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Methods of synthesis of deuterium-labelled lipids

N A Bragina, V V Chupin

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Abstract. The review deals with the methods of synthesis of deuterium-labelled hydrophobic and hydrophilic synthons of lipid molecules and the routes to selectively and perdeuterated phospholipids and their analogues. Deuterium-labelled lipids are used to study the structural organisation and functioning of biological membranes including NMR studies and neutron diffraction of lipid – lipid and lipid – protein interactions. The bibliography includes 145 references.

I. Introduction

The explosive development of molecular biology, particularly of biomembranology, has stimulated research aimed at the synthesis of deuterated lipids. The preparation of selectively and perdeuterated lipids has permitted studies of structural organisation and functioning of biological membranes. Deuterium can be selectively incorporated into any methyl, methylene, or methine group of the lipid molecule. In these transformations, the properties of lipid molecules remain unchanged, which makes the deuterium label superior to spin or fluorescent labels.

Originally, the deuterium-labelled lipids were used only in mass spectrometry for the analysis of pathways of fragmentation of lipid molecules.[1,2] In the 70 – 80's, novel procedures for the study of the structural organisation of membranes were developed, such as solid-state ^{2}H NMR[3–6] and neutron diffraction.[7–9]

However, these methods are not very sensitive and require large quantities of lipid samples (tens and even hundreds of milligrams). Thus, the development of convenient (in the preparative sense) methods of synthesis of deuterium-labelled lipids that would permit preparation of these lipids in the amounts required is still urgent.

Another trend in the use of deuterated lipids is related to studies into the structure of membrane proteins. Secondary and tertiary structures of membrane proteins are determined by the traditional method of high-resolution two-dimensional ^{1}H NMR spectroscopy.[10] Obviously, such studies require perdeuterated detergents that are 'transparent' for the ^{1}H NMR method. Organic solvents or micelles obtained from lipid-like detergents are generally used as the membrane environment.[11–14] Yet no ^{1}H NMR studies into the structure of membrane proteins in lipid bilayers have thus far been carried out because, in contrast to small-sized micelles, the movement in bilayers is anisotropic, which results in significant broadening of signals in the NMR spectra. The principal conditions for the study of secondary and tertiary structures of proteins in lipid bilayers have now been established. The possibility of obtaining high-resolution two-dimensional ^{1}H NMR spectra of lipid bilayers using the magic-angle spinning method has been demonstrated.[15,16] Studies into the structure of intramembrane proteins are being scheduled. The accessibility of perdeuterated lipids is a prerequisite for conducting these studies.

The present review describes the methods of synthesis of deuterium-labelled hydrophobic and hydrophilic precursors of lipid molecules and of selectively perdeuterated phospholipids and their analogues.

The methods for the incorporation of deuterium are based on the use of commercially available deuterium-containing chemical reagents,[17] such as heavy water (D_2O), alcohols (MeOD), acids (D_2SO_4), or bases (NaOD). Enolisable protons of lipids can be specifically exchanged for deuterium under conditions of acid or base catalysis. Molecular deuterium in combination with platinum or palladium catalysts and Raney nickel is mostly employed for conducting a non-specific exchange.

$LiAlD_4$, $NaBD_4$ and their derivatives are most often used for the specific reduction of particular functional groups. The use of molecular deuterium has not found wide application in the catalytic reduction of double and triple bonds, since the reduction is often accompanied by a non-selective proton – deuterium exchange.

Bacterial strains that are unable to synthesise certain lipid components and that are grown on media containing these components with deuterium labels are used for specific incorporation of deuterium. Non-specific deuteration is achieved by growing bacteria on culture media in which heavy water is used instead of H_2O.

The final assembly of deuterium-containing fragments into synthons needed for phospholipid synthesis is performed by

N A Bragina, V V Chupin M V Lomonosov Moscow State Academy of Fine Chemical Technology, prosp. Vernadskogo 86, 117571 Moscow, Russian Federation. Fax (7-095) 430 79 83. Tel. (7-095) 437 19 25

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methods of organic synthesis. Phospholipids are synthesised by traditional methods employed in lipid chemistry.

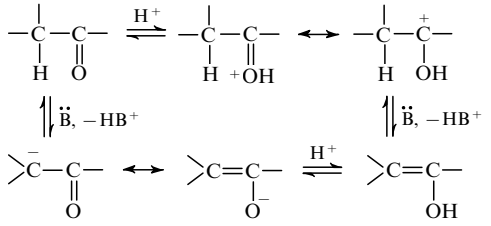
II. Synthesis of deuterated fatty acids

The simplest (in the preparative sense) methods for obtaining selectively and perdeuterated fatty acids are based on the reaction of proton–deuterium exchange.[17] A prerequisite for conducting such reactions is the presence of a medium with readily dissociating deuterons and a catalyst enabling the dissociation of C–H bonds of fatty acids.

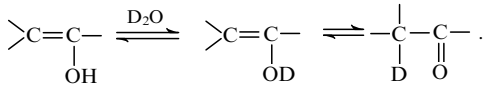
As a rule, such reactions are carried out at sufficiently high temperatures; fatty acids with C–D bonds are obtained after removal of the catalyst. Under conditions close to physiological ones, they are stable and do not enter into the reactions of reverse exchange. If fatty acid molecules contain functional groups that increase the acidity of vicinal C–H bonds, the exchange can be carried out selectively.

In particular, protons at the α-position to a carbonyl or a carboxyl group can be easily exchanged for deuterium. A tautomeric transformation shown in Scheme 1 is characteristic of carbonyl compounds.

Scheme 1



The formation of an enol tautomer occurs either in the presence of bases *via* the enolate anion or in the presence of acids *via* the carbocation.[18] If these transformations are carried out in an excess of a solvent containing readily dissociating deuterons, α-protons exchange for deuterium.



Let us consider some examples of such a selective exchange. The presence of a carboxyl group in palmitic acid **1** increases the acidity of α-protons; selective exchange of α-protons for deuterium gives compound **2** in D_2O (the source of mobile deuterons) in the presence of a base as the catalyst.

$$\underset{\mathbf{1}}{Me(CH_2)_{14}COOH} \xrightarrow{D_2O/NaOD} \underset{\mathbf{2}}{Me(CH_2)_{13}CD_2COONa}.$$

In case of long-chain fatty acids, the reaction proceeds under relatively drastic conditions (200 °C, 72 h, 1% NaOD).[19] α-Protons of short-chain carboxylic acids undergo the exchange more readily.[20]

This approach can be used for the incorporation of deuterium into fatty acids with one double bond without the migration of this bond along the hydrocarbon chain.[21]

Fatty acid esters are more reactive than the acids themselves. Thus equilibrium conditions for the exchange of α-protons of methyl palmitate are reached within several hours of boiling in MeOD in the presence of NaOD.[22–24] With the same ease, the exchange proceeds with monoalkyl malonates **3**.

$$\underset{\mathbf{3}}{AlkCH(COOMe)_2} \xrightarrow{D_2O/NaOD} \underset{\mathbf{4}}{[AlkCD(COONa)_2]} \longrightarrow \mathbf{2}$$

Alk = $Me(CH_2)_{13}$.

The exchange takes place on boiling in D_2O (which is a more readily available reagent than MeOD) in the presence of NaOD as the catalyst.[24,25] Decarboxylation of the sodium dicarboxylate **4** results in a salt of the monocarboxylic acid **2** deuterated at the α-position.

In aldehydes, the exchange of α-protons occurs very readily, which can be used in exchange reactions aimed at deuterium incorporation at the α-position of relatively labile, unsaturated fatty acids. The synthesis of [2,2-$^2H_2$]-oleic acid **9** is an example.[26] Methyl oleate acid **5** served as the starting material. It was first reduced to the alcohol **6** which was then oxidised to the aldehyde **7** followed by the exchange reaction in D_2O/Py. [2,2-$^2H_2$]-Oleic acid **9** was obtained by the oxidation of the aldehyde **8** with silver oxide.

$$\underset{\mathbf{5}}{Me(CH_2)_7CH{=}CH(CH_2)_7COOMe} \xrightarrow{LiAlH_4} \underset{\mathbf{6}}{Me(CH_2)_7CH{=}CH(CH_2)_7CH_2OH} \xrightarrow{CrO_3\cdot Py} \underset{\mathbf{7}}{Me(CH_2)_7CH{=}CH(CH_2)_6CH_2CHO} \xrightarrow{D_2O/Py} \underset{\mathbf{8}}{Me(CH_2)_7CH{=}CH(CH_2)_6CD_2CHO} \xrightarrow{Ag_2O} \underset{\mathbf{9}}{Me(CH_2)_7CH{=}CH(CH_2)_6CD_2COOH}.$$

The multistep character of this approach is its obvious limitation.

Exchange reactions are also used for the substitution of non-enolisable protons for deuterium. Complete non-specific exchange of protons in palmitic acid **1** is achieved at high temperatures and pressure in the presence of Pt and Na_2O_2 as the catalysts.[27]

$$\mathbf{1} \xrightarrow[240\ ^\circ C,\ 24\ h]{D_2O/Na_2O_2,\ Pt} \underset{\mathbf{10}}{CD_3(CD_2)_{14}COONa}.$$

Under these conditions, partial decarboxylation of a fatty acid occurs, the yield of the deuterated product **10** is 80%–85%.

Fully deuterated acids can also be obtained by passing gaseous deuterium through a suspension of palladium black in a fatty acid.[28] The specificity of the exchange of non-enolisable protons is rather low. However, in some cases selective proton–deuterium exchange can be achieved. If the reaction is carried out under closely controlled conditions, it is possible to exchange predominantly the protons of the terminal methyl group of a carboxylic acid.[29] In this case, K_2PtCl_4 is used as the catalyst.

The obvious merit of the above methods is that the synthesis of deuterated fatty acids does not require complex multistage procedures. Moreover, it allows the use of available fatty acids as the initial material. The reactions occur with high yields. The final products are isolated by extraction without recourse to chromatographic purification. The extent of deuteration of a fatty acid depends on the number of repetitions of the exchange procedure, the nature of the deuterating reagent, and its excess. In order to increase the extent of deuteration of the target fatty acids and achieve a more rational use of expensive deuterated solvents, exchange reactions are usually carried out in 2–3 steps.

The preparation of selectively deuterated fatty acids requires the use of more sophisticated procedures. They combine the exchange reactions, selective reduction, and the synthesis of fatty acids from individual synthons. The synthesis of methyl laurate **12** having a deuterated methyl group at the end of the hydrocarbon chain has been described.[25] Methyl 12,12,12-trichlorododecanoate **11** was used as the starting material. Zinc dust in D_2O–dioxane specifically reduces the CCl_3 group of the ester **11** to CD_3 resulting in the product **12**.

$$\underset{\mathbf{11}}{CCl_3(CH_2)_{10}COOMe} \xrightarrow[D_2O/dioxane]{Zn} \underset{\mathbf{12}}{CD_3(CH_2)_{10}COOMe}.$$

The methyl ester **12** can be reduced to the corresponding alcohol and used to obtain labelled myristic acid.[30, 31]

Reduction with $LiAlD_4$ and $NaBD_4$ is extensively used for the selective incorporation of deuterium labels into different positions of hydrocarbon chains of fatty acids. In this case, deuterium incorporation is carried out in one step and the extent of deuteration of the final products depends exclusively on the extent of deuteration of $LiAlD_4$ or $NaBD_4$ used in this reaction. This method has been used for the synthesis of [3-$^2H_2$]-stearic acid **17** [19, 32] from methyl palmitate **13** as the starting compound. It was reduced to the alcohol **14** with $LiAlD_4$, and then the hydrocarbon chain was elongated by a malonate approach. To this end, the alcohol **14** was activated by its conversion into the mesyl derivative **15**, which was used as the alkylating reagent. The alkylation of the diethyl malonate under alkaline conditions is accompanied by saponification of ester groups; subsequent decarboxylation of the dicarboxylic acid **16** gives the acid **17** with a deuterium label at the β-position.

$$\underset{\mathbf{13}}{Me(CH_2)_{14}COOMe} \xrightarrow{LiAlD_4} \underset{\mathbf{14}}{Me(CH_2)_{14}CD_2OH} \xrightarrow{MeSO_2Cl} \underset{\mathbf{15}}{Me(CH_2)_{14}CD_2OSO_2Me} \xrightarrow{CH_2(COOEt)_2} \underset{\mathbf{16}}{Me(CH_2)_{14}CD_2CH(COOH)_2} \xrightarrow{-CO_2} \underset{\mathbf{17}}{Me(CH_2)_{14}CD_2CH_2COOH}.$$

Apparently, any oxo group in the hydrocarbon chain can be selectively reduced to the CD_2 group.[19, 33] Thus the methyl ester of the oxo acid **18** was selectively reduced with $NaBD_4$ to the hydroxy ester **19.** The latter was further converted into the tosylate **20** and reduced with $LiAlD_4$, which resulted in the alcohol **21** deuterated at positions 1 and 17. The oxidation of the alcohol **21** with chromium oxide was accompanied by the removal of deuterium from the position 1, eventually resulting in [17-$^2H_2$]-stearic acid **22**.

$$\underset{\mathbf{18}}{MeCO(CH_2)_{15}COOMe} \xrightarrow{NaBD_4} \underset{\mathbf{19}}{MeCD(OH)(CH_2)_{15}COOMe} \xrightarrow{TsCl} \underset{\mathbf{20}}{MeCD(OTs)(CH_2)_{15}COOMe} \xrightarrow{LiAlD_4} \underset{\mathbf{21}}{MeCD_2(CH_2)_{15}CD_2OH} \xrightarrow{CrO_3} \underset{\mathbf{22}}{MeCD_2(CH_2)_{15}COOH}.$$

The same scheme can be used for the stereospecific incorporation (if required) of one deuterium atom into the fatty acid molecule.[33]

In addition to the tosylates of the type **20**, the synthesis of deuterated fatty acids makes use of mesylates and iodides, which are also reduced with $LiAlD_4$.[34, 35] Primary and secondary bromides are not reduced,[35, 36] which makes it possible to obtain the necessary synthons for the fatty acid synthesis. However, the selectivity of $LiAlD_4$ is rather low. Therefore, another reducing agent, $NaBD_3CN$, has been recommended.[37] This compound selectively reduces tosylates, mesylates, and iodides in the presence of ester groups in aprotic solvents.

The CD_2 groups are obtained by the Clemmensen reduction of oxo groups (Zn/DCl).[32, 38] However, the presence of mobile deuterons in the reaction medium results in the exchange of the protons at the α-position relative to the oxo group, which leads to the formation of the $(CD_2)_3$ fragment in the hydrocarbon chain. A versatile procedure has been developed [39] for the substitution of the CH_2 group by CD_2 at any position of the saturated fatty acid. This method consists in the reduction of the corresponding ester of the carboxylic acid **23** with $LiAlD_4$. The alcohol formed **24** is converted into the bromide, and the bromide is used to obtain the Grignard reagent **25**. The interaction of the Grignard reagent **25** with a salt of a bromo-substituted carboxylic acid **26** in the presence of Li_2CuCl_4 as a catalyst gives the carboxylic acid **27** with a deuterated methylene group. At this stage, the reaction proceeds in high yields (77% – 85%) which tend to decrease with the elongation of the hydrocarbon chain in the compound **25.**

$$\underset{\mathbf{23}}{Me(CH_2)_9COOMe} \xrightarrow{LiAlD_4} \underset{\mathbf{24}}{Me(CH_2)_9CD_2OH} \xrightarrow[2.\ Mg]{1.\ HBr} \underset{\mathbf{25}}{Me(CH_2)_9CD_2MgBr} \xrightarrow[Li_2CuCl_4]{Br(CH_2)_5COOMgCl\ (\mathbf{26})} \underset{\mathbf{27}}{Me(CH_2)_9CD_2(CH_2)_5COOH}.$$

By varying the length of the hydrocarbon chain of the ester **23** and the bromo-substituted acid **26**, one can obtain fatty acids with variable lengths of the hydrocarbon chain and variable positions of the CD_2 group.

Lithium dimethylcuprate, $LiCuMe_2$, was used for the substitution of the tosyl group by the methyl group in the synthesis of methyl [13-$^2H_2$]-myristate.[40]

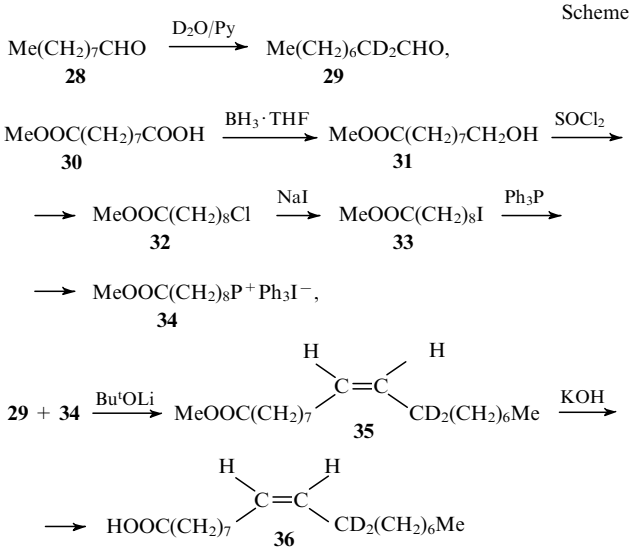
Vicinally deuterium-labelled fatty acids are synthesised by the reduction of multiple bonds with gaseous deuterium on platinum and palladium catalysts.[41, 42] This method for deuterium labelling is rarely used due to a number of disadvantages, such as the necessity to use a large excess of deuterium, drastic reaction conditions, and, which is the most important, because of the side reactions of isotope exchange. The use of the homogeneous Wilkinson catalyst, [tris(triphenylphosphine)rhodium chloride], allows one to avoid side reactions to some extent.[43 – 46] Double and triple bonds can be reduced on a deuterated Raney nickel.[47, 48] However, the most popular procedure for the catalytic hydrogenation of double and triple bonds with molecular deuterium is the deuteration of triple bonds on the Lindlar catalyst. The lower activity of the Lindlar catalyst in comparison with other catalysts makes it possible to selectively deuterate triple bonds. This reaction proceeds with a high degree of stereoselectivity; the content of the *cis*-isomer (the natural isomer in unsaturated fatty acids) in dideuterated alkenes amounts to 98% – 99%. This method was used in the synthesis of octadeuterioarachidonic acid [49] and a series of unsaturated CD = CD-fatty acids.[49 – 53] Reduction on the Lindlar catalyst is also used in 'alkyne' syntheses of unsaturated fatty acids with deuterium-labelled methylene fragments, since no reverse deuterium – proton exchange takes place under conditions of catalytic hydrogenation.[21]

Selective deuteration of the double bonds of unsaturated fatty acids is also performed with deuteriohydrazine.[32, 54, 55] This reaction is carried out only in the presence of small amounts of an oxidising agent (oxygen, sodium metaperiodate, hydrogen peroxide, copper(II) salts, *etc.*). First, the diimide DN = ND is formed *in situ* from deuteriohydrazine in the presence of the oxidiser; its *syn*-form ensures the *cis*-addition of deuterium to the double bond.[56]

The synthesis of deuterium-labelled unsaturated acids is based on the use of the Wittig reaction.[44, 57 – 59] An example of such synthesis is shown in Scheme 2.

According to this scheme, deuterium is incorporated *via* the exchange reaction into the α-position of nonanal **28** upon boiling the aldehyde in a D_2O/pyridine mixture. The second component of the Wittig reaction is obtained by the selective reduction of monomethyl azelate **30** to the alcohol **31**; the hydroxyl group is then consecutively substituted by chlorine (compound **32**) and iodine (compound **33**), and the iodide **33** alkylates triphenylphosphine. The key step in this synthesis is the Wittig condensation of the aldehyde **29** and the phosphonium salt **34**. Initially, it was proposed to use sodium ethoxide in this reaction.[58] However, very often the condensation proceeded in very low yields.[60] Later,

Scheme 2



Bu^tOLi was recommended as a base.[59] The latter was obtained by the interaction of equimolar amounts of *tert*-butyl alcohol and hexyllithium. In this case, the condensation of compounds **29** and **34** proceeded with a sufficiently high degree of stereoselectivity, the yield of [11-$^2H_2$]-oleic acid (**36**) and its *trans*-isomer, [11-$^2H_2$]-elaidic acid, reached ~70%. The ratio of *cis*- and *trans*-isomers was 95 : 5. The formation of *cis*- and *trans*-isomers in the Wittig reaction is a serious limitation of this method, since the separation of geometric isomers of fatty acids requires special chromatographic procedures.[57] The ratio of *cis*- and *trans*-isomers depends largely on the reaction conditions.[60] For example, by performing the reaction with potassium bis(trimethylsilyl)amide, one can increase the content of the *cis*-isomer up to 98%[61] which is comparable with the stereopurity of alkenes obtained by hydrogenation of acetylenic compounds on the Lindlar catalyst.

In addition to the above-described methods of elongation of hydrocarbon chains (malonate synthesis, condensation with the use of copper catalysts, the Wittig reaction), other approaches are employed in the synthesis of deuterium-labelled acids that are traditionally used in fatty acid chemistry, such as the 'alkyne' synthesis,[21, 35, 43, 62, 63] enamine synthesis,[19, 34, 64] and Kolbe electrolytic synthesis.[33, 65–67] Synthesis of various deuterium-labelled acids has been considered in a number of reviews.[30, 31]

III. Synthesis of deuterium-labelled hydrophilic components of phospholipids

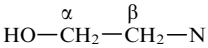
The hydrophilic part of phospholipid molecules includes choline, ethanolamine, serine, glycine, inositol, and some other residues.[68, 69]

To study the structural peculiarities of the polar region of a lipid bilayer, various methods of synthesis of phospholipids with different deuterium-labelled polar portions of phospholipid molecules have been developed.

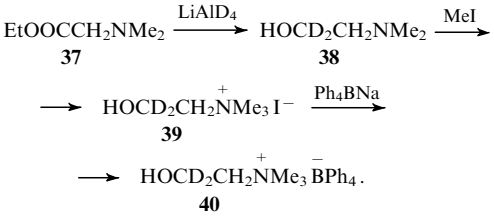
1. Synthesis of deuterated cholines

One of the most common phospholipids of biological membranes are phosphatidylcholines.[68] They are the favourite object for biophysical and biochemical studies. This is due to their ability to form stable bilayers within a broad range of temperatures, pH, and ionic strength. The quadrupole splitting of signals of deuterium labels in the ^2H NMR spectra of choline is extremely sensitive to changes in the surface electric potential of the lipid bilayer,[70] which opens new opportunities for the study of electric properties of membranes. It is not surprising therefore that a great deal of attention has been given to the synthesis of deuterated cholines or their chemical precursors.

The developed methods to date permit the incorporation of deuterium into any part of the choline molecule. In order to define the positions in the choline residue, we shall use the system of designations proposed by Seelig *et al.*:[70]



In order to obtain choline labelled with deuterium at the α-position, the ethyl ester of *N,N*-dimethylglycine (**37**) is reduced with lithium aluminium deuteride, after which the *N,N*-dimethylaminoethanol (**38**) formed is subjected to methylation. This reaction gives [α-$^2H_2$]-choline iodide (**39**),[71] which is then condensed with 1,2-dipalmitoyl-*sn*-glycerophosphodichloridate to give 1,2-dipalmitoyl-*sn*-glycerophospho-[*a*-$^2H_2$]-choline:

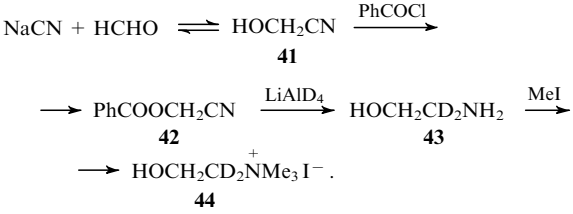


It should be noted that the condensation of phosphatidic acid or phosphodichloridate with choline is an important step in the synthesis of phosphatidylcholines. This reaction is carried out in organic solvents (usually, in pyridine, chloroform, dichloromethane, *etc.*). However, the solubility of choline iodide in organic solvents is very low, which is the reason for low yields at the stage of condensation of the phosphatidic acid and choline iodide. To overcome this problem, it was proposed to use choline acetate.[72] However, the yields varied within a very broad range and were 40% – 60% at best. This is also due to the relatively low solubility of choline acetate in organic solvents and its high hygroscopicity.

The synthesis of perdeuterated phosphatidylcholine was carried out with the use of a complex of choline iodide with iodine.[73] The drawback of this approach is the fact that this complex is unstable and loses iodine on storage or drying *in vacuo*. Moreover, after condensation it is necessary to remove the large excess of free iodine, whereas the yield of phosphatidylcholine is as low as 25% – 40%.

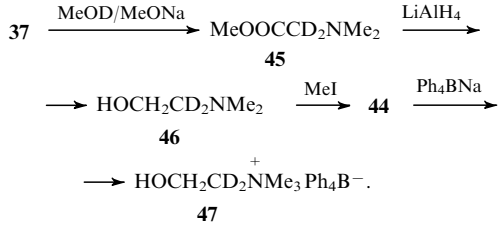
An effective approach to the synthesis of phosphatidyl choline has been proposed by Harbison and Griffin[74] who have used choline tetraphenylborate. A salient advantage of this method is that this salt is virtually insoluble in water, and choline can be easily and quantitatively isolated from aqueous solutions. The fact that choline tetraphenylborate is non-hygroscopic and is readily soluble in organic solvents, such as pyridine, allows one to achieve sufficiently high yields in its condensation with phosphatidic acid. [α-$^2H_2$]-Choline tetraphenylborate (**40**) was prepared as follows. After methylation, the organic solvent was removed *in vacuo*, the residue was treated with aqueous alkali, insoluble aluminates were filtered off, and [α-$^2H_2$]-choline was precipitated with an aqueous solution of sodium tetraphenylborate.

The synthesis of β-deuterated choline[71] is based on the method earlier proposed for the synthesis of ^{14}C-labelled ethanolamine.[75] According to this method, condensation of sodium cyanide with formaldehyde gives hydroxyacetonitrile (**41**), which is further benzoylated to give the nitrile **42**. The latter is reduced to [β-$^2H_2$]-ethanolamine (**43**). Exhaustive N-methylation of [β-$^2H_2$]-ethanolamine **43** gives [β-$^2H_2$]-choline iodide (**44**):

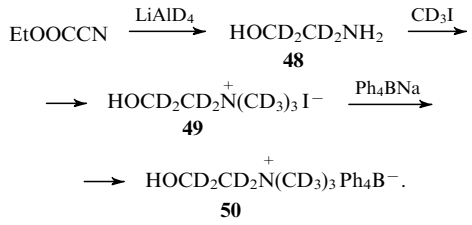


Yet another approach[76] consists in the use of the ethyl ester of *N,N*-dimethylglycine (**37**) as the starting compound. The mobile

α-protons of the compound **37** are exchanged for deuterium in MeOD in the presence of sodium methoxide. This exchange occurs at a high rate, and equilibrium conditions are reached within 1 h at room temperature. The deuterium exchange is accompanied by transesterification of the ethyl ester to give the compound **45**. The progress of the exchange reaction is easily monitored by following the disappearance of the signals corresponding to the CH_2 group and the appearance of signals of CHD and CD_2 groups in the ^{13}C NMR spectra. The reduction of the methyl ester **45** to the corresponding alcohol **46**, its further methylation, and the preparation of [β-$^2H_2$]-choline tetraphenylborate (**47**) are carried out in much the same way as the synthesis of the compound **40,** except that lithium aluminium hydride is used instead of lithium aluminium deuteride.

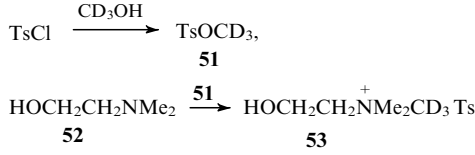


Per-C-deuterated choline [74] is obtained by reduction of ethyl cyanoacetate with lithium aluminium deuteride with subsequent exhaustive N-deuteromethylation of the deuterated ethanolamine **48**. The resulting choline iodide (**49**) can be converted into the acetate,[72] the complex with iodine,[73] or tetraphenylborate (**50**).[74] The latter seems to be the most convenient form of choline for its subsequent condensation with phosphatidic acid. The total yield of choline tetraphenylborate **50** exceeds 40%.[74]



In addition to this method, per-C-deuterated choline can be obtained by quaternisation of [$^2H_9$]-trimethylamine with 1,1,2,2-[$^2H_4$]-2-bromoethanol as in the synthesis of perdeuterated dodecylphosphocholine (see below).[77] Perdeuterated β-bromoethanol can be used in those schemes of phosphatidylcholine syntheses that envisage the formation of the choline residue at the last stage, by the reaction of bromo(chloro)alkanes with trimethylamine.[78]

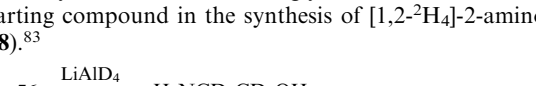
Choline with deuterated methyl groups is synthesised by the methods based on methylation of ethanolamine or its partly methylated derivatives. Methylation of ethanolamine followed by precipitation of choline as tetraphenylborate was used to obtain [γ-$^2H_9$]-choline in a practically quantitative yield.[74] Choline tosylate is yet another convenient derivative for the phosphatidylcholine synthesis.[79] However, this choline salt is readily soluble in water. Moreover, choline tosylate cannot be obtained by a direct ion-exchange reaction between choline iodide and *p*-toluenesulfonic acid. The conversion of choline iodide into tosylate requires an intermediate synthesis of choline acetate.[24] *p*-Toluenesulfonyl chloride is the starting compound in the synthesis of [γ-$^2H_3$]-choline tosylate (**53**).[24] Its reaction with CD_3OH yields methyl tosylate (**51**), which is further used for the deuteriomethylation of 2-*N,N*-dimethylethanolamine (**52**). Choline tosylate **53** is obtained in a quantitative yield:



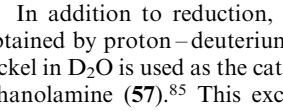
2. Synthesis of deuterium-labelled ethanolamines

Ethanolamine is the structural element of phosphatidylethanolamines, which, like phosphatidylcholines, are zwitterionic phospholipids. However, in contrast to phosphatidylcholines, phosphatidylethanolamines manifest basically different self-organisation properties. Under physiological conditions, phosphatidylethanolamines form in water inverted hexagonal structures rather than an extended bilayer.[80,81] It is believed that the ability of phosphatidylethanolamines to form non-bilayer structures strongly affects many membrane functions.

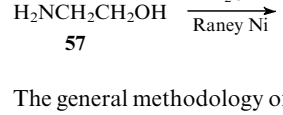
To obtain ethanolamines with deuterium labels in different positions, the reduction of glycine derivatives [82–84] with metal deuterides is generally used. Thus the reduction of glycine ethyl ester (**54**) with lithium aluminium deuteride [83] results in ethanolamine (**55**) with a deuterium label at position 1.



An analogous procedure is used to synthesise ethanolamine **43** with a deuterium label at position 2. However, in this case the deuterated ethyl ester of glycine (**56**) and lithium aluminium hydride are used as the starting compounds.[83]

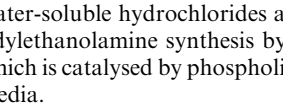


[2-$^2H_2$]-2-Aminoethanol **43** can also be obtained by the methods employed in the synthesis of deuterated cholines.[71,75] The ethyl ester of deuterated glycine **56** can also serve as the starting compound in the synthesis of [1,2-$^2H_4$]-2-aminoethanol (**48**).[83]



The latter was also obtained by the reduction of ethyl cyanoacetate according to the procedure for the synthesis of perdeuterated choline.[74]

In addition to reduction, deuterated ethanolamines can be obtained by proton–deuterium exchange reactions. Thus Raney nickel in D_2O is used as the catalyst in the exchange of protons in ethanolamine (**57**).[85] This exchange is non-specific despite the higher activity of position 2. The indisputable advantage of this approach is undoubtedly the accessibility of the initial reagents.



The general methodology of the preparation and utilisation of 'deuterated' Raney nickel for proton exchange in aliphatic and aromatic compounds has been described by Pojer.[86]

Deuterium-labelled ethanolamines are obtained in the form of water-soluble hydrochlorides and are routinely used in phosphatidylethanolamine synthesis by enzymic transphosphatidylation, which is catalysed by phospholipase D and takes place in aqueous media.

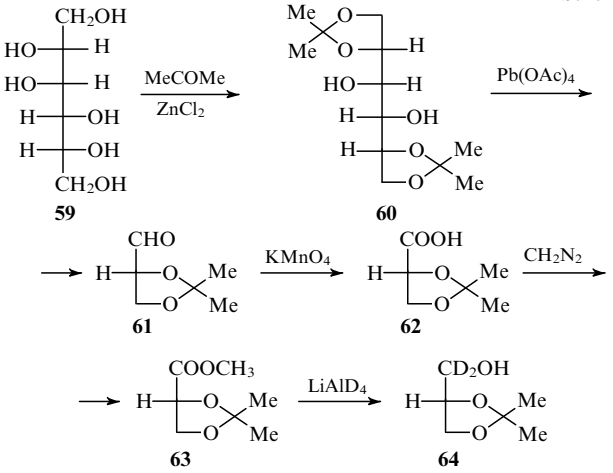
3. Synthesis of deuterium-labelled glycerols

Glycerol (**58**) is a structural element of all glycerophospholipids and a constituent of the polar region of the negatively charged phospholipid phosphatidylglycerol, which, in turn, is the structural component of cardiolipin (diphosphatidylglycerol). The interest in the synthesis of deuterated glycerols is associated not only with their use in the synthesis of labelled lipids, but also with studies of glycerol as a substrate and intermediate of many biosynthetic processes. In this connection, the synthesis of various deuterium-labelled glycerols has become the subject of a large number of publications.[73,87–95] The methods elaborated to date allow one to obtain glycerols with a deuterium label at any

position of the molecule. Non-protected deuterium-labelled glycerols are used as substrates in enzymic syntheses of phospholipids, and their isopropylidene derivatives are used in chemical syntheses of phosphatidylglycerols. The necessary stereoconfiguration of isopropylideneglycerols is provided by the original use of specific optically active reagents. Deuterium is introduced into the glycerol molecule by reduction with lithium aluminium deuteride, sodium borodeuteride, or their derivatives,[73, 87–92] as well as by enzymic methods.[87, 93, 94]

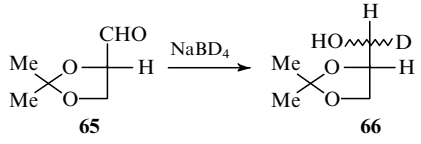
Let us consider the syntheses of various deuterated glycerols and their isopropylidene derivatives. 1,2-*O*-Isopropylidene-*sn*-[3-$^2H_2$]-glycerol (**64**) has been synthesised by the method traditionally employed in lipid chemistry.[86] This method is based on the conversion of D-mannitol (**59**) into the 1,2 : 5,6-di-*O*-isopropylidene derivative **60** followed by scission of the vicinal diol group with lead tetraacetate. To remove the proton remaining at position 1, the protected D-glyceraldehyde **61** is oxidised to the corresponding glyceric acid **62**, which can further be reduced to the alcohol. For more optimal utilisation of the relatively expensive lithium aluminium deuteride, the acid **62** is converted into the methyl ester **63** prior to reduction to obtain 1,2-*O*-isopropylidene-*sn*-[3-$^2H_2$]-glycerol **64** (Scheme 3).[87]

Scheme 3



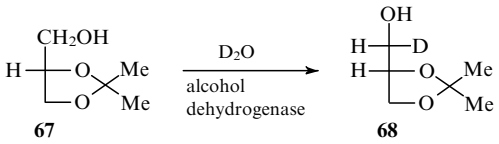
An approach, which is very similar to that described above, was used [88] in the synthesis of **64**, except that the oxidation of the vicinal diol **60** was performed with sodium periodate, the aldehyde **61** was oxidised with chromic acid, and the resulting acid **62** was directly reduced with lithium aluminium deuteride. Along with D- and L-mannitol, D- and L-serine are also used as the original optically active compounds.[90, 91]

The synthesis of 2,3-*O*-isopropylidene-*sn*-[1-^{2}H]-glycerol (**66**) has been described.[87] The aldehyde **65**, an enantiomer of the aldehyde **61**, was prepared from L-mannitol, which, in turn, was obtained by the reduction of L-mannose. The reduction of the aldehyde **65** with sodium borodeuteride gives the isopropylidene derivative **66** containing one deuterium atom at position 1.



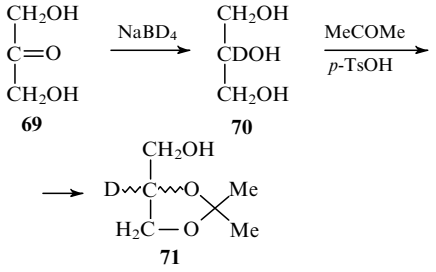
Unfortunately, the reduction of the aldehyde **65** is non-stereospecific, and the compound **66** is a mixture of two diastereomers.

The stereospecific incorporation of deuterium into glycerol is carried out by both chemical [91] and enzymic [87, 93, 94] methods. An original procedure [87] based on the enzymic reaction of proton–deuterium exchange has been proposed. In the presence of horse alcohol dehydrogenase, one of the protons of 1,2-*O*-isopropylidene-*sn*-glycerol (**67**) is stereospecifically exchanged in D_2O to give 1,2-*O*-isopropylidene-(3*R*)-*sn*-[3-^{2}H]-glycerol (**68**).

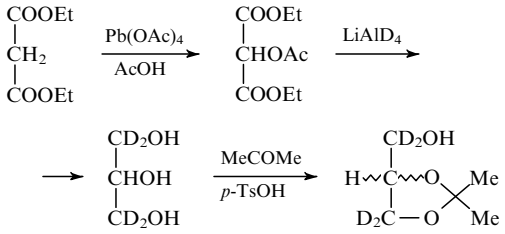


Apparently, this reaction is of general character and can be used for stereospecific exchange of one proton for deuterium in any primary alcohol. A combination of chemical and enzymic methods gave (1*R*, 2*R*)-[1,1-^{2}H,^{3}H]-glycerol stereospecifically labelled with both deuterium and tritium.[93]

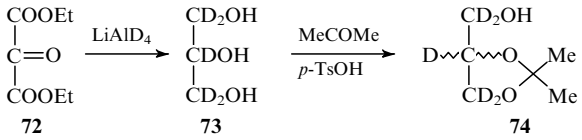
In order to incorporate deuterium into position 2 of the glycerol molecule, the reduction of dihydroxyacetone (**69**) with sodium borodeuteride is used.[87] When required, glycerol (**70**) can be converted into the isopropylidene derivative by the reaction with acetone in the presence of *p*-toluenesulfonic acid. However, this reaction gives racemic isopropylideneglycerol (**71**):



Selective incorporation of deuterium into positions 1 and 3 of the glycerol molecule is achieved using the sequence of reactions depicted in the following scheme:[87]



Perdeuterated glycerol **73** (mobile protons of the hydroxy groups are not taken into consideration) is prepared by the reduction of diethyl oxomalonate (**72**) with lithium aluminium deuteride.[73] Enzymic phosphorylation of the deuterated glycerol **73** ensures the necessary stereoconfiguration in subsequent intermediates of glycerophospholipid synthesis.[73] The introduction of the protecting isopropylidene group into the glycerol **73** gives racemic 1,2-*O*-isopropylidene-*rac*-[1,2,3-$^2H_5$]-glycerol (**74**).[87]



Deuterium-labelled glycerols and their isopropylidene derivatives are used in the synthesis of deuterium-labelled phosphatidylglycerols and perdeuterated phosphatidylcholine. The incorporation of deuterium into position 3 of 1,2-di-*O*-alkylglycerol has been described.[92] The latter was oxidised to the dialkyl derivative of glyceric acid using the Jones reagent. The resulting acid was reduced with lithium aluminium deuteride to give 1,2-di-*O*-alkyl-[3-$^2H_2$]-glycerol. The diglyceride component can further be used for the synthesis of phospho- or glycolipids. However, this method is inapplicable to the synthesis of diacyl diglycerides due to the reduction of ester groups.

4. Other deuterium-labelled hydrophilic synthons of lipid molecules

Deuterium-labelled [$^2H_2$]-serine is commercially available and can be used in enzymic or chemical syntheses of deuterium-labelled

phosphatidylserines. A synthesis of L-serine stereospecifically labelled with deuterium from labelled asparagine has been described.[95] A number of publications have been dedicated to the synthesis of glycolipids with deuterium labels in the carbohydrate part.[92, 96–103] Usually, deuteration of carbohydrate fragments is carried out by catalytic exchange in the presence of Raney nickel in D_2O, which results in the exchange of protons geminal to the hydroxyl groups of primary and secondary alcohols. [96] Glucopyranosides subjected to such exchange accept deuterium labels at positions 2, 3, 4, 6, and 6′. The rate of exchange depends on steric factors and differs markedly for different positions in the carbohydrate moiety. The progress of the proton–deuterium exchange is monitored by ^{13}C NMR spectroscopy. In case of glycosyldiglycerides,[98] dialkyl diglycosydiglycerides,[99] and other glycolipids[101] devoid of ester groups, the exchange is carried out directly in the glycolipids, which do not undergo destruction. In case of diacyl glycosyldiglycerides, the presence of relatively labile ester bonds precludes the application of this approach. Therefore, deuterium is first incorporated into the carbohydrate fragments, which are further bound to diglycerides by traditional chemical methods.[97] Deuterium can be incorporated into carbohydrates by reduction with metal deuterides.[103]

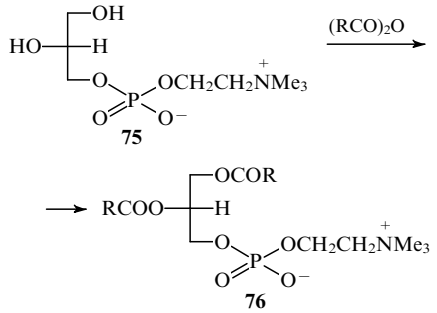
IV. Synthesis of phospholipids with deuterated fatty acids

The synthesis of phospholipids with deuterated fatty acids is carried out by traditional methods of lipid chemistry. The synthetic procedures for obtaining phospholipids have been described in manuals,[69, 104] monographs,[104–106] and reviews.[107, 108] As a rule, original papers dealing with the synthesis and applications of deuterium-labelled phospholipids do not contain the description of experimental techniques, and only references to publications dedicated to the synthesis of non-labelled lipids are given. The experimental procedures that are most commonly employed in lipid chemistry are summarised and reviewed in monographs.[105, 106]

The relatively high cost of deuterium-labelled fatty acids accounts for their incorporation into phospholipid molecules at final stages of the synthesis. The general chemical approaches that are most widely employed for the solution of this task are presented below.

The starting material for the phosphatidylcholine synthesis is *sn*-glycerophosphocholine (**75**) and/or its cadmium adduct. The former is easily prepared by alkaline hydrolysis of natural phosphatidylcholines [most commonly, egg phosphatidylcholine (lecithin)],[106–108] and is devoid of functional groups that need protection in the acylation. By selecting the deuterium-labelled fatty acids, one can synthesise the desired phosphatidylcholine **6** in one step (Scheme 4):

Scheme 4



75 76

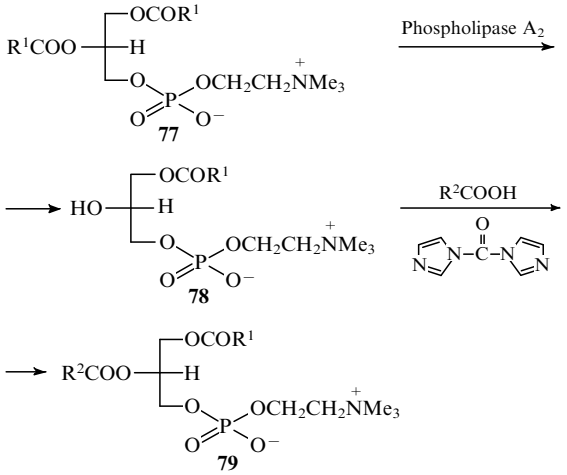
The acylation is performed with acid chlorides,[107] anhydrides,[109–112] imidazolides,[113] or other activated derivatives of fatty acids.[104] The acylation of *sn*-glycerophosphocholine **75** can be accompanied by the formation of large amounts of side products, mostly, cyclic phosphate. Therefore, this reaction requires that the preselected experimental conditions were strictly met. Then the yields at the stage of acylation of *sn*-glycerophosphocholine **75** reach 60% – 80% irrespective of the acylating agent used.

The most convenient in the experimental sense are the methods of acylation of *sn*-glycerophosphocholine deuterium-labelled fatty acid with anhydrides and imidazolides. In earlier studies, fatty acid salts have been used as the catalysts for acylation with acid anhydrides;[111] later, these were replaced by 4-*N,N*-dimethylaminopyridine,[108] sodium methylsulfenylmethanide,[109] and 4-pyrrolidinopyridine.[110] Acylation in these cases occurs under milder conditions and is less time-consuming, which is especially important in the synthesis of unsaturated phosphatidylcholines. Fatty acid anhydrides are prepared by treating fatty acids with dicyclohexylcarbodiimide.

Fatty acid imidazolides are obtained upon interaction of the acid with carbonyldiimidazole.[105, 106, 113] The imidazolide method is advisable for the synthesis of phospholipids containing expensive labile labelled fatty acids (including spin-labelled ones),[113] since exhaustive acylation with imidazolides requires lesser amounts of the fatty acid than the anhydride method. After acylation has been completed, the excess of the non-consumed fatty acid can be isolated at the stage of chromatographic purification of phosphatidylcholine.

Let us consider the synthesis of phosphatidylcholines with the use of the anhydride method. As can be seen from Scheme 4, in this case phosphatidylcholine synthesis gives only phosphatidylcholines **76** that contain the same fatty acid residues at positions 1 and 2 of glycerol. This is a serious pitfall of this method, since natural phospholipids contain, as a rule, non-identical fatty acids.

The synthesis of these phospholipids rests on the use of lysophosphatidylcholine **78** as the starting compound, which can be easily obtained by enzymic hydrolysis of phosphatidylcholine **77** with phospholipase A_2.[104, 108, 114]



77 78 79

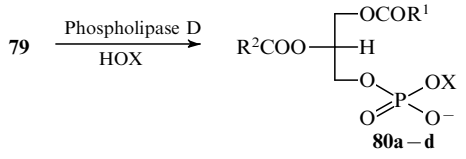
The hydrolysis of phosphatidylcholines does not necessarily require purified preparations of phospholipase A_2. They can be successfully replaced by whole snake or bee venoms. The hydrolysis of phosphatidylcholine with phospholipase A_2 occurs with a high degree of stereoselectivity and can be used for separation of racemic mixtures of synthetic phospholipids.[104, 108, 114] The acylation of lysophosphatidylcholines is carried out by the same methods as that of *sn*-glycerophosphocholine, *e.g.*, with a fatty acid imidazolide. By combining various fatty acids in the synthesis of phosphatidylcholines **77** and **79**, one can obtain phosphatidylcholine **79** with any desired set of fatty acid residues including isotopically labelled fatty acids.

These methods of phosphatidylcholine synthesis by acylation of glycerophosphate derivatives have turned out to be very convenient in the preparative sense and they have nearly ousted total chemical synthesis, at least in the case of diacylphosphatidylcholines. The acylation of barium *sn*-glycerophosphate has also been successfully used in the synthesis of perdeuterated

phosphatidic acid, which was further used to obtain perdeuterated phosphatidylcholine.[73]

However, the synthesis of other phosphoglycerides by acylation of glycerophosphate derivatives is a far more complex problem. Synthesis of such phospholipids as phosphatidylethanolamine, -glycerol, -inositol, and -serine requires that all the functional groups that can be acylated (with the exception of the hydroxy groups of glycerol) be selectively protected and the protecting groups removed in subsequent stages with the preservation of the structure of the synthetic glycerophospholipids. This is rather difficult to accomplish, which is the reason for the low yields of the target product.

A way to overcome this problem is the extensive use of a combination of chemical and enzymic methods and available phosphatidylcholines as the starting material. The phosphatidylcholines **79** with any desired set of fatty acids can be obtained by the above methods and further converted into other glycerophospholipids **80** by the transphosphatidylation reaction catalysed by phospholipase D.[104–106, 114] A prerequisite for this reaction is a large excess of the alcoholic component (glycerol, ethanolamine, serine, inositol), to which the phosphatidyl group is transferred. The yields of the glycerophospholipids **80a–d** make up to 80%. The only side product of this reaction is phosphatidic acid **80a**. It is impossible to avoid the formation of phosphatidic acid, since this enzymic reaction is carried out in aqueous media, and water competes with an alcohol for the binding with the phosphatidyl residue.



80a–d

X = H (**a**); $CH_2CH_2\overset{+}{N}H_3$ (**b**); $CH_2CH(OH)CH_2OH$ (**c**); $CH_2CH(\overset{+}{N}H_3)COO^-$ (**d**).

The experimental procedures based on the use of lipolytic enzymes in lipid chemistry have been considered in detail in some monographs.[105, 106, 114]

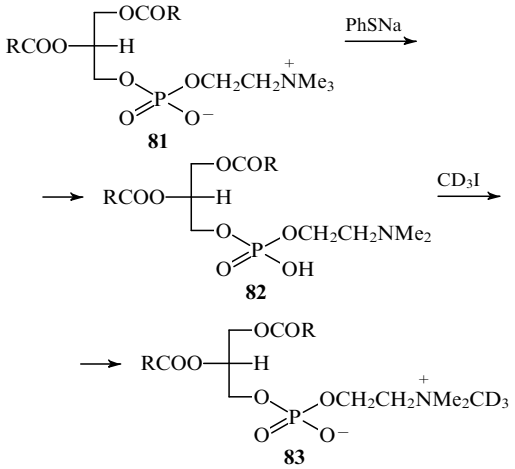
A simple one-step procedure of deuterium incorporation into fatty acid residues of phospholipids is based on the catalytic reduction of double bonds of unsaturated phospholipids.[115, 116] However, this approach has not found wide use in the synthesis of deuterium-labelled phospholipids, since the reduction of double bonds results in saturated phospholipids the labelled methylene groups of which contain only one deuterium atom. To ensure identical sensitivity in further membrane studies, the content of lipids with CHD labels in the samples must be increased twofold in comparison with lipids containing analogous CD_2 labels.

To obtain lipids with deuterium-labelled hydrophobic substituents, total chemical synthesis is employed.[117] Normally, this is applied to obtain lipids of non-natural structure. Thus Sunamoto *et al.*[117] described the synthesis of 1,2-[8-$^2H_2$]-dimyristoylamino-1,2-dideoxyphosphatidylcholine, a phospholipid in which ester groups have been substituted by amide ones.

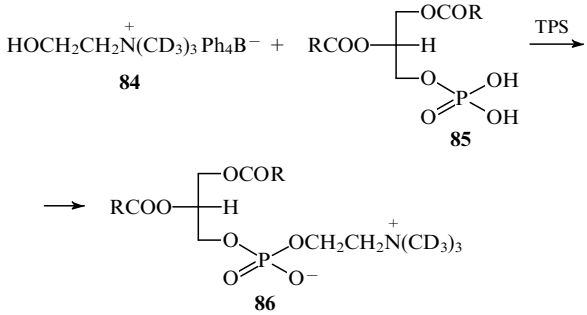
V. Synthesis of phospholipids with deuterium labels in the hydrophilic part of the molecule

As in the case of phospholipids with labelled fatty acids, the synthesis of phospholipids with deuterium labels in the hydrophilic part of the molecule is carried out by traditional methods of lipid chemistry. At the same time, the introduction of deuterium-labelled hydrophilic groups into lipid molecules has a number of distinctive features.

An original procedure for the introduction of a labelled methyl group into the γ-position of choline has been described by Stoffel *et al.*[118] In this method one of the methyl groups of choline is selectively removed upon treatment of the phosphatidylcholine **81** with sodium thiophenoxide. The *N,N*-dimethylphosphatidylethanolamine **82** formed was alkylated with trideuteriomethyl iodide to give the phosphatidylcholine **83** with one deuteriomethyl group at the γ-position of choline. It should be borne in mind that alkylation with methyl iodide of amino groups of unsaturated phospholipids can be accompanied by modification of double bonds,[119] apparently due to the presence of free iodine in the reaction medium.

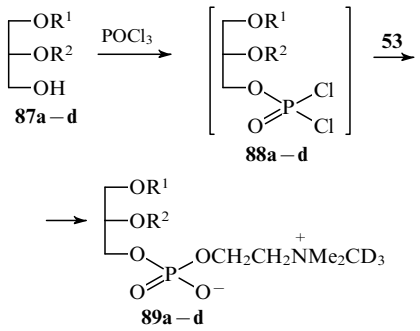


The incorporation of deuterium labels into phospholipids is also carried out at the stage of formation of the phosphodiester bond. The tetraphenylborate of deuterated choline [$HOCH_2CH_2N(CD_3)_3BPh_4$, **84**], which is soluble in organic solvents, is phosphorylated with phosphatidic acid **85** in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride as the condensation agent.[74] The reaction is carried out in pyridine. The yield of phosphatidylcholine **86** is 70%. Reactions with other deuterated cholines, **40** and **50**, occur analogously. Phosphatidic acid **85** is an accessible phospholipid, which can be obtained by phosphorylation of the corresponding diglycerides with phosphorus oxychloride[79, 120] followed by hydrolysis of phosphodichlorides or by enzymic hydrolysis of phosphatidylcholines with phospholipase D.[114] Choline tetraphenylborates with different deuterium labels are obtained according to the schemes described earlier.[74]



R = $(CH_2)_{12}Me$; TPS = 2,4,6-$Pr^i_3C_6H_2SO_2Cl$.

Phospholipids of a particular structure or phospholipids having several different deuterium labels are obtained by using procedures based on total chemical synthesis. Chudinov *et al.*[24] described the synthesis of a series of diacyl, dialkyl, alkylacyl, and acylalkyl phosphatidylcholines **89a–d**. The synthesis of phosphatidylcholines **89a–d** involved the total chemical synthesis of the deuterium-labelled diglycerides **87a–d** and their subsequent phosphorylation with phosphorus oxychloride; the phosphodichlorides **88a–d** formed in this reaction were directly (without isolation) brought into the reaction with choline tosylate **53** using the method described by Brockerhoff and Ayengar.[79] The yield of the product at the stage of phosphorylation was 70%–80%.



$R^1 = COCD_2(CH_2)_{13}Me$, $R^2 = CO(CH_2)_{14}Me$ (**a**);

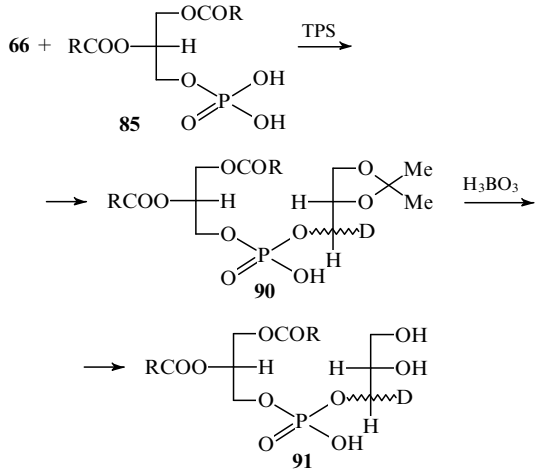
$R^1 = CH_2CD_2(CH_2)_{13}Me$, $R^2 = CO(CH_2)_{14}Me$ (**b**);

$R^1 = CD_2(CH_2)_{14}Me$, $R^2 = (CH_2)_{15}Me$ (**c**);

$R^1 = COCD_2(CH_2)_{13}Me$, $R^2 = (CH_2)_{15}Me$ (**d**).

The chemical synthesis of deuterium-labelled phosphatidylglycerols **91** has been described.[87,89] The deuterium-labelled glycerol **66** in which two hydroxy groups were blocked by isopropylidene protection was phosphorylated with phosphatidic acid **85** in the presence of a condensation agent. At the last stage, the isopropylidene protecting group in the compound **90** was removed by relatively mild treatment with boric acid. Depending on the structure of the isopropylideneglycerol used for phosphorylation, one can obtain phosphatidylglycerols with labels at any position in glycerol and of any desired stereoconfiguration. However, the yield of phosphatidylglycerols is rather low (30%) (Scheme 5).

Scheme 5



Yet another method for the introduction of deuterium-labelled glycerol into phosphatidylglycerols is based on the use of the transphosphatidylation reaction catalysed by phospholipase D, much as is the case of the compound **80**. The transphosphatidylation is also used to obtain other glycerophospholipids. It should be noted that this reaction requires a large excess of the alcoholic component. Thus in the synthesis of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-[1,1-$^2H_2$]-ethanolamine and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-[2,2-$^2H_2$]-ethanolamine, a 100-fold excess of the appropriate deuterium-labelled ethanolamine relative to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine was used.[83]

VI. Synthesis of fully deuterated lipids

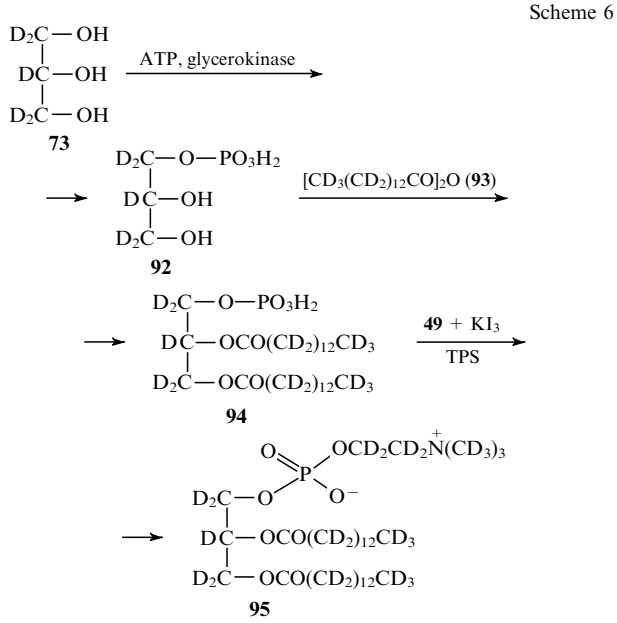
It has already been mentioned that perdeuterated lipids can be used as a transparent matrix for structural analyses of membrane proteins by high-resolution two-dimensional ^1H NMR. This has predetermined the interest in the synthesis of these compounds. There is only one publication in the literature that is dedicated to the synthesis of perdeuterated phospholipids.[73]

1. Synthesis of perdeuterated phosphatidylcholine

The synthesis of perdeuterated 1,2-dimyristoyl-*sn*-glycerophosphocholine (**95**) was carried out according to Scheme 6.[73] Perdeuterated glycerol **73** was obtained by the reduction of diethyl oxomalonate **72** with lithium aluminium deuteride. In order to obtain the desired stereoconfiguration of the substituted glycerol, an original approach has been developed, which was based on the phosphorylation of free glycerol with ATP in the presence of glycerokinase. This reaction results in high yield (66%) of glycerophosphate **92** with the same stereoconfiguration as that of the natural glycerophospholipids. Glycerophosphate **92** was isolated by precipitation from an aqueous solution as the barium salt, further converted into the pyridinium salt by ion-exchange chromatography, and then acylated with a perdeuterated myristic anhydride (**93**) in pyridine in the presence of 4-*N,N*-dimethylaminopyridine. The yield of 1,2-dimirystoyl-*sn*-glycero-3-phosphate (**94**) exceeded 90%. Perdeuterated myristic acid was obtained by the exchange on a platinum catalyst (similar to the compound **10**) and converted into the anhydride with dicyclohexylcarbodiimide. Phosphatidic acid **94** was condensed with perdeuterated choline in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride. To increase the solubility of choline in organic solvents, choline iodide **49** was converted into a complex with iodine by interaction with KI_3. The yield at the stage of the phosphorylation of choline with phosphatidic acid reaches 25% – 40%.[73]

The use of choline tetraphenylborate permits an increase in the yield of the phosphatidylcholine **95** up to 70%.[74]

Scheme 6



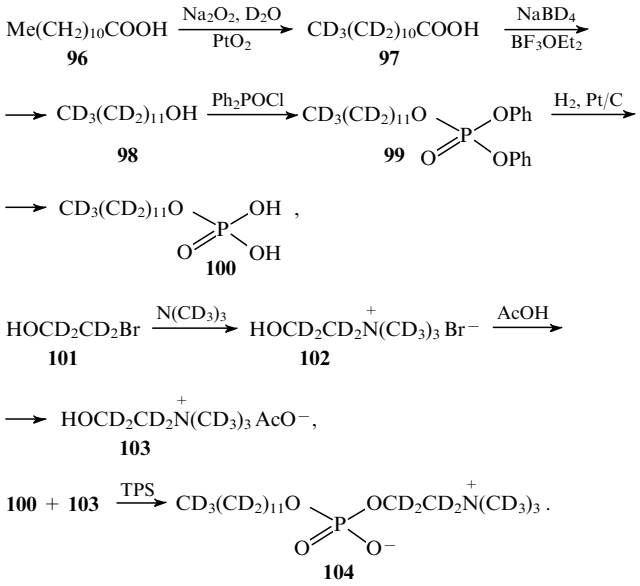
2. Synthesis of perdeuterated phospholipid-like detergents

In studies of secondary and tertiary structures of proteins in the membrane environment by two-dimensional ^1H NMR spectroscopy, perdeuterated sodium dodecyl sulfate is mostly used. The use of perdeuterated sodium dodecyl sulfate in these studies is motivated by its commercial availability. Meanwhile, sodium dodecyl sulfate is not a close structural analogue of natural phospholipids. A search for more close structural analogues of phospholipids has stimulated studies aimed at the synthesis of novel detergents.

It was proposed to use dodecyl phosphocholine as a phosphatidylcholine analogue. The structure of its polar region fully corresponds to that of phosphatidylcholine. At the same time, the presence of only one short-chain hydrocarbon residue accounts for the fact that dodecyl phosphocholine forms micelles in water. The critical micelle concentration is 1.1 mmol litre^{-1};[121] the micelle diameter is about 50 Å, and one micelle comprises 56±5 molecules of dodecyl phosphocholine.[122]

Dodecyl phosphocholine has been used in membrane studies for simulating the interaction between phospholipase A_2 and the lipid bilayer surface.[121] The synthesis of non-deuterated dodecyl phosphocholine involved the phosphorylation of dodecyl alcohol with 2-bromoethyl phosphodichloride and subsequent reaction of the bromoalkyl derivative with trimethylamine.[121] The first total chemical synthesis of perdeuterated dodecyl phosphocholine (**104**) was carried out in 1979.[77] The starting compound was lauric acid (**96**), which was subjected to total proton–deuterium exchange by the previously described method.[27] Perdeuterated lauric acid (**97**) was reduced to the alcohol (**98**). Perdeuterated dodecanol **98** was phosphorylated with diphenylphosphinoyl chloride. The protecting phenyl groups in the compound **99** were removed by catalytic hydrogenolysis to give dodecyl phosphate (**100**).

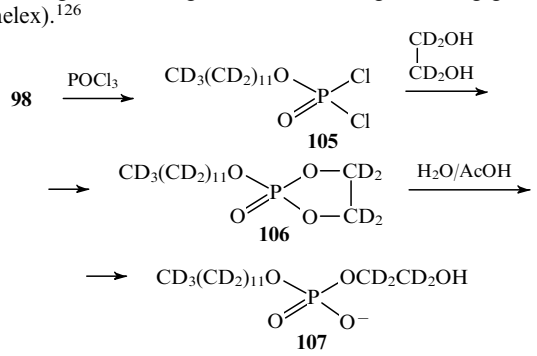
The choline component was obtained by quaternisation of perdeuterated trimethylamine with 2-bromoethanol (**101**); the resulting choline bromide (**102**) is converted into the acetate (**103**) in order to increase its solubility in organic solvents. Choline acetate **103** is phosphorylated with the phosphate **100** in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride to give perdeuterated dodecyl phosphocholine (**104**).



This scheme is multistep; the procedure of synthesis of perdeuterated choline was later modified.

Thus Dekker *et al.*[123] reduced perdeuterated lauric acid **97** with lithium aluminium deuteride to give the alcohol **98**, which was further phosphorylated with phosphorus oxychloride. The resulting phosphodichloride [$CD_3(CD_2)_{11}OP(O)Cl_2$, **105**] was hydrolysed with aqueous hydrochloric acid and dodecyl phosphate **100** was precipitated with acetone. Perdeuterated choline in the form of tetraphenylborate **40** was condensed with dodecyl phosphate in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride. The isolation of dodecyl phosphocholine (**104**) was associated with certain difficulties due to its good solubility in water, which excludes extraction. Perdeuterated dodecyl phosphocholine **104** is now commercially available.

Another representative of phospholipid-like detergents is dodecyl phosphoethylene glycol. It represents a structural analogue of the negatively charged phospholipid, phosphatidylglycerol. Together with dodecyl phosphocholine, perdeuterated dodecyl phosphoethylene glycol (**107**) was used to study the effect of lipid environment on the secondary structure of a mitochondrial signal peptide by high-resolution two-dimensional ^1H NMR spectroscopy.[124] The synthesis of perdeuterated dodecyl phosphoethylene glycol was carried out as described earlier.[125] Dodecanol **98** was phosphorylated with phosphorus oxychloride and the phosphodichloride (**105**) then reacted with perdeuterated ethylene glycol to give phospholane **106**. The latter was hydrolysed in the presence of acetic acid to give dodecyl phosphoethylene glycol **107**. All these reactions proceeded in high yields; intermediate products were not isolated. It is necessary to note that dodecyl phosphoethylene glycol possesses chelating properties and after conventional purification procedures it may contain polyvalent cations. It is therefore recommended to convert it into the sodium salt using ion-exchange resins containing chelating groups (*e.g.*, Chelex).[126]



VII. Other deuterium-labelled lipids

In addition to phospholipids, biological membranes of eukaryotic cells contain cholesterol, the content of which can make up to 25% of the total pool of membrane lipids.[127] Cholesterol plays an important role in the regulation of the phase state of the lipid bilayer.[128] To study its interaction with phospholipids by the ^2H NMR method, cholesterol with deuterium labels at different positions of the molecule and its derivatives have been synthesised.[129, 130] Syntheses of deuterium-labelled sterols are performed by methods analogous to those used in the synthesis of deuterium-labelled fatty acids.

In some cases, when chemical synthesis of deuterium-labelled lipids is a rather difficult task, microbiological synthesis can be applied. This is exemplified by the synthesis of deuterium-labelled plasmalogens.[131] The incorporation of specifically deuterated glycerols into cardiolipin is achieved by growing *E.coli* cells on culture media containing deuterium-labelled glycerol.[89, 90, 132, 133]

An analogous approach was used to incorporate deuterium-labelled oleic acid into cardiolipin and phosphatidylglycerol [134] as well as of deuterium-labelled palmitic acid into the *E.coli* membrane.[135] To this end, *E.coli* cells were grown on a culture medium containing the fatty acid and a detergent. Depending on the problem to be solved, different strains of *E.coli* were used.[89, 90, 132–135] Lipids were isolated from the culture mass by conventional methods, which included cell disintegration, extraction, and chromatographic purification. The resulting phospholipid preparations represented mixtures of homologues differing in the length and degree of unsaturation of hydrocarbon residues.

Deuterium-labelled fatty acids that are used as a nutrition source for bacteria can be involved in the biosynthesis of other fatty acids, being converted into other deuterium-labelled acids.[134, 136] Microbiological synthesis can be used for obtaining perdeuterated lipids. The synthesis of perdeuterated fatty acids by culturing of *Scenedesmus obliguus* cells on a culture medium containing heavy water has been described.[137]

Deuterium-labelled fatty acids of various structures and having labels at different positions of the hydrocarbon chain were incorporated into the membrane of a mycoplasm *A. laidlawii,* which made possible a detailed profile analysis of the order parameter of hydrocarbon chains.[138–142]

VIII. Analysis of deuterium-labelled lipids

In the synthesis of deuterium-labelled lipids, it is often necessary to monitor the isotopic purity of intermediate and final products and to establish the selectivity of the label incorporation. These

problems can be solved by mass spectrometry, high resolution NMR, gas-liquid, and liquid chromatography.[30, 31]

Mass spectrometry offers a direct and the most sensitive method of analysis of the content of deuterium in organic compounds. The main information about the use of this method in the study of lipids labelled with stable isotopes is given in the review by Rohwedder.[143] It is suggested that labelled and non-labelled molecules are ionised to the same degree. The measurements have to be performed under conditions that ensure minimum fragmentation of the molecular ion. Prior to analysis compounds with an enhanced tendency for fragmentation (unsaturated acids and alcohols) are converted into silyl derivatives.[36, 49]

The isotopic purity and the position of deuterium labels in the lipids and the intermediates used in the lipid synthesis can be established by ^{1}H NMR spectroscopy.[4–6] This method is used for the elucidation of the structure of intermediates and/or lipids that are synthesised. The position and content of deuterium labels is determined from a decrease in the integral intensity of the signal in ^{1}H NMR spectra corresponding to protons that are replaced by deuterium. However, many signals in the ^{1}H NMR spectra of lipids overlap, which affects the accuracy of the analysis.

In principle, these problems can be solved with the help of high-resolution ^{2}H NMR. In contrast to ^{1}H NMR, ^{2}H NMR spectra contain signals from deuterium labels alone, which significantly simplifies the spectra. However, the range of variations of absolute chemical shifts in the ^{2}H NMR spectra is about 6.5 times more narrow (which is in accord with the gyromagnetic proton-to-deuteron ratio) than in the ^{1}H NMR spectra. Moreover, in the recording of the ^{2}H NMR spectra one cannot use deuterium stabilisation of the magnetic field of the NMR spectrometer, which also diminishes the resolution. It is therefore rather difficult to differentiate signals from several groups with close chemical shifts despite the relative simplicity of the ^{2}H NMR spectra. To some extent, this problem can be overcome by using shift reagents, which made it possible to resolve deuteron signals in the α-, β-, γ-, and δ-positions of a fatty acid.[144]

^{13}C NMR spectroscopy is devoid of the shortcomings characteristic of ^{1}H NMR and ^{2}H NMR. The range of variations of the chemical shifts in the ^{13}C NMR spectra is one order of magnitude higher than that in the ^{1}H NMR spectra and, as a consequence, the majority of signals in the ^{13}C-NMR spectra of lipids are resolved. Another advantage of the ^{13}C NMR method is that the incorporation of deuterium induces a small isotopic upfield shift of the signal.[30, 31, 145] As a result, the spectrum simultaneously manifests resolved signals of carbon atoms covalently bound to deuterons and protons, which increases the accuracy of measurements of the isotopic purity. It should be kept in mind that precise measurements of the ratios of deuterated to non-deuterated products is possible only under conditions when ^{13}C NMR spectra are recorded under conditions of complete relaxation and in the absence of the Overhauser effect. The signals of carbon atoms bound to deuterium are readily recognised in the spectra because of the characteristic multiplicity caused by the spin-spin interaction with deuterium. The spin of deuterium $I = 1$, that is the signal of the CD-group is observed as a triplet with lines of equal intensity; the spin-spin coupling constant, J_{CD}, is about 20 Hz. A serious pitfall of the ^{13}C NMR method is its low sensitivity, which decreases even more upon substitution of protons for deuterium due to the elimination of the Overhauser effect.

Among other methods of analysis of deuterated fatty acids, special mention should be made of gas-liquid chromatography (GLC). The possibility of separation of deuterium-labelled and non-labelled fatty acids has long been known, but it was difficult to perform.[31] In the study of Rietveld *et al.*,[134] GLC was successfully used for the separation of fatty acids as methyl esters, including acids with one deuterium-labelled methylene group.

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