue dissolved in 20 ml ether and the solution washed twice with 20 ml 2M HCl and water, then dried over Na_2SO_4 and concentrated. The cholesteryl ester was purified by silicic acid chromatography (60 x 2 cm column) with petroleum ether/ether (99:1) as eluent. Yield: 80–90% of theory.

1) Synthesis of 5-azido-[8,9-$^3H_2$]palmitic acid (8)

The sodium salt of 3-methoxypropyne[9] and 1-bromoheptane were condensed to 1-methoxy-2-decyne (**1**) in liquid ammonia in 53% yield, bp_{12} = 96 °C. **1** was reacted with acetylbromide in dichloromethane and yielded 1-bromo-2-decyne (**2**), $bp_{0.1}$ = 78 °C, in 86% yield. The mass spectrum exhibited the doublet $M^{\oplus}$ at 216/218 due to the bromine isotopes.

2-(2-Decynyl)-1,3-cyclohexadione (**3**). 7.8 g (0.2 mol) Potassium was dissolved in 60 ml dry methanol. 22 g Dihydroresorcin (0.2 mol) was added followed by 43.5 g (0.2 mol) **2**. The reaction mixture was stirred and refluxed for 30 min. The solvent was evaporated and the residue dissolved in 300 ml 3% NaOH. The aqueous solution was extracted twice with ether and the pH adjusted to pH 4 with 4M HCl. 2-(2-Decynyl)-1,3-cyclohexadione (**3**) precipitated and was obtained in 65% yield (0.13 mol, 32.2 g).

5-Oxo-8-hexadecynoic acid (**4**). 32.2 g (0.13 mol) **3** was refluxed in 0.5M $Ba(OH)_2$ solution for 30 h. The solution was acidified and 5-oxo-8-hexadecynoic acid (**4**) extracted with ether. Recrystallisation of the acid from petroleum ether/ether 9:1 yielded 18.6 g (54% of theory) of the ketoacid. In mass spectroscopy fragments at $M^{\oplus}$ 266, *m/e* 167 $M^{\oplus} - [CH_2]_6-CH_3$, *m/e* 123 $CH_3-[CH_2]_6-C{\equiv}C^{\oplus}$ and the fragments at *m/e* 206, 194 and 60 originating from Mc Lafferty rearrangement were prominent.

$NaBH_4$-Reduction in methanol and esterification in methanolic HCl yielded *methyl 5-hydroxy-8-hexadecynoate* (**5**) the structure of which was proved by mass spectroscopy: prominent ions at $M^{\oplus}$ 282, *m/e* 264 $M^{\oplus} - H_2O$, *m/e* 208 originating from Mc Lafferty rearrangement.

Methyl 5-hydroxy-[8,9-$^3H_2$]palmitate (**6**). 1.0 g (3.5 mmol) **5** was dissolved in 10 ml dry ethyl acetate and catalytically reduced over PtO_2 in a tritium atmosphere. Specif. radioactiv. 820 Ci/mol.

Methyl 5-(methylsulfonyloxy)-[8,9-$^3H_2$]palmitate (**7**). 1.0 g (3.5 mmol) of the tritiated hydroxyester **6** dissolved in 20 ml absolute pyridine was reacted with 0.5 g (4.1 mmol) mesylchloride over 6 h at room temperature. The solvent was evaporated, 25 ml of water added and the reaction product extracted with ether.

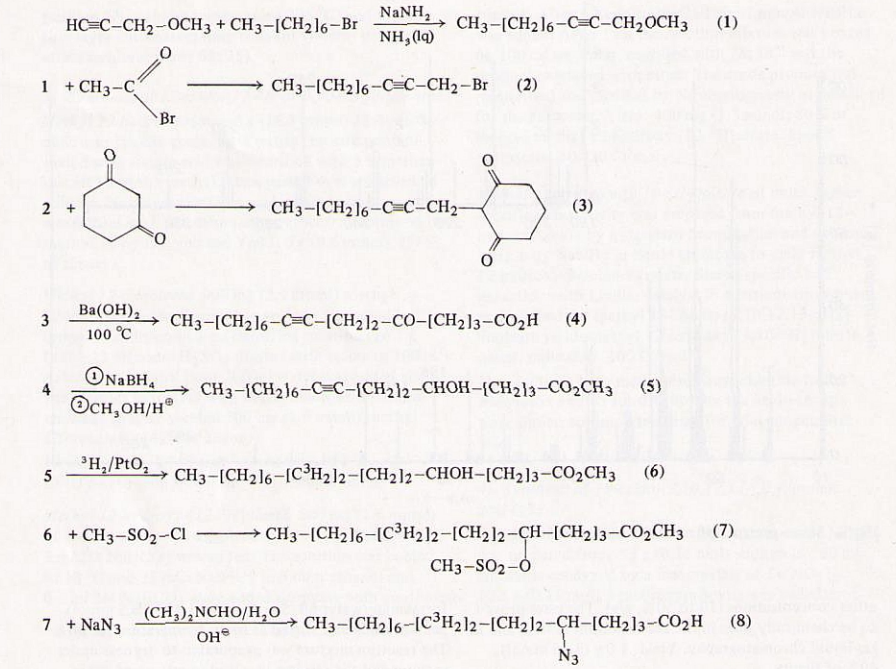


Fig. 1. Flow diagram of the chemical synthesis of 5-azido-[8,9-$^3H_2$]palmitic acid (8).

Methyl 5-azido-[8,9-$^3H_2$]palmitate (methyl ester of 8). 1.2 g of the crude product was immediately dissolved in 60 ml of a mixture of dimethylformamide/water 10:1 (v/v), 0.3 g (4.6 mmol) sodium azide was added and the mixture stirred for 48 h at room temperature with the exclusion of light. The solvent was evaporated and the product extracted with petroleum ether/ether 19:1. Yield: 0.53 g (1.7 mmol) methyl 5-azido-[8,9-$^3H_2$]-palmitate. The typical azido absorption band at 2120 cm^{-1} was present in the IR spectrum. In Fig. 2, the mass spectrum of methyl 5-azidopalmitate, the following typical fragmentation ions characterize the compound: *m/e* 282 $M^{\oplus} - (N_2 + H)$; *m/e* 269 $M^{\oplus} - N_3$; *m/e* 252 $M^{\oplus} - (N_2 + OCH_3)$; *m/e* 170 $M^{\oplus} - {}^{\bullet}CH_2-CH(N_3)-[CH_2]_3-CO_2CH_3$ (β-cleavage); *m/e* 156 α-cleavage product; *m/e* 74 originating from Mc Lafferty rearrangement.

5-Azido-[8,9-$^3H_2$]palmitic acid (8) was obtained almost quantitatively by alkaline hydrolysis of the methyl ester under standard conditions.

2) Synthesis of 16-azido-[9,10-$^3H_2$]palmitic acid

Methyl 16-hydroxypalmitoleate. 3.0 g (8.9 mmol) 16-bromopalmitoleic acid (CH-9470 Fluka) and 50 ml 20% KOH were refluxed for 72 h and the progress of the reaction followed by thin-layer chromatography (solvent system: petroleum ether/ether/acetic acid 70:30:1). The reaction mixture was cooled and acidified with 6M HCl and the 16-hydroxypalmitoleic acid extracted with ether. The combined ether extracts were washed with water and dried over Na_2SO_4. Evaporation of the solvent yielded 3.0 g of the crude acid, which was esterified with 5% methanolic HCl at 80 °C for 6 h.

The ester was purified by silicic acid chromatography with petroleum ether/ether mixtures with increasing

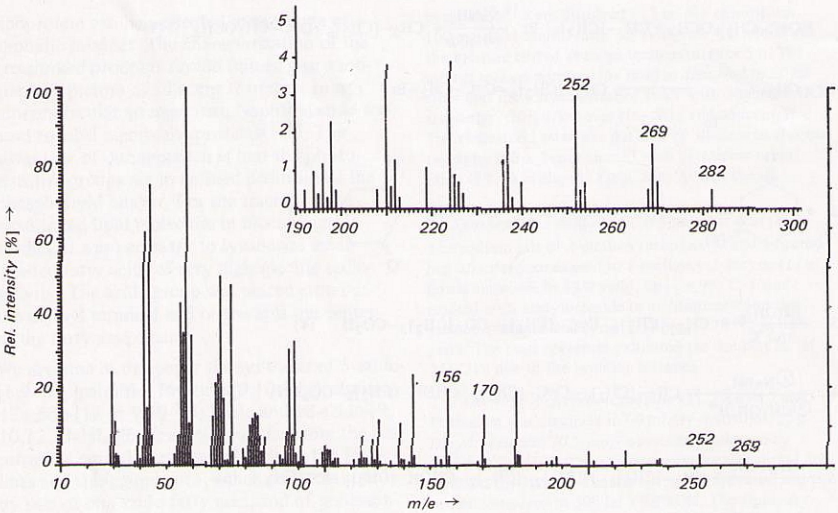


Fig. 2. Mass spectrum of methyl 5-azidopalmitate.

ether concentrations (10 to 50%, v/v). The ester proved to be chemically pure in the above solvent system and in gas-liquid chromatography. Yield: 1.0 g (3.65 mmol); 40% of theory.

Methyl 16-hydroxy-[9,10-$^3H_2$]palmitate. 1.00 g (3.52 mmol) methyl 16-hydroxypalmitoleate was dissolved in 25 m*l* ethyl acetate and catalytically (PtO_2) hydrogenated in an atmosphere of tritium. The ester proved to be radiochemically pure in thin-layer chromatography with dichloroethane as solvent. Yield: 0.97 g (3.42 mmol); (97% of theory); specif. radioactiv. 100 Ci/mol.

Methyl 16-(methylsulfonyloxy)-[9,10-$^3H_2$]palmitate. 0.97 g (3.42 mmol) methyl 16-hydroxy-[9,10-$^3H_2$]-palmitate was dissolved in 20 m*l* dry pyridine and 0.48 g (4.17 mmol) methanesulfonyl chloride was added. The mixture was stirred for 3 h at room temperature. Pyridine was evaporated at room temperature under vacuum and the residue extracted three times with ether. The combined ether extracts were washed with water, dried over Na_2SO_4 and concentrated to dryness at the rotary evaporator and finally at oil pump vacuum. Yield: 1.15 g (3.3 mmol); (97% of theory).

Methyl 16-azido-[9,10-$^3H_2$]palmitate. 1.15 g (3.3 mmol) of the above ester was dissolved in 60 m*l* of dimethylformamide/water 60:5 containing 1.1 g (16.5 mmol) sodium azide and stirred at room temperature for 60 h. The reaction mixture was evaporated to dryness under vacuum and the residue dissolved in water and ether. The ether extracts were thoroughly washed with water, dried over Na_2SO_4 and concentrated to dryness. Yield: 0.89 g (2.86 mmol); (87% of theory).

16-Azido-[9,10-$^3H_2$]palmitic acid. 0.89 g (2.86 mmol) of the methyl ester was hydrolyzed in 1M methanolic KOH for 24 h at room temperature. One volume of 2M HCl was added for acidification and the free acid extracted with ether. The extracts were washed with water, dried over Na_2SO_4 and the solvent evaporated. Yield: 0.85 g (2.88 mmol, 100% of theory). The strong absorption band at 2120 cm^{-1} in the IR spectrum represents the azido group. The following fragments can be assigned to the ions at *m/e* 283 $M^{\oplus} - N_2$; *m/e* 280 $M^{\oplus} - OCH_3$; *m/e* 252 $M^{\oplus} - (N_2 + OCH_3)$; *m/e* 129 $[CH_2]_5$-CO_2CH_3; *m/e* 74 originating from Mc Lafferty rearrangement (Fig. 3).

It should be noted that in ^{13}C-NMR-spectroscopy a strong down field shift of the C-16 (δ = 126.7 ppm) and of the C-15 resonance (21.2 ppm) is induced by the azido-substitution at C-16. The methyl ester proved to be homogenous in gas-liquid chromatography (1% SE 30,

purified N_2, column temperature 205 °C) and in radio thin-layer chromatography (solvent system: petroleum ether/dichloroethane 65 : 35).

3) Synthesis of 12-azido-[12-^{3}H or 9,10-$^3H_2$]oleic acid

Methyl 12-hydroxyoleate. 5 g (16.8 mmol) 12-hydroxyoleic acid (techn. grade, 80% purity) mostly contaminated with stearic acid was esterified with 5% methanolic HCl and the methyl esters passed over a silicic acid column. Non-hydroxy fatty acid methyl esters were eluted first with petroleum ether/ether 9 : 1 followed by methyl 12-hydroxyoleate. Yield: 3 g (9.6 mmol), (57% of theory).

Methyl 12-oxooleate. 900 mg (2.9 mmol) methyl 12-hydroxyoleate dissolved in acetone is reacted by dropwise addition of 1 ml oxidizing solution (26.7 g CrO_3, 23 ml conc. H_2SO_4 diluted with water to 100 ml). After an additional hour 200 ml water was added and the product extracted with ether. Silicic acid column chromatography yielded 500 mg (1.6 mmol) methyl 12-oxooleate (62% of theory).

IR absorption: 2740 cm^{-1} C=O ester; 2720 cm^{-1} C=O β-keto group; 730 cm^{-1} *cis*-double bond.

Methyl 12-hydroxy-[12-^{3}H]oleate. 500 mg (1.6 mmol) of the ketoester was dissolved in 30 ml methanol and 3 ml 2M $NaHCO_3$ was added. The solution was cooled to 10 °C and 25 mCi NaB^3H_4 in 5 ml methanol and 0.5 ml 2M $NaHCO_3$ were added dropwise with continuous stirring. After 3 h additional 250 mg (7 mmol) $NaBH_4$ was added. After 15 h the reaction mixture was poured on 100 ml ice water, acidified with 2M HCl and the product extracted with ether. The crude product was reesterified and purified by chromatography as described for the ketoester. Yield: 400 mg (1.3 mmol; 80% of theory) methyl 12-hydroxy-[12-^{3}H]oleate. Specif. radioactiv. 10–20 Ci/mol.

Methyl 12-hydroxy-[9,10-$^3H_2$]oleate of much higher specific radioactivity was prepared from methyl 12-hydroxyoleate by a standard bromination and debromination by $NaNH_2$ in liquid ammonia to yield methyl 12-hydroxy-9-octadecynoate. Stereo-specific half reduction with Lindlar catalyst in a tritium atmosphere as described for methyl 18-chloro-[9,10,12,13-$^3H_4$]-linoleate yields methyl 12-hydroxy-[9,10-$^3H_2$]oleate, specif. radioactiv. 100 Ci/mol.

The reactions with methanesulfonyl chloride to the mesylester and its substitution by the azido group were similar to those described for 16-azidopalmitic acid.

4) Synthesis of 18-azido-[9,10,12,13-$^3H_4$]linoleic acid (13)

8-Chloro-1-methoxy-2-octyne (9). A solution of $NaNH_2$ was prepared from 3.5 g (0.15 mol) sodium in 100 ml ammonia catalyzed by a few crystals of $Fe(NO_3)_3$. 10.5 g (0.15 mol) 3-methoxypropyne was added drop-

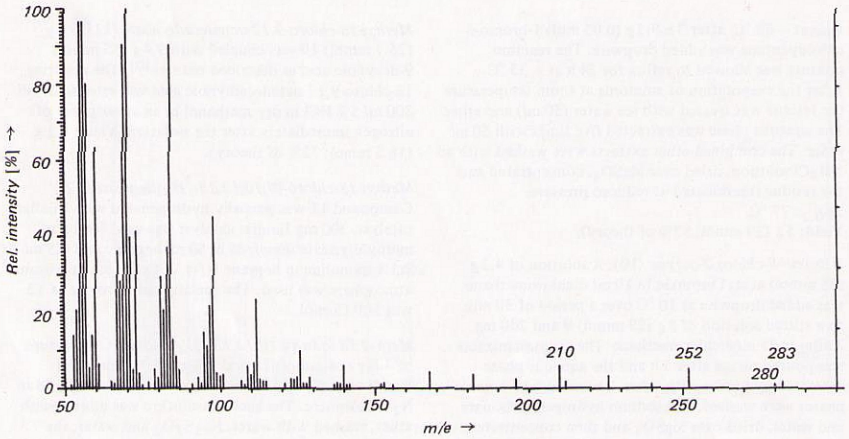


Fig. 3. Mass spectrum of 16-azidopalmitate.

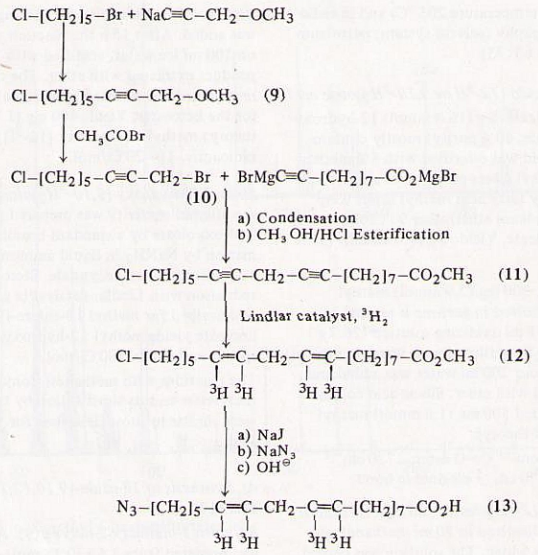


Fig. 4. Flow diagram of the chemical synthesis of 18-azido-[9,10,12,13-$^3H_4$]linoleic acid (**13**)

wise at – 68 °C, after 3 h 9.3 g (0.05 mol) 1-bromo-6-chloropentane was added dropwise. The reaction mixture was allowed to reflux for 24 h at – 33 °C. After the evaporation of ammonia at room temperature the residue was treated with ice water (50 m*l*) and ether. The aqueous phase was extracted five times with 50 m*l* ether. The combined ether extracts were washed with an NH_4Cl solution, dried over $MgSO_4$, concentrated and the residue fractionated at reduced pressure.
$Bp_{0.2}$: 77 °C.
Yield: 5 g (29 mmol; 57% of theory).

1-Bromo-8-chloro-2-octyne (**10**). A solution of 4.3 g (35 mmol) acetyl bromide in 10 m*l* dichloromethane was added dropwise at 10 °C over a period of 30 min to a stirred solution of 5 g (29 mmol) **9** and 200 mg $ZnBr_2$ in 25 m*l* dichloromethane. The reaction mixture was poured on ice after 5 h and the aqueous phase extracted five times with ether. The combined organic phases were washed with sodium hydrogen carbonate and water, dried over $MgSO_4$ and then concentrated. **10** distilled at $Bp_{0.2}$ 92–93 °C. Yield: 5.7 g (25.7 mmol; 90% of theory).

Methyl 18-chloro-9,12-octadecadiynoate (**11**). 5.7 g (25.7 mmol) **10** was coupled with 9.4 g (55 mmol) 9-decynoic acid as described before[10]. The resulting 18-chloro-9,12-octadecadiynoic acid was esterified with 300 m*l* 5% HCl in dry methanol in an atmosphere of nitrogen immediately after the isolation. Yield: 6.1 g (18.5 mmol; 72% of theory).

Methyl 18-chloro-[9,10,12,13-$^3H_4$]linoleate (**12**). Compound **11** was partially hydrogenated with Lindlar catalyst. 300 mg Lindlar catalyst was used for 1 g of methyldiynoate dissolved in 50 m*l* heptane and 0.5 m*l* 2.5% quinoline in heptane (v/v). A hydrogen or tritium atmosphere was used. The specific radioactivity of **12** was 500 Ci/mol.

Methyl 18-iodo-[9,10,12,13-$^3H_4$]linoleate. A mixture of 4.6 g (14 mmol) **11** and 2.3 g (15.5 mmol) NaI dissolved in 20 m*l* acetone was refluxed overnight in an N_2-atmosphere. The acetone solution was diluted with ether, washed with water, $Na_2S_2O_3$ and water, the organic phase dried over $MgSO_4$, concentrated and immediately used for the next step.

Methyl 18-azido-[9,10,12,13-$^3H_4$]linoleate. The crude iodo-compound was dissolved in 100 ml dimethylformamide, 70 mmol sodium azide was added and then the mixture stirred at room temperature for 20 h. 100 ml water was added and the product extracted with ether. The combined ether extracts were washed with a saturated NaCl solution and water, dried over $MgSO_4$ and concentrated.

18-Azido-[9,10,12,13-$^3H_4$]linoleic acid (**13**). The methyl ester was dissolved in 100 ml 5% methanolic KOH and stirred at room temperature with protection from light for 15 h. The solution was acidified with 2M HCl and the free acid extracted three times with ether. The ether extracts were washed with water, dried over Na_2SO_4 and concentrated.

The acid migrates as a single band in thin-layer chromatography (solvent system: petroleum ether (30–60 °C)/ether/acetic acid 70:30:1). Yield: 4 g (12.4 mmol; 90% of theory). The IR spectrum shows the strong absorption band at 2120 cm^{-1} of the azido group and the mass spectrum (Fig. 5) the following fragmentation ions: *m/e* 293 $M^{\oplus} - N_2$; *m/e* 206 $M^{\oplus} - (N_2 + [CH_2]_3{-}CO_2H)$; *m/e* 164 $M^{\oplus} - (N_2 + [CH_2]_6CO_2H)$; *m/e* 150 $M^{\oplus} - (N_2 + [CH_2]_7{-}CO_2H)$; *m/e* 110 $M^{\oplus} - (N_2 + CH_2{-}CH{=}CH{-}[CH_2]_7{-}CO_2H)$.

5) Synthesis of 18-aminostearic acid

Methyl 18-chlorostearate. 500 mg (1.5 mmol) methyl 18-chloro-9,12-octadecadiynoate was hydrogenated in a hydrogen atmosphere and PtO_2 as catalyst in quantitative yield.

Methyl 18-iodostearate resulted from the halogen exchange reaction analogous to the dienoic acid and *methyl 18-azidostearate* by the substitution of iodine against the azido group.

Methyl 18-aminostearate. 450 mg (1.3 mmol) methyl 18-azidostearate was dissolved in 25 ml ethyl acetate and reduced in the presence of 100 mg PtO_2 to the amino-ester. Yield: 420 mg (1.1 mmol; 92% of theory).

6) Synthesis of methyl 18-[1-hexyl-11-(methoxycarbonyl)undecylamino]stearate (dimethyl 12,18'-imidodistearate)

200 mg (0.64 mmol) methyl 18-aminostearate and 300 mg (0.96 mmol) methyl 12-oxostearate were dissolved in 20 ml absolute toluene, about 50 mg *p*-toluenesulfonic acid added and refluxed for 2 h using a water separator. The Schiff base remaining after the evaporation of the solvent was dissolved in methanol and catalytically reduced with PtO_2 and H_2 or $NaBH_4$ to the secondary amine. The crosslinking product was purified by thin-layer chromatography. Excess methyl 12-oxostearate separates from the reaction product in the solvent system methylene chloride (R_F = 0.8). The product remained at the origin but migrated in the solvent system methylene chloride/methanol 8:2 with R_F = 0.6. The product was eluted from the silica gel for further characterization by mass spectroscopy.

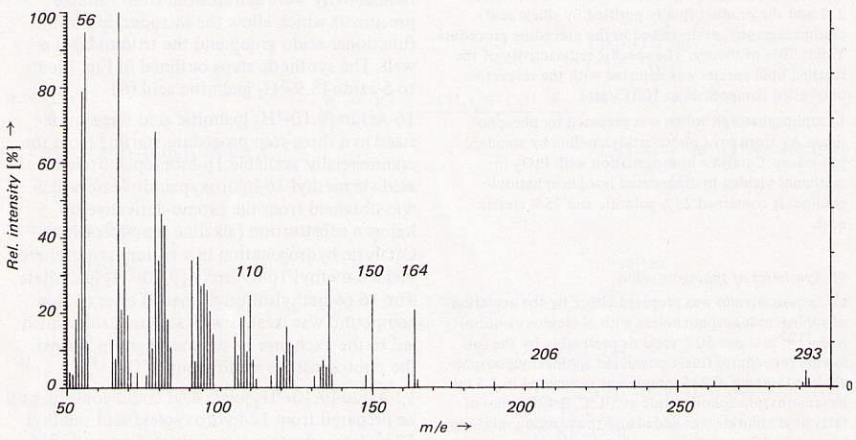


Fig. 5. Mass spectrum of 18-azidolinoleic acid.

7) General procedure for the synthesis of symmetrical phosphatidylcholines

Finely powdered 3-*sn*-glycerophosphocholine (1 mmol) was suspended in 5 ml hexamethylphosphotriamide[11] and heated to 50 °C in a Sovirel tube with magnetic stirring. A four molar excess of acyl chloride was added and the reaction mixture stirred at 50 °C overnight under a nitrogen atmosphere in the closed reaction vessel. After this time the reaction was completed as demonstrated by thin-layer chromatographic analysis (solvent system: chloroform/methanol/water 65 : 25 : 4). The mixture was then immediately purified by silicic acid column (2.5 × 30 cm) chromatography, equilibrated with chloroform. Excess fatty acid, acyl chloride and triamide were eluted with chloroform and the phosphatidylcholine subsequently with chloroform/methanol mixtures 3 : 1 and 1 : 1. Yields ranged between 60–90% of theory. The labelled fatty acids could be recovered from the chloroform phase by alkaline hydrolysis and acidification.

8) General acylation procedure for the synthesis of asymmetrical phosphatidylcholines

0.2 mmol 2-Lysophosphatidylcholine [1-palmitoyl- or (1-stearoyl)glycero-3-phosphocholine] and 0.8 mmol 4-(dimethylamino)pyridine[7,8] were dissolved in 8 ml dry chloroform and 0.3 mmol fatty acyl chloride dissolved in 2 ml dry chloroform was added. The mixture was stirred at room temperature for 15 h. The reaction mixture was washed with 50 ml 2M HCl and water and the solvent evaporated under vacuum, traces of water were azeotropically evaporated with benzene/ethanol 2 : 1 and the product finally purified by silicic acid chromatography, as described in the preceding procedure. Yield: 70% of theory. The specific radioactivity of the labelled lipid species was adjusted with the respective unlabelled compounds to 100 Ci/mol.

Lysophosphatidylcholine was prepared by phospholipase A_2 from soya phosphatidylcholine by standard procedure. Catalytic hydrogenation with PtO_2 in methanol yielded hydrogenated lysophosphatidylcholine. It contained 25% palmitic and 75% stearic acid.

9) Syntheses of sphingomyelins

a) *Sphingomyelin* was prepared either by the acylation of sphingenylphosphocholine with *N*-(acyloxy)succinimide[12] in about 50% yield or preferably by the following procedure: finely powdered sphingenylphosphocholine (100 mg, 0.215 mmol) was suspended in 1.5 ml hexamethylphosphotriamide at 50 °C. 0.430 mmol of fatty acyl chloride was added and the reaction mixture kept at 50 °C with stirring until no more sphingenylphosphocholine could be detected by thin-layer chromatography (solvent system: chloroform/methanol/water 65 : 25 : 4).

The products were isolated as described for phosphatidylcholine, followed by a mild alkaline hydrolysis (0.5M methanolic KOH, 37 °C, overnight) to hydrolyze 3-*O*-acyl sphingomyelin. Sphingomyelin was purified by silicic acid chromatography and obtained in 70–80% yield.

b) *Sphingenylphosphocholine* was obtained by acid hydrolysis (butanol/HCl) of sphingomyelin from beef brain[13]. 98% of the long chain bases was sphingenine.

Liposomes were prepared by concentrating the benzene solution of the phospholipid (5 mg/ml) in a stream of nitrogen. The lipid film was suspended by Vortex mixing in 0.025M Tris/HCl buffer, pH 7.4 and ultrasonication with the macrotip of the Branson sonifier at 80 W for 30 min in a temperature-controlled vessel (4 °C) in a nitrogen atmosphere.

The *photochemical reaction* was carried out with a high pressure UV-lamp HPK 125 W, Philips. The radiation below 310 nm was absorbed by a Pyrex filter. It was essential that the solution was deaerated by alternating evacuation and flushing with purified argon gas.

Results

Synthesis of azido-labelled radioactive fatty acids and phospholipids

Four azido-labelled fatty acids of high tritium radioactivity were synthesized from suitable precursors which allow the introduction of the functional azido group and the tritium label as well. The synthetic steps outlined in Fig. 1 led to 5-azido-[8,9-$^3H_2$]palmitic acid (8).

16-Azido-[9,10-$^3H_2$]palmitic acid was synthesized in a three-step procedure starting from the commercially available 16-bromopalmitoleic acid via methyl 16-hydroxypalmitoleate which was obtained from the bromo-derivative by halogen substitution (alkaline saponification). Catalytic hydrogenation in a tritium atmosphere yielded methyl 16-hydroxy-[9,10-$^3H_2$]palmitate. The 16-*O*-methylsulfonyl (mesyl) ester of this compound was treated with sodium azide which led to the exchange of the mesyl group against the photosensitive azido group.

12-Azido-[9,10-$^3H_2$]oleic acid could conveniently be prepared from 12-hydroxyoleic acid: methyl 12-hydroxyoleate was brominated to methyl 9,10-dibromo-12-hydroxystearate, which was

debrominated with $NaNH_2$ in liquid ammonia to methyl 12-hydroxy-9-octadecynoate. Half hydrogenation with Lindlar catalyst in a tritium atmosphere yielded methyl 12-hydroxy-[9,10-$^3H_2$]-oleate, with PtO_2 methyl 12-hydroxy-[9,10-$^3H_4$]-stearate. The hydroxy groups of the two intermediates were transformed into the mesyl ester which reacted with sodium azide to methyl 12-azido-[9,10-$^3H_2$]oleate and methyl 12-azido-[9,10-$^3H_4$]stearate.

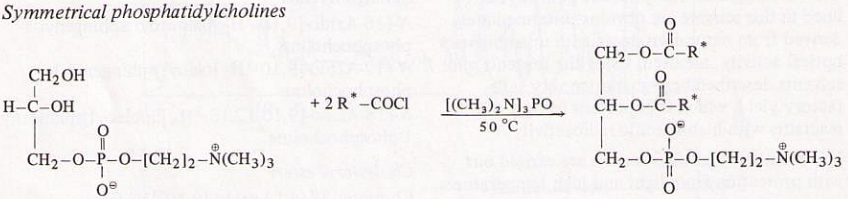
18-Azido-[9,10,12,13-$^3H_4$]linoleic acid (**13**) required the synthetic steps described previously for radioactive normal polyunsaturated fatty acids[10] except that the acetylenic precursor corresponding to the methyl end of the fatty acid was 8-chloro-1-methoxy-2-octyne (**9**) instead of 1-bromo-2-octyne. 9-Decynoic acid was the other reactant. The reaction steps are summarized in Fig. 4.

The structures of these azido-labelled fatty acids were proven by their mass spectra (Figs. 2, 3 and 5). Furthermore the strong bands at 2120 cm^{-1} of the IR spectra proved the presence of the azido groups.

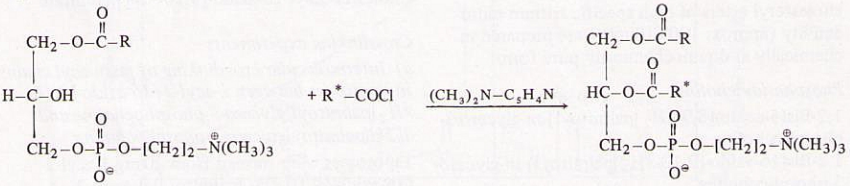
Chemical syntheses of photoactivatable phosphatidylcholines, sphingomyelins and cholesteryl esters

The synthesis of 25-azido-[25-^{3}H]27-norcholesterol has been described before[6]. The chemical synthesis of phosphatidylcholines, sphingomyelin and cholesteryl esters, substituted with the tritium-labelled photosensitive fatty acids (R*-COCl), are outlined in the following schemes.

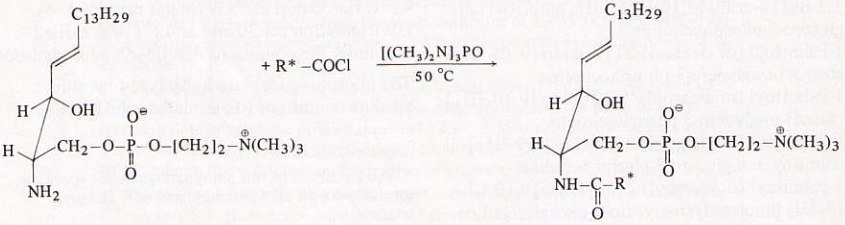
Symmetrical phosphatidylcholines



Asymmetrical phosphatidylcholines[7,8]

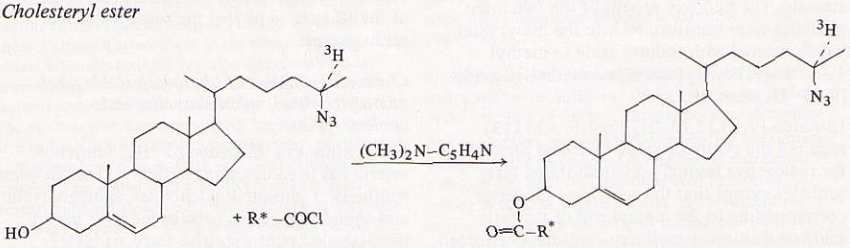


Sphingomyelins



The procedures described here are superior to any previously recommended acylation reaction. The esterification proceeds with retention of the optical center as proven by the complete hydrolysis of the ester bond in position 2 by pancreatic phospholipase A_2 (unpublished results).

Cholesteryl ester



The advantages of the synthetic pathways outlined in this scheme are obvious: intermediates, derived from natural products with unambiguous optical activity, are used. Using the reagents and solvents described here guarantee very satisfactory yield, which is important in view of the reactants with high specific radioactivity.

It is essential, that all reactions are carried out with protection from light and high temperatures.

The following photosensitive phosphatidylcholines, sphingomyelins and cholesteryl or 27-norcholesteryl esters of high specific tritium radioactivity (approx. 100 Ci/mol) were prepared in chemically and radiochemically pure form:

Phosphatidylcholines

1,2-Bis(5-azido-[8,9-$^3H_2$]palmitoyl)-*sn*-glycero-3-phosphocholine
1,2-Bis(16-azido-[9,10-$^3H_2$]palmitoyl)-*sn*-glycero-3-phosphocholine
1,2-Bis(12-azido-[9,10-$^3H_2$]oleoyl)-*sn*-glycero-3-phosphocholine
1,2-Bis(18-azido-[9,10,12,13-$^3H_4$]linoleoyl)-*sn*-glycero-3-phosphocholine
1-Palmitoyl (or stearoyl)-2-(12-azido-[9,10-$^3H_2$]-stearoyl-*sn*-glycero-3-phosphocholine
1-Palmitoyl (or stearoyl)-2-(12-azido-[9,10-$^3H_2$]-oleoyl)-*sn*-glycero-3-phosphocholine
1-Palmitoyl (or stearoyl)-2-(5-azido-[8,9-$^3H_2$]-palmitoyl)-*sn*-glycero-3-phosphocholine
1-Palmitoyl (or stearoyl)-2-(18-azido-[9,10,12,-13-$^3H_4$]linoleoyl)-*sn*-glycero-3-phosphocholine

Sphingomyelins

N-(16-Azido-[9,10-$^3H_2$]palmitoyl)sphingenyl-1-phosphocholine
N-(12-Azido-[9,10-$^3H_2$]oleoyl)sphingenyl-1-phosphocholine
N-(18-Azido-[9,10,12,13-$^3H_4$]linoleoyl)sphingenyl-1-phosphocholine

Cholesteryl esters

Cholester-3β-yl 12-azido-[9,10-$^3H_2$]oleate
25-Azido-[25-^{3}H]27-norcholester-3β-yl oleate
Cholester-3β-yl 16-azido-[9,10-$^3H_2$]palmitate

Crosslinking experiments

a) Intermolecular crosslinking of fatty acyl chains in liposomes between 1-acyl-2-(16-azido-[9,10-$^3H_2$]palmitoyl)glycero-3-phosphocholine and 1,2-dipalmitoylglycero-3-phosphocholine

Liposomes were formed from 20 mg 1-acyl-2-(16-azido-[9,10-$^3H_2$]palmitoyl)glycero-3-phosphocholine* and 100 mg 1,2-dipalmitoylglycero-3-phosphocholine by sonication at 42 °C for 30 min at 75 W (molar ratio of 1 : 5). UV irradiation for 30 min at 42 °C was carried out under the conditions described under Methods.

The phospholipids were hydrolyzed by mild alkaline conditions (0.5 M methanolic KOH at

* The 1-position of this phosphatidylcholine species is substituted with 20% palmitoyl and 80% stearoyl residues.

37 °C overnight). One portion of the fatty acid mixture was esterified with methanolic HCl, the other transformed into the 4-bromophenacyl esters with crown ether or triethylamine in acetone as catalysts[14].

The complete mixture of *4-bromophenacyl esters* was separated by reversed phase (RP 18) high-performance liquid chromatography (column dimensions 0.4 x 50 cm and 0.8 x 30 cm) with a linear gradient of acetonitrile in water (80–100%

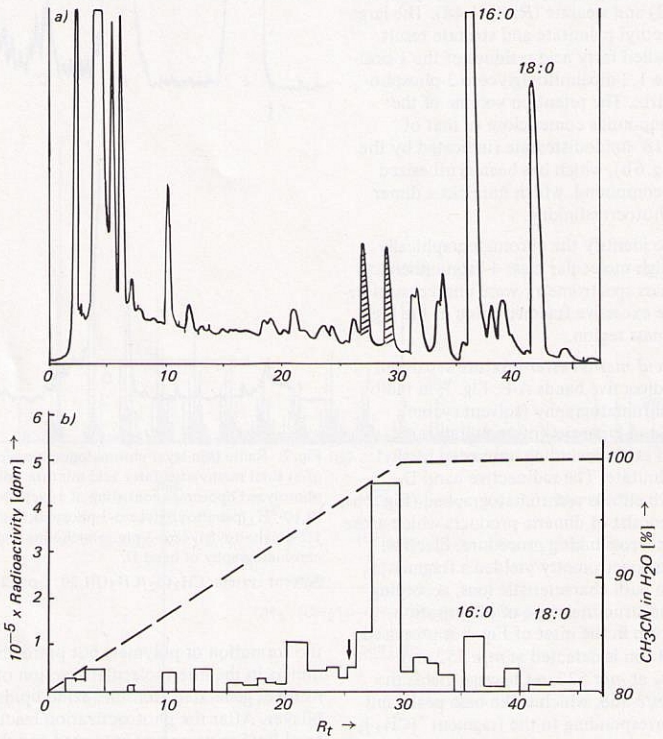


Fig. 6. High-performance liquid chromatographic separation of bromophenacyl esters of the fatty acid mixture isolated from liposomes consisting of 1-acyl-2-(16-azido-[9,10-$^3H_2$]-palmitoyl)glycero-3-phosphocholine and 1,2-dipalmitoylglycero-3-phosphocholine after irradiation.

The liposomes were hydrolyzed under alkaline conditions and the free acids esterified with *p*-bromophenacyl-bromide and 16-crown ether as catalyst. The arrow indicates retention time of synthetic product dimethyl 12,18'-imidodistearate ($NaBH_4$-reduced Schiff base between methyl 18-aminostearate and methyl 12-oxostearate). Conditions: RP 18 column (0.4 x 50 cm); acetonitrile/water gradient; flow rate 1.5 ml/min.

a) Absorbance at 280 nm. b) Fractions were collected and aliquot samples analyzed for radioactivity. Radioactive bands (hatched areas) at R_t 26.87 and 28.81.

CH_3CN) as indicated in Fig. 6. Fig. 6b presents the distribution of the radioactivity in the fractions. The radioactivity injected on the column was completely recovered and concentrated mainly in bands with R_t 26.87 and 28.81 (hatched areas of Fig. 6a). The radioactivity eluted before methyl 16-azidopalmitate (R_t = 33.71), palmitate (R_t = 36.12) and stearate (R_t = 41.48). The large bands of methyl palmitate and stearate result from unlabelled fatty acid residues of the 1-position and the 1,2-dipalmitoylglycero-3-phosphocholine matrix. The retention volume of the labelled compounds comes close to that of methyl 12,18′-imidodistearate (indicated by the arrow in Fig. 6b), which has been synthesized as a model compound, which mimicks a dimer arisen by photocrosslinking.

Attempts to identify the chromatographically separated high molecular mass 4-bromophenacyl esters by mass spectrometry were unsuccessful because of the excessive fragmentation in the high molecular mass region.

The *fatty acid methyl ester* mixture separated into five radioactive bands A-E, Fig. 7, in radio thin-layer chromatography (solvent system: CH_2Cl_2). Band E consists of unaltered fatty acid methyl esters including unreacted methyl 16-azidopalmitate. The radioactive band D (Fig. 7a), which was rechromatographed (Fig. 7b), proved to consist of dimeric products which arose by the photocrosslinking procedure. Electron impact mass spectrometry yielded a fragmentation pattern with characteristic ions, according to which the structural type of dimerisation products given in the inset of Fig. 8 is proposed. The $M^{\oplus} - 1$ ion is detected at m/e 552 and $M^{\oplus} - OCH_3$ at m/e 522. α-Cleavage yields the fragments m/e 368, which is the base peak, and m/e 185 corresponding to the fragment $^{\cdot}[CH_2]_9\text{-}CO_2CH_3$.

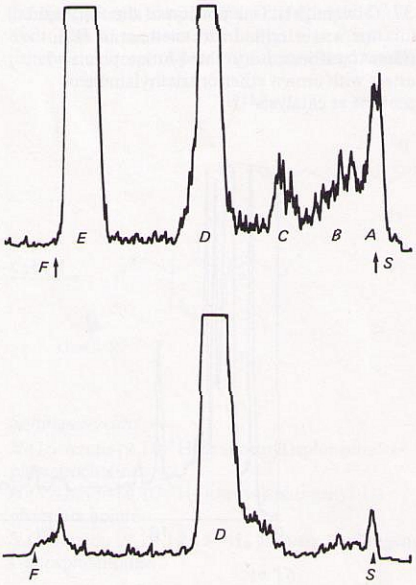


Fig. 7. Radio thin-layer chromatographic separation of a) total methylated fatty acid mixture isolated from photolyzed liposomes consisting of 1-acyl-2-(16-azido-[9,10-$^3H_2$]palmitoyl)glycero-3-phosphocholine and 1,2-dipalmitoylglycero-3-phosphocholine and b) rechromatography of band D.
Solvent system: CH_2Cl_2/CH_3OH 20:1 or CH_2Cl_2.

b) Intermolecular crosslinking in liposomes of fatty acyl chains between 1,2-bis(18-azido-[9,10,12,13-$^3H_4$]linoleoyl)glycero-3-phosphocholine and 1,2-dilinoleoylglycero-3-phosphocholine

Mixed vesicles consisting of 1,2-bis(18-azidolinoleoyl)glycero-3-phosphocholine and 1,2-dilinoleoylglycero-3-phosphocholine in a molar ratio of 1:10 were prepared in order to prevent the formation of polymers but preferably form dimers in the intermolecular reaction of the nitrenes generated from the azido lipids in the bilayer. After the photoactivation reaction the total lipid mixture was extracted and the fatty acid methyl esters were formed by transesterification with methanolic HCl.

The products were catalytically reduced in order to obtain easily interpretable mass spectra. Fig. 9 demonstrates that an almost complete photoactivation with crosslinking must have occurred because no radioactive bands corresponding to fatty acid methyl esters were detectable in the radio thin-layer chromatogram, which should have the R_F value of spot 3 corresponding to

the normal fatty acid methyl esters. Two major radioactive bands (1 and 2) were isolated and analysed by mass spectroscopy. Whereas band 1 represented higher polymers with no conclusive mass spectral informations, band 2 was characterized by the mass spectrum given in Fig. 10. The fragmentation pattern of this mass spectrum agrees with each of the four possible reaction products shown in Fig. 11.

They all have a molecular mass of either 609, if the nitrene has inserted into a C-C or C-H bond or 607, if an aziridine has been formed. A high resolution mass analysis has been recorded for *m/e* 608 ($M^{\oplus} - 1$). The sum formula of

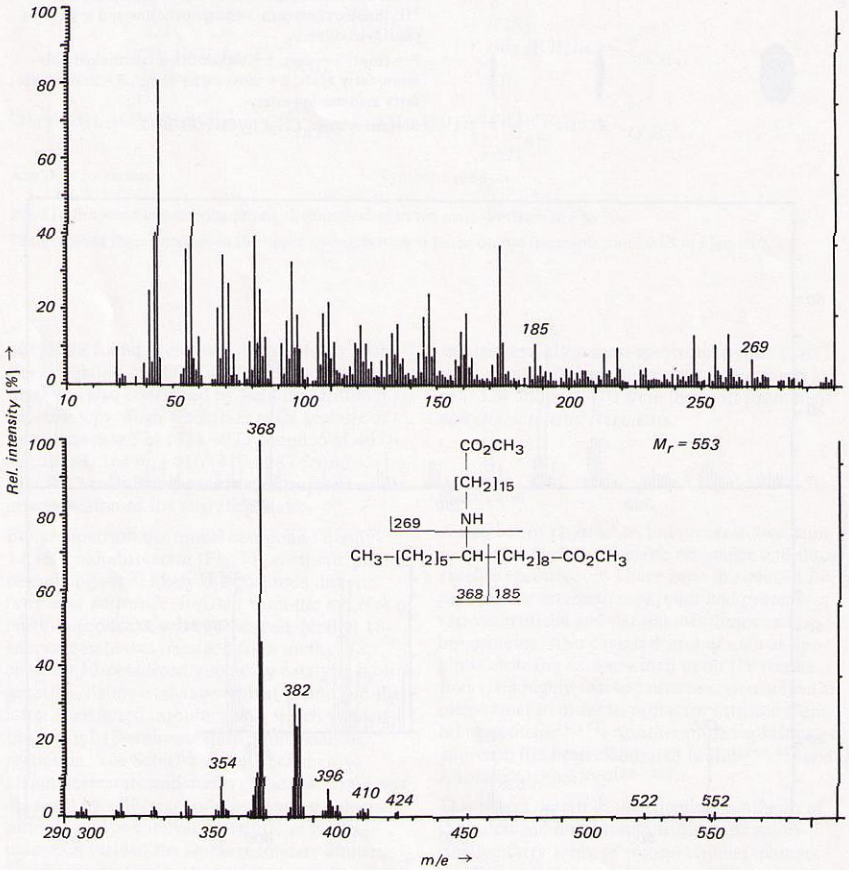


Fig. 8. Mass spectrum of methyl ester fraction D (crosslinking products) isolated and purified by thin-layer chromatography.

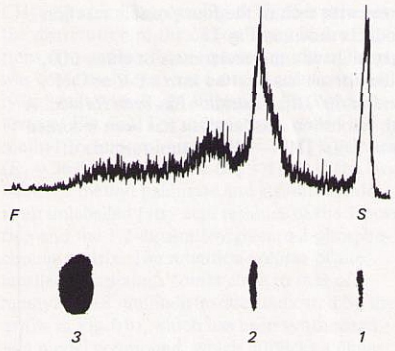


Fig. 9. Radio thin-layer chromatogram of hydrogenated fatty acid methyl esters isolated from photolyzed liposomes consisting of 1-acyl-2-(18-azido-[9,10,12,13-$^3H_4$]linoleoyl)glycero-3-phosphocholine and soya phosphatidylcholine.

F = front, S = start, 1 = unidentified crosslinked polymeric fatty acids, 2 = crosslinked dimer, 3 = monomeric fatty acid methyl ester.

Solvent system: CH_2Cl_2/CH_3OH 20:1.

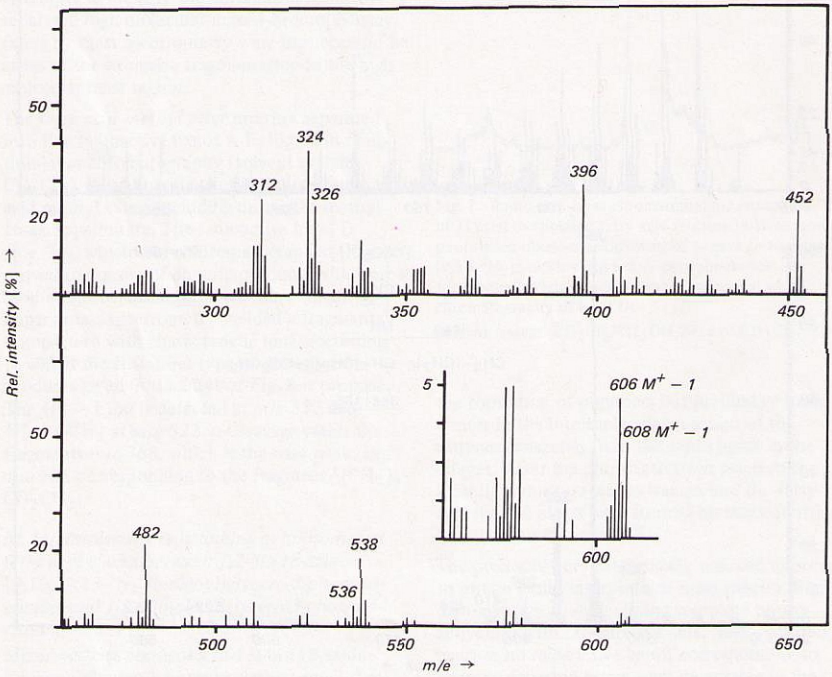


Fig. 10. Mass spectrum of crosslinked catalytically hydrogenated fatty acid methyl esters gained from Band 2 in Fig. 9.

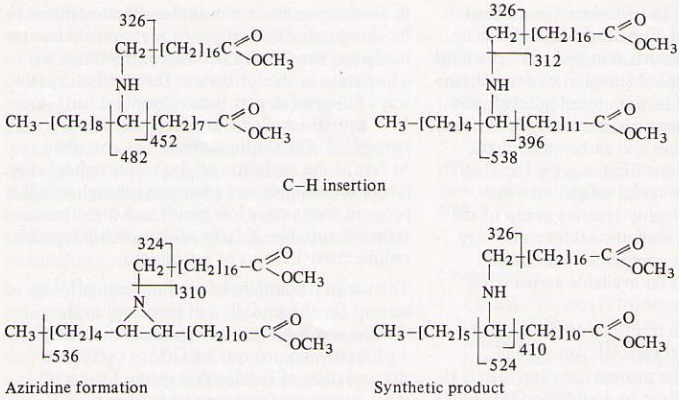


Fig. 11. Proposed crosslinking products consistent with the mass spectrum in Fig. 10.
The proposed fragmentation in the upper two structures is based on the fragments numbered in Figure 10.

608.5598 found agrees very satisfactorily with the calculated one 608.5617. This molecular mass was also confirmed by field desorption mass spectroscopy. High resolution mass analysis of fragments *m/e* 524 (524.4673 found; 524.4678 calculated) and *m/e* 410 (410.3987 found; 410.3987 calculated) gave further support of the interpretation of the analytical data.

For comparison the model compound methyl 12,18′-imidodistearate (Fig. 11, synthetic product) resembling very closely the proposed dimeric fatty acid derivative, isolated from the mixture of reaction products, was synthesized. Methyl 18-aminostearate was obtained from methyl 18-chloro-9,12-octadecadiynoate by catalytic hydrogenation, halide exchange against iodine and the latter transferred into the azide, which yielded the methyl 18-aminostearate upon catalytic reduction. The Schiff base between methyl 18-aminostearate and methyl 12-oxostearate was obtained by refluxing in toluene and *p*-toluenesulfonic acid as catalyst. Catalytic or $NaBH_4$-reduction yielded the stable secondary amine. The fragmentation pattern of this compound was very similar to those presented in Fig. 11 for the proposed crosslinking products.

In high resolution mass spectroscopy the mass peak at *m/e* 609 and the α-cleavage products at *m/e* 524 and *m/e* 410 were the most prominent and characteristic fragments.

Discussion

A number of physical techniques (electron spin resonance, nuclear magnetic resonance and fluorescence spectroscopy) have been introduced for probing the interaction of lipids and proteins in various artificial and natural membranes and lipoproteins. Also chemical probes such as lipophilic aromatic azides, which upon UV irradiation yield highly reactive nitrenes, were added to membranes in order to probe for intrinsic membrane proteins[5,15]. Another more sophisticated approach has been elaborated in this[16–22] and in Khorana's laboratory[23–26].

This report describes the chemical syntheses of saturated and unsaturated tritium and azido-labelled fatty acids, of phospholipids, sphingomyelins and cholesteryl esters substituted with these photoactivatable acyl residues and of 25-azido-[25-^{3}H]27-norcholester-3β-yl[6] oleate.

The azido group can be introduced in most of the desired positions of the fatty acyl chains by chemical synthesis, which thus become "chemical rulers" of the hydrophobic region of a membrane and therefore useful tools in topological studies of membrane and lipoprotein structures. Different from aromatic nitrenes and carbenes aliphatic nitrenes have very short life times ($\sim 10^{-11}$ s)[2] and should therefore reveal insight into the environment of the highly reactive group of the lipid molecule. Also aliphatic azides cause very little perturbation as compared to aromatic nitrenes and most so far available carbene precursors.

The literature on the reactions of aliphatic nitrenes is rather controverse[1]. Among the possible reactions of a nitrene insertion into C-H and C-C bonds, addition to double bonds yielding aziridines, hydrogen abstraction and dimerisation, it has been reported that aliphatic nitrenes would not undergo the insertion reactions. This statement however has been made for gas phase reactions and in solution. The systems described in this paper and the general field of application in membrane and lipoprotein structural analysis deals with two phase system of rather high order, in which the azido group is emburried in the hydrocarbon environment of the lipid bilayer or adjacent to hydrophobic sequences of polypeptide chains.

Besides the chemical syntheses of some useful azido fatty acids and new procedures for their chemical introduction into phospholipids and sphingolipids, chemical and mass spectroscopic evidence is presented for crosslinking between phospholipid molecules in a lipid bilayer (liposomes) consisting of saturated [1,2-dipalmitoylglycero-3-phosphocholine and 1-acyl-2-(16-azidopalmitoyl)glycero-3-phosphocholine] and unsaturated phospholipids [1-acyl-2-(18-azidolinoleoyl)glycero-3-phosphocholine and polyenephosphatidylcholine]. Furthermore, crosslinking products between the fatty acyl chains of phosphatidylcholine were isolated and characterized by mass spectroscopy.

The synthesis of the azido-substituted saturated, mono- and polyunsaturated fatty acids followed standard procedures elaborated in this field over the past years. The photosensitive precursor had to be labelled with high specific radioactivity (^{3}H) in an inexpensive way. Suitable precursors had to be chosen, mostly olefinic or acetylenic intermediates, which were tritiated catalytically at a late stage in the syntheses. The synthetic pathways followed in part those described for ^{13}C-, ^{14}C- and ^{3}H-labelled fatty acids described before[10]. The synthetic reactions described so far for the acylation of glycerophosphocholine, lysophospholipids and sphingosylphosphocholine proceed with rather low yields and therefore are rather unsuitable, if fatty acids with high specific radioactivity have to be introduced.

The use of hexamethylphosphotriamide[11] as solvent for the acylation of glycerophosphocholine and sphingosylphosphocholine and of 4-(dimethylamino)pyridine[7,8] as catalyst in the acylation of lysolecithin, proved to be of great advantage for these and further studies.

In this study, mass spectroscopic evidence is also presented for the intermolecular crosslinking between photoactivated azido-labelled fatty acids and acyl chains of the same molecule or neighbouring phospholipid molecules.

The crosslinking of aliphatic azides upon photoactivation has been interpreted as a radical reaction with unsaturated fatty acyl chains and not nitrene reaction[15,27] by C-H insertion[16,17] into the aliphatic chains. The experiments and analytical data reported here clearly prove that both saturated and unsaturated aliphatic azido-substituted fatty acids in phospholipids cross-react in yields up to 50%. Yields have been derived from the analysis in high-performance liquid and thin-layer chromatography.

Two systems were studied: a) phosphatidylcholine substituted with 16-azidopalmitic acid in 1,2-dipalmitoylglycero-3-phosphocholine vesicles, a fully saturated system, and b) phosphatidylcholine substituted with 18-azidolinoleic acid in vesicles of soya phosphatidylcholine, a highly unsaturated phospholipid matrix.

The decomposition of the aliphatic azido group occurred with high yield under the irradiation conditions used in the experiments described.

16-Azidopalmitic acid in 1,2-dipalmitoylglycero-3-phosphocholine vesicles reacted upon photoactivation up to 50% with intra- and intermolecular crosslinking and no starting material was

detected with 18-azidolinoleic acid as photoactivatable group in the vesicles. The analysis of the reaction products was facilitated because of the radio-labelled probes. For mass spectrometry the fatty acyl chains, monomeric or dimerized by photocrosslinking, were hydrolyzed from the glycerophosphocholine backbone, their methyl esters prepared and separated by thin-layer chromatography into monomeric, unaltered fatty acid methyl esters, dimeric and, to a small extent, high molecular mass polymeric crosslinking products.

In the fully saturated system with 16-azidopalmitate as photoactivatable probe, mass spectrometric data of the main crosslinked fatty acid derivates (Fig. 7, band D) shown in Figure 8 were obtained which clearly lend support for dimeric structure between a photoactivated 16-azidopalmitic acid and a neighbouring palmitoyl chain. The $M^{\oplus} - 1$ ion at 552 is observed and the characteristic α-cleavage fragments at m/e 354, 368, 382, 396, 410 indicate that a nitrene C-H insertion with crosslinking had occurred mainly towards the ω-end of the neighbouring palmitoyl chain, indicated in the insert of Figure 8.

The region of carbon atom 11 of a palmitoyl chain appears to be the predominant neighbour of the photoactivatable 16-azido group of 1-acyl-2-(16-azidopalmitoyl)glycero-3-phosphocholine in the liposomes above the phase transition temperature. The reasonable assumption is made that the fragmentation energy for α-cleavage of homologous residues ($^{\cdot}[CH_2]_n$-CO_2CH_3) is alike. Other crosslinks by C-H insertions with carbon atoms (C-12 to C-15) towards the methyl end are also indicated by fragments m/e 382, 396 and 410.

The same conclusion can be drawn from the unsaturated system with 18-azidolinoleate as acyl group in phosphatidylcholine and a highly unsaturated phosphatidylcholine matrix.

The molecular mass of the dimerization products and their main characteristic fragments have been analyzed by high resolution electron impact mass spectrometry. All data allow only the structure of a product which is formed upon C-H insertion or aziridine formation by nitrene addition to a double bond.

The subsequent paper[22] describes the application of these photoactivatable lipid probes in studies on lipid-protein interactions exemplified with high-density apolipoproteins and ^{3}H-labelled azidophosphatidylcholine, pursuing previous studies in more detail[16–21].

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