Biochemical and Biophysical Research Communications 453 (2014) 277–281

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Dolichol biosynthesis: The occurrence of epoxy dolichol in skipjack tuna liver

Terumi Ishiguro [a], Yuiko Morita-Fujimura [b], Yoshihiro Shidoji [c], Hiroshi Sagami [a,*]

[a] Institute of Multidisciplinary Research for Advanced Materials (IMRAM), Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan
[b] Institute of Development, Aging and Cancer (IDAC), Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
[c] Graduate School of Human Health Sciences, University of Nagasaki, 1-1-1 Academy Hills, Nagayo, Nagasaki 851-2195, Japan

ARTICLE INFO

Article history:
Received 2 May 2014
Available online 24 May 2014

Keywords:
Dolichol
Isoprenoid
Epoxide
Midbrain
Betulaprenol
Glycoprotein

ABSTRACT

Polyisoprenoid alcohols from the livers of temperate sea fish (skipjack tuna, chub mackerel, red sea bream and rainbow trout) were analyzed by using 2D-TLC, electrospray ionization (ESI) mass spectrometry and NMR methods. Dolichols (Dols) were detected in all the fish livers, and they were composed of 19–22 isoprene units with Dol-20 as the predominant prenolog. In addition, Dol-like family compounds were found by using 2D-TLC on skipjack tuna samples. These compounds were found to have a larger molecular mass than the Dol family by 16 mass units. NMR analysis indicated that the Dol-like compounds were consistent with the terminal epoxide structure of Dols (the ω-oxirane derivatives of Dols). ESI analysis also revealed the occurrence of dehydro molecules in both Dols and epoxy Dols (Dol-like) fractions. The occurrence of epoxy Dols in fish is discussed in context with the biosynthesis of Dols, which is responsible for forming Dol phosphate, which lead to Dol-PP-oligosaccharide.

1. Introduction

A naturally occurring isoprenoid alcohol with a greater carbon-chain length (dolichol, Dol) was first found in animal tissues in 1960 [1], and it was shown to act as a sugar carrier lipid during the biosynthesis of glycoproteins [2]. The Dol structure consists of two trans-isoprenes, several cis-isoprenes and α-saturated isoprene parts, and its biosynthetic pathway is largely divided into two steps (I and II). Step I is a backbone synthesis that is catalyzed by farnesyl diphosphate synthase followed by dehydrodolichyl diphosphate synthase. The initial farnesyl diphosphate synthesizing pathway is identical to the pathways that biosynthesize cholesterol, ubiquinone and farnesylated proteins, and the next pathway involves the *cis*-isoprene double bond formation of dehydrodolichyl diphosphate. Step II is a functional group conversion involving the reduction of the α-isoprene double bond. Several hypotheses for the step II have been proposed, and one was recently confirmed by identifying an enzyme (SRD5A3) that catalyzes the conversion of dehydroDol to Dol [3]. This study also noted the occurrence of another route for step II that led to the formation of Dol. This route might involve the NADPH-dependent reduction of dehydrodolichal to dolichal which was already proposed in yeast [4].

With regards to the terminal reactions in the Dol biosynthetic pathway [5], CTP- dependent kinase catalyzes the conversion of Dol to dolichyl phosphate (Dol-P), which is responsible for sugar-carrier lipids such as Dol-P-sugar, Dol-PP-sugar and Dol-PP-oligosaccharide for *N*-type glycoprotein synthesis and glycosyl phosphatidyl inositol-anchored proteins, and Dol-P phosphatase catalyzes the reverse reaction. However, fatty acid CoA transferase catalyzes the conversion of Dol to Dol ester, and esterase catalyzes the reverse reaction. The Dol derivatives are likely to be involved in the glycoprotein biosynthesis, but other Dol derivatives such as dolichoic acid (Dol-CA) have been found in ester form with Dol in bovine thyroids [6]. Furthermore, Dol-associated proteins [7] and Dol-associated glycerolipids [8] have also been found. Dol-CA and Dol have recently been found in the substantia nigra, which is located in the mid-brain region [9,10], and further oxidized Dol and Dol-CAs such as Dol (+16), Dol (+32), Dol (+48) and Dol (+64) and Dol-CA (+16) [11] were also found in similar brain regions. Because an unusually long hydrophobic chain portion of Dol or Dol derivatives is expected to affect the bilayer membrane fluidity and permeability by skewing the fatty acid structures [12], the variations in the Dol and oxidized Dol derivative content would influence brain activity. Interestingly, increased Dol content is observed with aging in the human hippocampus, but the level is unchanged or decreased in Alzheimer's disease [13,14]. The accumulation of Dol in older tissues has also been proposed as a biomarker of aging [15]. Thus, it seems that Dol derivatives play

* Corresponding author.
E-mail address: yasagami@tagen.tohoku.ac.jp (H. Sagami).

http://dx.doi.org/10.1016/j.bbrc.2014.05.079
0006-291X/

multiple roles in cells. In this respect, it may be very important to learn multiple routes for Dol synthesis.

The Dol carbon-chain length varies depending on the organisms, and humans and Antarctic sea fish have Dol-19 to 20 and Dol-20 to 21 [16]. We identified the Dol chain length of other fish living in the temperate sea and we found that fish contained Dols that were longer by one isoprene unit than those of humans [1]. Unexpectedly, we found Dol-like family compounds in the livers of one fish (*Katsuwonus pelamis*) and identified them as the ω-oxirane derivatives of Dols which had the same carbon-chain length as those of Dols. This article communicates these results, and their biosynthetic pathways are discussed in context with multiple dolichol pathways.

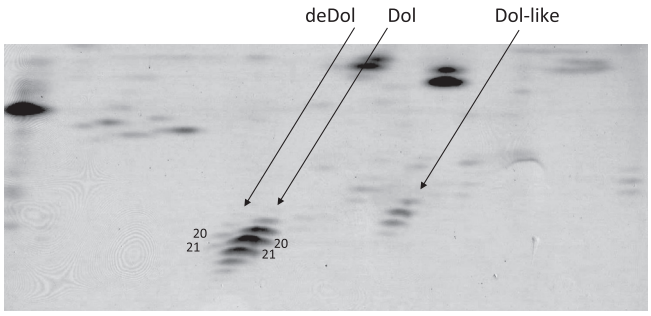

Fig. 1. 2D-thin-layer chromatograms Hexane extracts of skipjack tuna liver obtained by saponification with KOH were subjected to 2D-TLC. The first (right to left direction) and second (bottom to top direction) chromatography analyses were performed on a silica-gel plate using toluene: ethylacetate (4:1) and on a reversed-phase C18 silica-gel plate using acetone, respectively. Dol, dolichol and deDol, dehydrodolichol. The numbers (20 and 21) indicates the isoprene units of Dol and Dol-like compounds.

2. Materials and methods

The fresh livers of skipjack tuna, chub mackerel (*Scomber japonicus*), red sea bream (*Pagrus major*) and rainbow trout (a 6-month-old male *Oncorhynchus mykiss*) were locally obtained. DehydroDol (DeDol) with the predominant 17 and 18 isoprene units was prepared from gingko (*Ginkgo biloba*) leaves. Silica-gel 60 and RP-18 silica-gel thin-layer glass plates were obtained from Merck. Polyisoprenoid alcohol analyses were performed by nuclear magnetic resonance (NMR, JNM-ECA700) and electrospray ionization (ESI, Burker Daltonix Solarix) methods. Dol-CA was prepared according to a modified version of the method in [17]. Pyridinium dichromate (2 g) in DMF (5 ml) was slowly added dropwise to Dol (250 mg, a mixture of C_{90} and C_{95} purified from chicken liver) in dry acetone (15 ml). After stirring the mixture overnight at room temperature, the products were extracted with 50 ml of diethylether or ethylacetate after adding water (50 ml). The Dol-CA was purified by silica-gel 60 column chromatography with hexane: ethylacetate (9: 1) to give a pure Dol-CA (approximately 50% yield).

2.1. Preparing and purifying Dol and Dol-like compounds from skipjack tuna liver

Livers (1 g) were washed with physiological saline (0.9%w/v), cut into small pieces, and saponified after adding 1.0 ml of a mixture containing KOH 0.1125 g, ethanol 0.5 ml, and H_2O 0.5 ml with or without 0.5% (w/v) pyrogallol at 65 °C for more than 3 h. Unsaponifiable lipids were extracted with hexane and the hexane solution was washed three times with saturated saline. The hexane extracts were dried over Na_2SO_4 and analyzed by 2D-TLC [18]. Large quantities of Dol and Dol-like compounds were prepared for structure determination by using skipjack tuna livers (256 g)

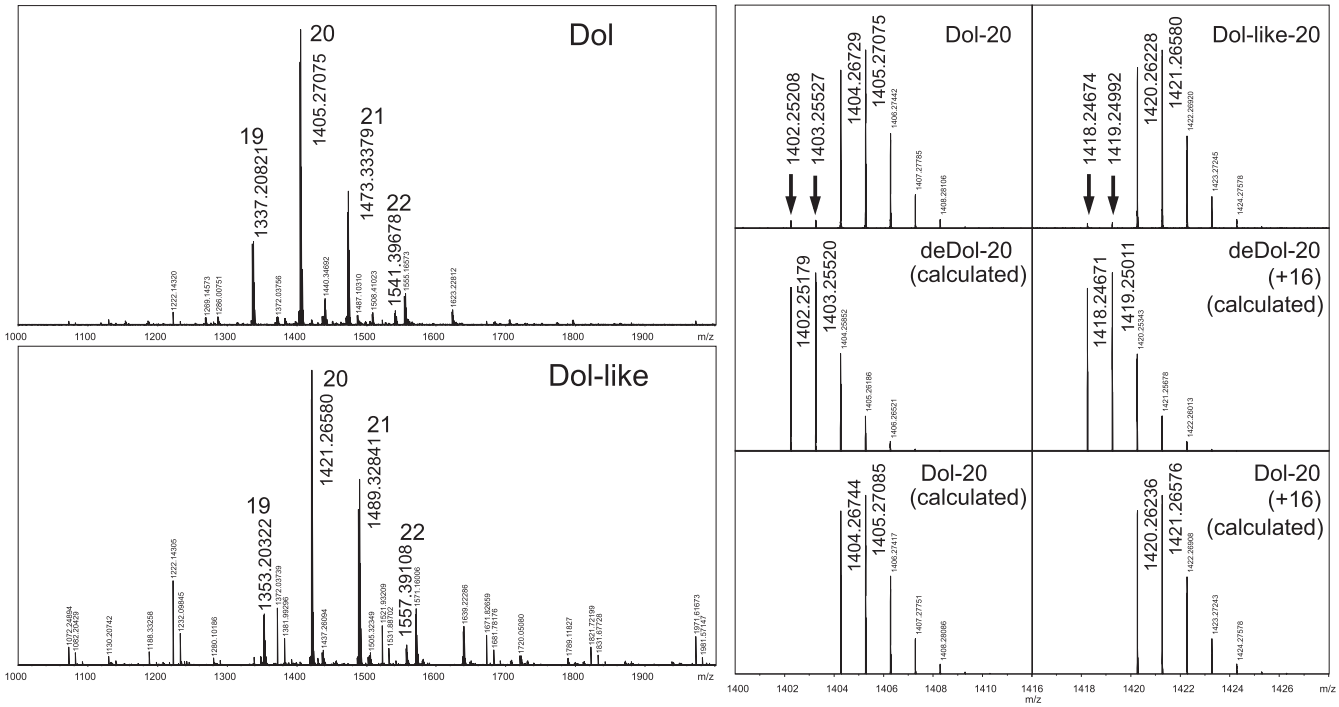

Fig. 2. The electrospray ionization spectra of Dol and Dol-like fractions and the occurrence of dehydroDol and dehydroDol-like compounds. Left panel: The Dol and Dol-like family compounds (Fig. 1) of hexane extracts were purified as described in Section 2 and analyzed by electrospray ionization mass spectrometry. Purified Dol (upper) and purified Dol-like compounds (lower). The numbers (19–22) indicates the isoprene units of Dol and Dol-like compounds. Right panel: Magnified ESI mass spectra of Dol-20 and Dol-like-20 compounds are shown in the left-top panel and the right-top panel, respectively. ESI spectra (calculated) of dehydroDol-20 and dehydroDol-20 (+16) compounds are shown in the middle panel and similar spectra (calculated) of Dol-20 and Dol-20 (+16) compounds are shown in the bottom panel. Arrows indicate the presence of dehydroDol-20 and dehydroDol-20 (+16) compounds.

followed by the addition of a 128 ml H_2O, 128 ml ethanol and 28.8 g KOH mixture at 65 °C overnight. The mixture was extracted with 250 ml of hexane, and the hexane extracts (483 mg) were applied to a silica gel 60 column (100 g, 4.5 × 12 cm) that was equilibrated with hexane: ethylacetate (9: 1). Fractions (frs. 38–58) containing Dol and fractions (frs. 68–78) containing Dol-like compounds were pooled and evaporated to dryness to yield 39.7 mg and 21.9 mg, respectively. The two samples were then applied to a seven-connection RP-18 Sep-Pak column equilibrated with acetone. Two ml fractions were collected. Fraction 9 was pure Dol and Fraction 7 and 8 were pure Dol-like compounds; they were each pooled and evaporated to dryness to give 7.3 mg and 0.8 mg, respectively.

2.2. Analyzing Dol and Dol-like compounds

Each sample was mixed with or without 1 μg of ginkgo deDols to make internal-standards and then subjected to the 2D-TLC [18]. Purified Dol and Dol-like compounds from skipjack tuna were analyzed by ^{1}H-NMR spectroscopy. The samples were each dissolved in chloroform-d or benzene-d_6.

3. Results

We first analyzed unsaponifiable lipids from skipjack tuna, mackerel, sea bream and rainbow trout livers by 2D-TLC chromatography, and the predominant Dols in four fish were identified as C_{100} and C_{105}, with contents (μg/g tissue) of 18.5, 18.4, 34.0 and 23.5, respectively. To examine whether deDols, a precursor of Dols, are detectable, higher quantities of the skipjack tuna Dol preparation were subjected to 2D-TLC. As shown in Fig. 1, a clearly separated deDol family was detected as an oblique ladder on the left-upper part of the major Dol family (Dol-20 and Dol-21). Unexpectedly, a Dol-like family was detected on the right-upper section of the Dol family. We tried to purify Dol and Dol-like compounds by silica-gel and RP-18 silica-gel chromatography, and the two purified preparations were analyzed by an ESI mass spectrometry (Fig. 2, left panel). In the case of purified Dols, positive ion primary peaks (upper) had *m/z* values $[M + Na]^+$ of 1337.2082, 1405.2708, 1473.3338 and 1541.3968 corresponding to $C_{95}H_{156}O$ (Dol-19), $C_{100}H_{164}O$ (Dol-20), $C_{105}H_{172}O$ (Dol-21) and $C_{110}H_{180}O$ (Dol-22), respectively. The positive ion primary peaks of purified Dol-like compounds were observed at *m/z* values $[M + Na]^+$ of 1353.2032, 1421.2658, 1489.3284 and 1557.3911 corresponding to $C_{95}H_{156}O_2$, $C_{100}H_{164}O_2$, $C_{105}H_{172}O_2$ and $C_{110}H_{180}O_2$, respectively (lower). Each peak from Dol and Dol-like compounds was accompanied by smaller peaks corresponding to the peaks of deDol and deDol-like compounds. Dol-20 and Dol-like-20 ESI peaks are shown in Fig. 2 (right panel). Two peaks (1402.2521 and 1403.2553) indicated by two arrows in the left corresponded to deDol-20 (calculated) and two peaks (1418.2467 and 1419.2499) indicated by two arrows in the right corresponded to deDol-20 (+16) (calculated). These results indicate that the Dol and Dol-like compounds differ from one another by 16 mass units. This is also true for the difference between deDol and deDol-like compounds. The difference of 16 mass units raised the possibility that Dol-like compounds are either epoxy Dols, hydroxy Dols or deDol-CA. We chemically synthesized Dol-CA from chicken Dol (Dol-18 and Dol-19) and compared the chromatographic behavior between Dol-like compounds and Dol-CA. The Dol-like compounds migrated faster than the Dol-CA on a silica-gel 60 thin-layer plate with toluene:ethylacetate (9:1), suggesting that Dol-like compounds contain no carboxylic group. To see whether Dol-like compounds are epoxy Dols or hydroxy Dols, we next analyzed both Dol and Dol-like compounds by NMR. The NMR results for Dol in $CDCl_3$ showed that absorption occurred at 0.907 and 0.898 ppm (3H doublet, α-methyl group) and 1.607, 1.598 and 1.594 ppm (three 3H singlets, trans allylic methyl groups). In the case of Dol-like compounds in $CDCl_3$, absorption occurred at 0.907 and 0.898 (3H doublet, α-methyl group), 1.614 and 1.606 (two 3H singlets, trans allylic methyl groups), 1.298 ppm and 1.256 ppm (two ω-terminal methyl groups), suggesting the presence of an epoxy structure at the Dol ω-double bond. To confirm the epoxy structure, we again purified Dol and Dol-like compounds by RP-18 silica-gel chromatography and measured their NMR in C_6D_6, because the C_6D_6 condition enables a clear separation of trans allylic methyl groups [19]. As shown in Fig. 3 (upper), the NMR spectra of Dol showed absorption at 1.683 (3H singlet, ω-cis-methyl group), and at 1.632, 1.616 and 1.571 ppm (three 3H singlets, trans allylic methyl groups). However, the NMR spectra of the Dol-like compound (lower) exhibited absorption at 1.630 and 1.561 (two 3H singlets, trans allylic methyl groups), at 1.157 and 1.111 (two 3H singlets, ω-terminal two-methyl groups), and at 2.572 ppm (1H triplet epoxide H). These analyses indicate that the Dol-like compounds found in the unsaponifiable lipids of skipjack tuna livers are ω-oxirane derivatives of Dol as shown in Fig. 3 (lower).

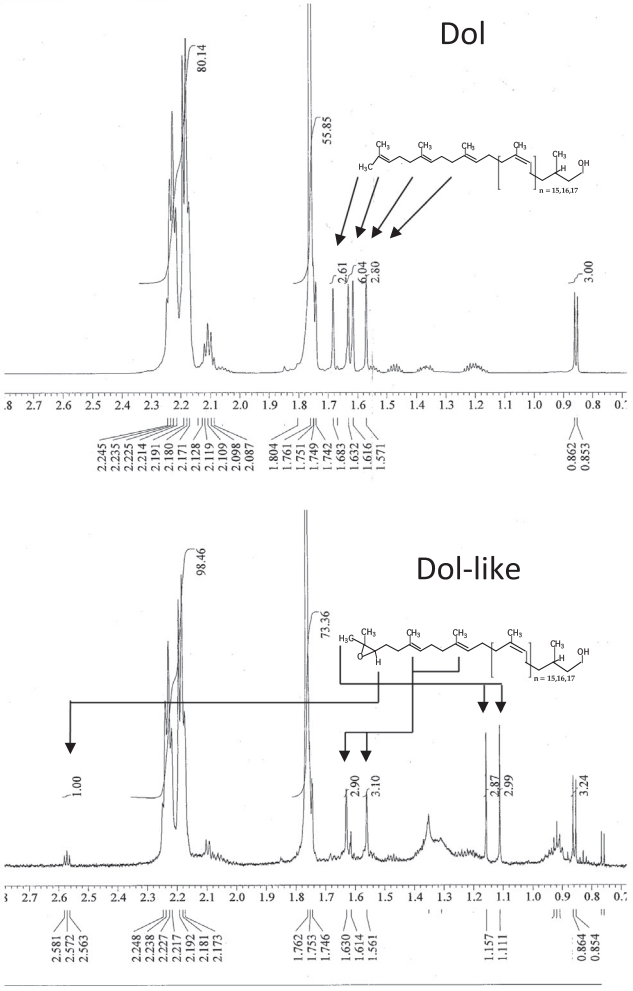

Fig. 3. ^{1}H-NMR spectra of Dol and Dol-like compounds. Dol and Dol-like compounds shown in Fig. 2 were purified once more by RP-18 chromatography and analyzed by ^{1}H-NMR in C_6D_6. Four arrows (upper panel, left to right) indicate one methyl group and three methyl groups at the Dol ω-terminal structure. Five arrows (lower panel, left to right) indicate the one epoxy proton, two trans-methyl groups, and two terminal methyl groups of the epoxy structure.

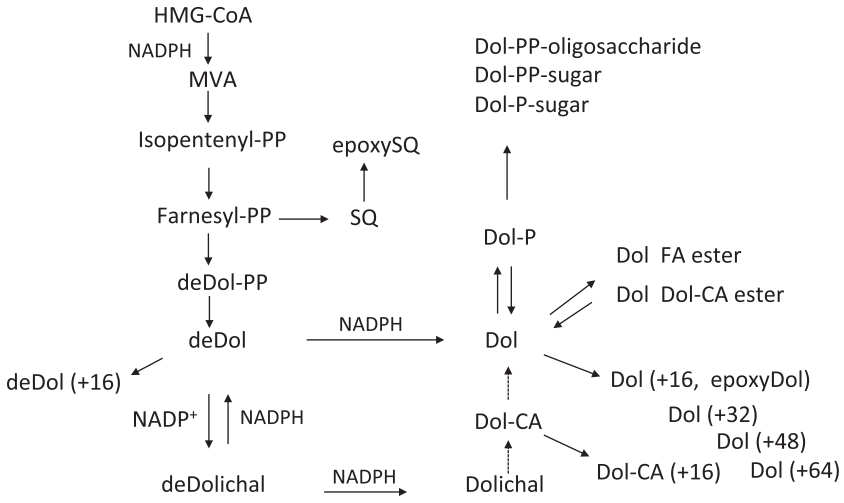

Fig. 4. The biosynthestic pathway of Dol and Dol derivatives. HMGCoA, 3-hydroxy-3-methylglutaryl Coenzyme A and deDol, dehydrodolichol.

4. Discussion

The initial purpose of the present study was to understand the dolichol carbon chain length of fish Dols, because all fish tissues are known to be rich in polyunsaturated fatty acid components such as EPA and DHA. Some fish living in an Antarctic sea contain the predominant isoprenologs Dol-20 and Dol-21 [16]. However, trout in temperate waters [20] contain Dol-18 and Dol-19. This limited information on fish Dols led us to analyze several temperate fish that could be obtained locally in Japan. The major isoprenologs of skipjack tuna, mackerel, sea bream and rainbow trout livers were all Dol-20 and Dol-21. It seems that fish contain Dol-20 and Dol-21 as major molecular species irrespective of their living environments such as the Antarctic or temperate sea, although there was a discrepancy in the chain length between trout in the literature [20] and rainbow trout in the present study. There may be a correlation in the carbon-chain lengths between Dols and fatty acids to control the membrane fluidity and permeability in each organism.

In the present study unexpected Dol-like compounds were detected in the skipjack tuna livers. The Dol-like compounds had a terminal-epoxide structure, so it might be possible to assume the oxidized Dol that was detected in the brain [11] resembles the structure found in fish. If so, it is interesting to know whether the oxidized Dols are enzymatically or non-enzymatically synthesized in either skipjack tuna liver or human brains. In the non-enzymatic case, it seems that oxidized compounds such as the Dol (+16), Dol-CA (+16) and epoxy Dol from the present study may contain no newly-formed chiral carbon. However, it seems that those molecules may have a newly-formed chiral carbon. In that case, Dol (+16), Dol-CA (+16), and epoxy Dol exhibit chirality in two-thirds of the carbons from the α-terminus [21] and from the ω-terminus. Such novel molecules are expected to have some function, because other compounds with a terminal-epoxy structure such as juvenile hormones and a chemoprophylactic agent (14,15-epoxygeranylgeraniol) [22] are biologically active.

In Fig. 4 summarizes the Dol biosynthetic pathway from 3-hydroxy-3-methylglutaryl CoA together with Dol derivatives. The conversion of deDol to Dol by the SRD5A3 action [3] has been elucidated with regards to the reduction of α-isoprene double bond in the pathway. Because the enzymatic conversion of dehydrodolichal to dolichal [4] has been suggested in yeast and because it is plausible that dolichal is oxidized to Dol-CA, it is possible to assume that the second pathway leads to the formation of Dol from deDol through dehydrodolichal, dolichal, Dol-CA and a putative Dol-CA CoA thioester. The final conversion of the thioester CoA structure to the alcohol structure is similar to that of the reduction pathway from 3-hydroxy-3-methylglutaryl CoA to mevalonate in the presence of NADPH [23].

References

[1] J.F. Pennock, F.W. Hemming, R.A. Morton, Dolichol: a naturally occurring isoprenoid alcohol, Nature 186 (1960) 470–472.
[2] N.H. Behrens, L.F. Leloir, Dolichol monophosphate glucose: an intermediate in glucose transfer in liver, Proc. Natl. Acad. Sci. U.S.A. 66 (1970) 153–159.
[3] V. Cantagrel, D.J. Lefeber, B.G. Ng, Z. Guan, J.L. Silhavy, S.L. Bielas, L. Lehle, H. Hombauer, M. Adamowicz, E. Swiezewska, A.P. De Brouwer, P. Blümel, J. Sykut-Cegielska, S. Houliston, D. Swistun, B.R. Ali, W.B. Dobyns, D. Babovic-Vuksanovic, H. van Bokhoven, R.A. Wevers, C.R. Raetz, H.H. Freeze, E. Morava, L. Al-Gazali, J.G. Gleeson, SRD5A3 is required for converting polyprenol to dolichol and is mutated in a congenital glycosylation disorder, Cell 142 (2010) 203–217.
[4] H. Sagami, Y. Igarashi, S. Tateyama, K. Ogura, J. Roos, W.J. Lennarz, Enzymatic formation of dehydrodolichal and dolichal, new products related to yeast dolichol biosynthesis, J. Biol. Chem. 271 (1996) 9560–9566.
[5] T. Chojnacki, G. Dallner, The biological role of dolichol, Biochem. J. 251 (1988) 1–9.
[6] L. Steen, G. Van Dessel, M. De Wolf, A. Lagrou, H.J. Hilderson, D. De Keukeleire, F.A. Pinkse, R.H. Fokkens, W.S. Dierick, Identification and characterization of dolichyl dolichoate, a novel isoprenoic derivative in bovine thyroid, Biochim. Biophys. Acta 796 (1984) 294–303.
[7] J. Wejde, M. Hjertman, M. Carlberg, B. Egestad, W.J. Griffiths, J. Sjövall, O. Larsson, Dolichol-like lipids with stimulatory effect on DNA synthesis: substrates for protein dolichylation?, J Cell Biochem. 71 (1998) 502–514.
[8] M. Ishinaga, A. Ueda, Novel dolichyl derivatives in rat spleen, Biosci. Biotechnol. Biochem. 63 (1999) 449–451.
[9] H. Fedorow, R. Pickford, J.M. Hook, K.L. Double, G.M. Halliday, M. Gerlach, P. Riederer, B. Garner, Dolichol is the major lipid component of human substantia nigra neuromelanin, J. Neurochem. 92 (2005) 990–995.
[10] W.C. Ward, Z. Guan, F.A. Zucca, R.G. Fariello, R. Kordestani, L. Zecca, C.R. Raetz, J.D. Simon, Identification and quantification of dolichol and dolichoic acid in neuromelanin from substantia nigra of the human brain, J. Lipid Res. 48 (2007) 1457–1462.
[11] W.C. Ward, F.A. Zucca, C. Bellei, L. Zecca, J.D. Simon, Neuromelanins in various regions of human brain are associated with native and oxidized isoprenoid lipids, Arch. Biochem. Biophys. 484 (2009) 94–99.
[12] G.P. Zhou, F.A. Troy, NMR studies on how the binding complex of polyisoprenol recognition sequence peptides and polyisoprenols can modulate membrane structure, Curr. Protein Pept. Sci. 6 (2005) 399–411.
[13] C. Edlund, M. Söderberg, K. Kristensson, Isoprenoids in aging and neurodegeneration, Neurochem. Int. 25 (1994) 35–38.
[14] G.P. Hooff, W.G. Wood, W.E. Müller, G.P. Eckert, Isoprenoids, small GTPases and Alzheimer's disease, Biochim. Biophys. Acta 2010 (1801) 896–905.
[15] I. Parentini, G. Cavallini, A. Donati, Z. Gori, E. Bergamini, Accumulation of dolichol in older tissues satisfies the proposed criteria to be qualified a biomarker of aging, J. Gerontol. A Biol. Sci. Med. Sci. 60 (2005) 39–43.

[16] W. Jankowski, T. Chojnacki, Dolichols in the liver of antarctic vertebrates, Bull. Pol. Acad. Sci. 36 (1989) 207–214.
[17] E.J. Corey, G. Schmidt, Useful procedures for the oxidation of alcohols involving pyridinium dichromate in aprotic media, Tetrahedron Lett. 20 (1979) 399–402.
[18] H. Sagami, A. Kurisaki, K. Ogura, T. Chojnacki, Separation of dolichol from dehydrodolichol by a simple two-plate thin-layer chromatography, J. Lipid Res. 33 (1992) 1857–1861.
[19] N. Ohya, Y. Tanaka, Structural characterisation of rubber from lactarius mushrooms; 1H-NMR analysis of terminal groups in the rubber chain, J. Rubber Res. 1 (1998) 84–94.
[20] M. Bizri, J.F. Bouhours, Y. Bizri, R. Got, Separation and quantitative determination of dolichol and dolichyl phosphate in rat and trout liver, Comp. Biochem. Physiol. B 84 (1986) 565–569.
[21] W.L. Adair, S. Robertson, Absolute configuration of dolichol, Biochem. J. 189 (1980) 441–445.
[22] W.B. Mors, M.F. dos Santos Filho, H.J. Monteiro, B. Gilbert, J. Pellegrino, Chemoprophylactic agent in schistosomiasis: 14,15-epoxygeranylgeraniol, Science 157 (1967) 950–951.
[23] J.L. Goldstein, M.S. Brown, Regulation of the mevalonate pathway, Nature 343 (1990) 425–430.