Absolute configuration of dolichol

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A derivative of dolichol was formed and then chemically degraded to a small fragment containing the sole centre of asymmetry of the original molecule. Polarimetric comparison of this derivative with a standard prepared from (*R*)-citronellol showed dolichol to have an *S*-configuration at C-3. To determine the optical purity of dolichol a diastereoisomeric derivative was prepared and compared with standard diastereoisomers, which could be resolved by high-pressure liquid chromatography. Dolichols from pig liver, human liver and hen oviduct were analysed by this procedure and were all found to be >95% *S*-configuration.

The dolichols are a group of compounds in the polyprenol family that are characterized by the presence of a saturated α -isoprene unit. This class of compounds found in eukaryotes contains two internal *trans*-isoprene units, with the remainder being *cis* (Hemming, 1974). The saturated α -isoprene has been found to be biogenetically *cis* by the use of stereospecifically labelled mevalonic acid as a precursor (Gough & Hemming, 1970). Saturation of the α -isoprene unit produces a centre of asymmetry at C-3 and previous polarimetric measurements on pure dolichol have demonstrated a slightly negative optical rotation (Burgos *et al.*, 1963). The absolute configuration and optical purity of dolichol have not been determined, however.

A phosphorylated ester of dolichol, dolichyl phosphate, has been shown to participate in the assembly of heterosaccharide units destined for incorporation into nascent polypeptide chains (Parodi & Leloir, 1979). It has been suggested that dolichyl phosphate participates as a coenzyme, accepting and donating monosaccharide residues as well as the final heterosaccharide product (Hemming, 1977). An identical role for the somewhat similar molecule undecaprenyl phosphate has been advanced for the biosynthesis of peptidoglycans, lipopolysaccharides and teichoic acids in prokaryotes (Hemming, 1974).

The possible importance of a saturated α -isoprene unit to dolichyl phosphate function is suggested by the work of Mankowski *et al.* (1977). These investigators showed that synthetic substrates containing saturated α -isoprene units were four to five times as active as glycosyl acceptors as those of similar chain length that were fully unsaturated. It is possible, therefore, that saturation of the α -isoprene represents a final 'activation' of the

molecule before its utilization in glycoprotein biosynthesis.

To more fully investigate the role of the saturated α -isoprene unit in the functioning of dolichyl phosphate it was felt that a logical first step would be the determination of the absolute configuration of dolichol at C-3. It is to that end that the present paper is advanced.

Experimental

Materials

Triphenylphosphine, (*R*)- α -methylbenzylamine, (*R*)-pulegone and 2-chloro-1-methylpyridinium iodide were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). (*S*)-Citronellol was a gift from Firmenich Inc. (Princeton, NJ, U.S.A.). All other chemicals used were reagent grade or better.

High-pressure liquid chromatography was carried out with a Lab Data Control Constametric II G pump and a Spectro Monitor II variable-wavelength detector set at 210 nm. The chromatography column (0.4 cm $\times$ 25 cm) contained 5 μ Spherisorb silica. Solvents used were from Fisher Chemical Co. (Orlando, FL, U.S.A.) and were 'HPLC Grade'.

G.l.c. was performed with a Packard-Becker 417 gas chromatograph with a glass column (2 mm $\times$ 6 foot) containing SP2100 (Supelco, Bellefonte, PA, U.S.A.). A linear temperature program of 75–200°C at 20°C/min was employed. Detection was by flame ionization. Polarimetry was carried out in a 10 cm quartz microcell with a Perkin-Elmer model 241 polarimeter. Low-resolution mass spectra were obtained by using a Dupont Dima-Spec mass spectrometer, and high-resolution analysis was performed with an AEI MS30 double-beam mass spectrometer.

T.l.c. was carried out on 0.2mm layers of silica gel impregnated with fluors on plastic-backed plates (EM Laboratories, Englewood Cliffs, NJ, U.S.A.). Detection of polyprenyl derivatives was achieved by using an anisaldehyde spray (McSweeney, 1965), and aldehydes were detected by an ethanolic dinitrophenylhydrazine spray. Enhancement of detection with the latter was obtained by super-spraying with the anisaldehyde reagent followed by gentle heating.

Solvents used were: A, hexane/diethyl ether (8:2, v/v); B, acetonitrile; C, hexane/diethyl ether (3:7, v/v); D, hexane/diethyl ether/acetonitrile (5:2:0.8, v/v).

Methods

Preparation of 6-acetoxy-4-methylhexanal. Dolichol from pig liver was prepared and acetylated by the method of Burgos *et al.* (1963). (*R*)-Citronellol, prepared from (*R*)-pulegone by the method of Overberger & Kaye (1967), was acetylated by an identical procedure. Ozonolysis of the polyprenyl acetates was carried out at -70°C by using a Micro-ozonizer (Supelco) until completion was indicated by the starch/iodide test. After purging the system with N_2 , cleavage of the ozonate was achieved by adding an excess of solid triphenylphosphine and allowing the reaction to warm to room temperature. Purification of the product 6-acetoxy-4-methylhexanal was carried out by preparative t.l.c. in solvent C and quantified by g.l.c. Purity was estimated as $>95\%$ for all samples. Samples were dissolved in hexane for polarimetric analysis.

Preparation of polyprenyl aldehydes. Dolichol (50mg) from pig liver, human liver or hen oviduct was dissolved in 2ml of dry dichloromethane. To the mixture was added, with stirring, 12.5mg of pyridinium chlorochromate prepared by the method of Corey & Suggs (1975). After stirring for 60min at room temperature, t.l.c. in solvent A showed a single aldehyde-positive spot ($R_F = 0.59$) migrating substantially faster than dolichol ($R_F = 0.21$). The reaction mixture was diluted with 10ml of dichloromethane and poured on to a Florisil column (1cm $\times$ 5cm) equilibrated with the same solvent. Elution was continued with dichloromethane until the product emerged (about 20ml). Yield of dolichal: 42mg (84%). (*S*)-Citronellol was oxidized to (*S*)-citronellal by an identical procedure.

Preparation of polyprenoic acids (I). Dolichal (20mg) was dissolved in 1.0ml of dichloromethane/methanol (1:1, v/v). This was added to a stirred mixture of 3.8mg of NaCN and 19mg of AgO (argentic oxide) prepared by the method of Jirsa (1935). After stirring for 16h at room temperature, the mixture was diluted with ethanol and extracted twice with hexane to remove the product. The crude

acid (11.6mg, 58% yield), after evaporation of the hexane, had an $R_F = 0.20$ in solvent A and was used without purification in the next step. (*S*)-Citronellic acid was obtained by an identical procedure, whereas (*R*)-citronellic acid was prepared from (*R*)-pulegone (Overberger & Kaye, 1967).

Preparation of (*R*)- α -methylbenzylamides (II). Dolichoic acid (I) (10mg) was dissolved in 1.0ml of dichloromethane. To this mixture was added 5.1mg of 2-chloro-1-methylpyridinium iodide, 3.9mg of tributylamine and 5mg of (*R*)- α -methylbenzylamine. After stirring for 3h, the mixture was extracted with dilute aqueous HCl. T.l.c. of the organic phase showed a single u.v.-positive anisaldehyde-positive spot ($R_F = 0.36$, solvent C).

The (*R*)- and (*S*)-citronellamides were prepared in the same manner, giving their α -methylbenzylamides ($R_F = 0.22$, solvent C).

Preparation of 3-methyl-6-oxohexanamides (III). The amide (II) (5mg), dissolved in 5ml of dichloromethane, was ozonized and cleaved in a manner identical with the preparation of 6-acetoxy-4-methylhexanal above. Purification was carried out by preparative t.l.c. with three plates (20cm $\times$ 20cm) developed in solvent C. Bands corresponding to the desired product (2,4-dinitrophenylhydrazine-positive) were scraped and eluted with acetonitrile ($R_F = 0.51$). Mass-spectral analysis of the purified material gave m/e 247 (M^+ , the molecular ion), 204 ($M - \text{CH}_2 - \text{CHO}^+$), 190 ($M - [\text{CH}_2]_2 - \text{CHO}^+$), 163 ($M - (\text{CH}_3) - \text{CH}[\text{CH}_2]_2 - \text{CHO}^+$), 120 ($\text{C}_6\text{H}_5 - \text{CH} - (\text{CH}_3) - \text{NH}$), 105 ($\text{C}_6\text{H}_5 - \text{CH} - \text{CH}_3$, the base peak). High-resolution mass-spectral analysis gave m/e 247.15703 (calculated for $\text{C}_{15}\text{H}_{21}\text{NO}_2$: 247.15723).

Results and discussion

Initial efforts to determine the absolute configuration of dolichol used polarimetric analysis. To do this, it was necessary to convert dolichol into a form comparable with standards of known absolute configuration. The derivative chosen was 6-acetoxy-4-methylhexanal, which was obtainable from dolichol as well as from the standards (*R*)- and (*S*)-citronellol. The chemical conversion of dolichyl acetate (pig liver) and (*R*)-citronellyl acetate to 6-acetoxy-4-methylhexanal is shown in Scheme 1.

Comparison of polarimetric measurements on 6-acetoxy-4-methylhexanal derived from dolichyl acetate and from (*R*)-citronellyl acetate is shown in Table 1. It was clear from these results that dolichol was likely to have an absolute configuration predominantly *S* at C-3, but the optical purity could not be assessed due to the low absolute rotations observed.

To accurately measure the optical purity of dolichol, diastereoisomers were prepared by an unambiguous synthetic procedure shown in Scheme

Scheme 1. Preparation of 6-acetoxy-4-methylhexanal from citronellyl acetate and dolichyl acetate

Table 1. Optical-rotatory-dispersion data

Rotations were measured at 23° in hexane with an Hg lamp. Each sample concentration was 0.1 g/100 ml.

Wavelength (nm)	(R)-6-Acetoxy-4-methylhexanal* (α)	6-Acetoxy-4-methylhexanal† (α)
365	+12.3	-12.4
404	+9.7	-13.5
434	+8.1	-7.2
546	+5.3	-5.2
579	+4.2	-2.0

* From (R)-citronellol.

† From pig liver dolichol.

2. Preparation of polyprenic acids (I) involved intermediate oxidation to the aldehydes by the method of Corey & Suggs (1975) followed by argentic oxide oxidation to polyprenic acids (I) by the method of Corey *et al.* (1968). A two-step protocol was employed since other procedures tested (Jones reagent, Sarett reagent) gave either low yields or excessive amounts of by-products. In addition, dolichoic acid is extremely difficult to separate from dolichol by t.l.c. Coupling of the polyprenic acids (I) to the optically active amine [(R)-α-methylbenzylamine] to form the diastereoisomeric amides (II) used 2-chloro-1-methylpyridinium iodide as a coupling agent, a method developed previously by Bald *et al.* (1975). Attempted syntheses using dicyclohexylcarbodi-imide or carbonyldi-imidazole as coupling reagents gave substantially lower yields of amide. Ozonolysis of the amide (II) and isolation of the product (III) by t.l.c. preceded successful high-pressure-liquid-chromatographic resolution of the (R)-acid, (R)-amine and (S)-acid, (R)-amine diastereoisomeric amide standards prepared from (R)- and (S)-citronellic acid (Fig. 1a). Previous successful high-pressure-liquid-chromatographic separations of diastereoisomeric amides of citronellic acid (Scott *et al.*, 1976; Bergot *et al.*, 1978) had used optically active α-methyl-*p*-nitrobenzylamine (not commercially available) or 1-(1-naphthyl)-ethylamine, which is sensitive to ozone. We were able to separate the α-methylbenzylamides by

Scheme 2. Preparation of the (R)-α-methylbenzylamides of 3-methyl-6-oxohexanoic acid

high-pressure liquid chromatography through the injection of small quantities of sample (5 μg or less) and by detecting at 210 nm where the benzylamides absorb the strongest.

When the diastereoisomer derived from dolichol was chromatographed alone or with pure (R,R)-

Fig. 1. High-pressure-liquid-chromatographic resolution of diastereoisomeric amides (III). Chromatographic conditions were: elution solvent D, 1.0 ml/min. Detection was at 210 nm; full-scale deflexion 0.16 A unit; chart speed 20 cm/h (8 in/h). (a) Diastereoisomeric amides (III) prepared from (*R*)- and (*S*)-citronellol; (b) amide (III) prepared from pig liver dolichol; (c) mixture of amide (III) from pig liver dolichol and (*R*)-citronellol.

(III), the results shown in Figs. 1(b) and 1(c) were obtained. Co-injection of the dolichol-derived diastereoisomer with pure (*S,R*)-(III) gave a single peak with substantially increased amplitude. It was thus clear that pig liver dolichol had an *S* configuration at C-3, confirming the polarimetric results. Optical purity was estimated as >95%.

It should be noted that previous investigations of similar prenoic acid diastereoisomers using high-pressure liquid chromatography reported that the (*R,R*)-diastereoisomer emerged before the (*S,R*)-diastereoisomer, the reverse of what was found by us. Bergot *et al.* (1978) reported that the (3*S*)-7-methoxycitronellamide of (*R*)-1-(1-naphthyl)-ethylamine emerged anomalously earlier than its opposite diastereoisomer. These results, in combination with our own present findings, suggest that it is possible that a polar group distal to the diastereoisomeric centre induces this change in elution pattern.

The determination of the absolute configuration of dolichol should facilitate studies on the enzymic biosynthesis of the molecule, since it permits the unambiguous identification of the product. Recent studies on the enzymic synthesis of dolichyl phosphate (Daleo & Pont Lezica, 1977; Daleo *et al.*, 1977; Grange & Adair, 1977) suggest the requirement of a long-chain prenyl transferase and an α -isoprene reductase. The long-chain prenyl transferase has been solubilized from hen oviduct membranes and partially characterized (Grange & Adair, 1977), but nothing is known of the reductase, its subcellular location, polyprenyl substrate or

reducing cofactor. The only reported enzymological studies of α -isoprene reduction have been on the conversion of geraniol into citronellol in extracts of rose petals (Dunphy & Allcock, 1972). The enzyme that catalysed that reaction was found to be cytosolic in origin, utilized the free alcohol as a substrate and employed NADPH as a reducing cofactor. Recent results in our laboratory (P. T. Bates & W. L. Adair, unpublished work) suggest that the substrate for the mammalian α -isoprene reductase is 2,3-didehydrololichyl pyrophosphate and the hydrogen donor is NADPH. Thus the mammalian α -isoprene reductase appears to differ from the plant enzyme in utilizing a pyrophosphate ester as a substrate rather than the free alcohol.

When dolichols from human liver and hen oviduct were analysed in a manner identical with that for pig liver, it was found that all three dolichols were *S* in configuration (>95% optical purity in all cases). The fact that dolichols from both mammals and birds have the same absolute configuration is intriguing since it supports the concept of a function for the saturated α -isoprene unit, which has been preserved in the same stereochemical orientation throughout millions of years of evolution. To more rigorously test the hypothesis of functionality for the chiral saturated α -isoprene requires the synthesis of (*R*)-dolichyl phosphate and the testing of this compound as a glycosyl acceptor and donor.

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