Determination of arrangement of isoprene units in pig liver dolichol by ^{13}C-n.m.r. spectroscopy

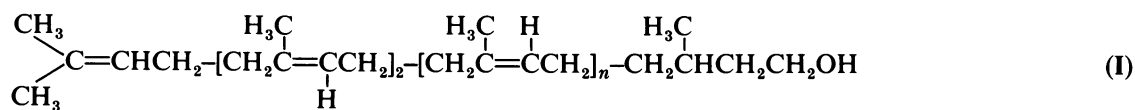Yasuyuki TANAKA, Hisaya SATO, Akira KAGEYU* and Toshihiko TOMITA
Department of Material Systems Engineering, Faculty of Technology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184, Japan

The arrangement of isoprene units in pig liver dolichol-18, -19 and -20 was determined by ^{1}H- and ^{13}C-n.m.r. spectroscopies. The alignment of *trans* and *cis* isoprene units was found to be in the order: dimethylallyl unit, two *trans* units, a sequence of 14–16 *cis* units, and a saturated isoprene unit terminated with a hydroxyl group, which verified the presumed chemical structure of dolichol. The absence of geometric isomers was confirmed. A slight amount of impurity was detected in each reversed-phase h.p.l.c. fraction of dolichol obtained by a conventional method. Detailed assignments of the ^{13}C-n.m.r. spectrum were given for these dolichols by using model compounds and INEPT (insensitive nuclei enhanced by polarization transfer) measurement. The chemical structure of synthetic dolichol-19, which was prepared by the addition of a saturated isoprene unit to the polyprenol-18 isolated from *Ginkgo biloba*, was confirmed to be identical with that of pig liver dolichol-19.

INTRODUCTION

Dolichols are a series of long chain polyisoprenoid alcohols found in many mammals, yeasts, and higher plants. In these organisms, the phosphate esters of dolichols function as glycosylated intermediates in the transfer of sugar to peptides. The dolichols generally consist of 14–24 isoprene units with a saturated isoprene

absolute configuration of the saturated isoprene terminal unit (α-terminal) was found to be an S-configuration in dolichols from pig liver, human liver, and hen oviduct (Adair & Robertson, 1980). On the basis of the proposed structure of mammalian dolichols, synthetic dolichol was prepared by the addition of an optically active saturated isoprene unit to the polyprenol acetate isolated from leaves of *Ginkgo biloba* (Suzuki *et al.*, 1983).

$$\underset{\substack{CH_3 \\ | \\ CH_3}}{>}C{=}CHCH_2{-}[CH_2\underset{\substack{H_3C \\ | \\ H}}{C}{=}CCH_2]_2{-}[CH_2\underset{\substack{H_3C \ H \\ | \ | }}{C}{=}CCH_2]_n{-}CH_2\underset{\substack{H_3C \\ | }}{C}HCH_2CH_2OH \qquad (I)$$

unit terminated with a hydroxyl group (Hemming, 1983). Two of the internal isoprene units have been determined to be in *trans* configuration and the others in *cis* configuration for pig liver and human dolichols by ^{1}H-n.m.r. analysis (Burgos *et al.*, 1963; Feeney & Hemming, 1967). The presence of two internal *trans* units was further confirmed biochemically by using double-labelled radioactive mevalonate (Martin & Thorne, 1974; Gough & Hemming, 1970). Unfortunately, these methods provide no direct information on their precise position in the dolichol molecule. It was found that the dolichols are formed from all-*trans* farnesyl pyrophosphate by biosynthesis *in vitro* in rat liver slices (Wong *et al.*, 1982). This was also confirmed in part by biosynthesis *in vitro* of a long chain polyprenyl phosphate with a particulate enzyme from hen oviduct (Grange & Adair, 1977). These facts strongly support the idea that the two *trans* isoprene units are linked to the dimethylallyl units (ω-terminal) and that dolichols have the arrangement of isoprene units as shown by I (Gough & Hemming, 1970; Butterworth & Hemming, 1968). The

The polyprenols thus far found are classified into three groups according to the number of *trans* units in the molecule: (*a*) two-*trans* and poly-*cis* type, (*b*) three-*trans* and poly-*cis* type, and (*c*) all-*trans* type (Hemming, 1979). On the other hand, the presence of isomers containing 1.2–3.0 *trans* units has been suggested for polyprenols isolated from *Magnolia campbellii* by ^{1}H-n.m.r. analysis (Sasak *et al*, 1977). In the case of dolichols the presence of geometric isomers has not been suspected. A small amount of C_{55} dolichols containing three *trans* units was isolated from pig liver. This was proposed to have arisen from plant polyprenols present in the diet (Mańkowsky *et al*, 1976).

We have proposed a new ^{13}C-n.m.r. method to characterize the arrangement of the *trans* and *cis* isoprene units in polyprenol molecules (Tanaka *et al*, 1982*a*). The method was successfully applied to the determination of the detailed structure of ficaprenol-11 (Tanaka & Takagi, 1979), polyprenols from *Ginkgo biloba* (Ibata *et al.*, 1983*a*, 1984) and polyprenols from a family of pinaceae (Ibata *et al*, 1983*b*). The present

---

paper reports the determination of the arrangement of isoprene units in pig liver dolichol, and explores the possibility of the existence of geometric isomers by using [1]H- and ^{13}C-n.m.r. spectroscopies, and also presents the comparison of the structure with that of synthetic dolichol.

EXPERIMENTAL

Dolichol was isolated from pig liver according to the method of Burgos et al. (1963). Dolichol fractions were separated from unsaponifiable lipid by preparative gel permeation columns (21.2 mm int. diam. × 60 cm × 3) packed with styrene/divinylbenzene gel (Tanaka et al., 1982b). Commercially obtained pig liver dolichol (Sigma; dolichol Grade I, approx. purity 98%) was also used without further purification. These dolichol mixtures were isolated into each component by h.p.l.c.

H.p.l.c. separation was carried out using a JASCO TRIROTAR II as a high-pressure pump and a Waters R 401 differential refractometer as a detector. Preparative separation was done using 600 mm × 20.0 mm int. diam. or 600 mm × 21.2 mm int. diam. stainless steel columns with a flow rate of 4–6 ml/min using ODS-silica gel of 5 μm diameter as a stationary phase and ethanol as an eluent.

The ^{13}C-n.m.r. spectra were obtained at 50.1 MHz with a JEOL FX-200 spectrometer. Measurements were made at room temperature in C^2HCl_3 solution (5%, w/v). Chemical shifts were referred to TMS as an internal standard. The accuracy of the chemical shifts was ±0.01 p.p.m.

RESULTS AND DISCUSSION

Distribution of chain length

Fig. 1 shows the distribution of chain length in the dolichol isolated from fresh pig liver determined by reversed-phase h.p.l.c. The central three fractions gave a field-desorption mass spectrum with peaks at 1244, 1312 and 1380, respectively, which are in accord with the theoretical values for dolichols -18, -19 and -20. The other peaks in Fig. 1 were assigned to dolichols -17, -21 and -22 by using the calibration curve between the chain length and elution volume. The percentages by weight of dolichols -17 to -22 were found to be 4.0, 21.8, 42.5, 24.0, 6.3, and 1.4% respectively. H.p.l.c. of commercially
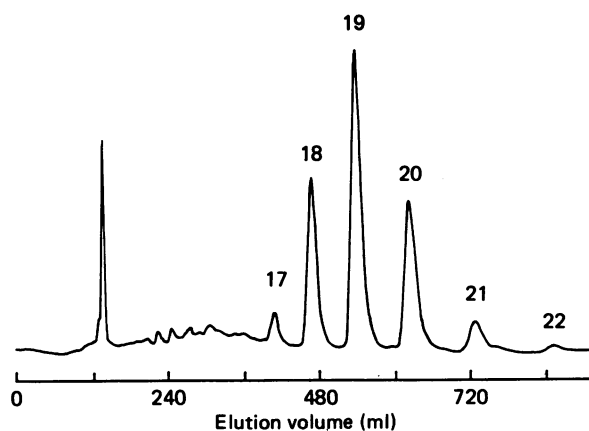

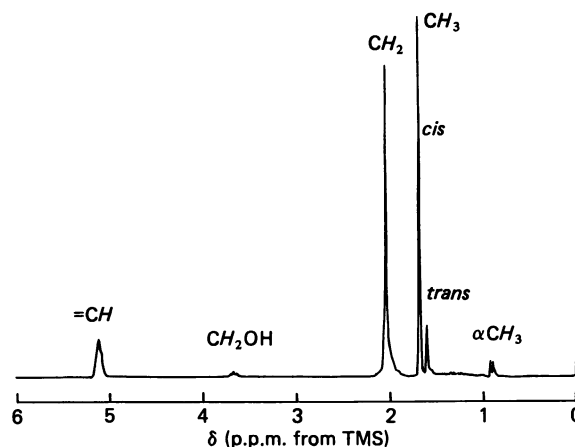Fig. 1. Reversed-phase h.p.l.c. of dolichols from pig liver

Sample size, 5 mg.

Fig. 2. [1]H-n.m.r. spectrum of dolichol-19

obtained dolichol gave a slightly different distribution of chain length, i.e. 0.4, 2.0, 11.7, 36.9, 32.6, 13.6 and 2.8% for dolichols -16 to -22. The isolated central three fractions showed the same field-desorption mass spectra as those of fresh dolichols -18 to -20. The purities of the dolichols -18, -19, and -20 were found to be 99.3, 99.8, and 100% by analytical h.p.l.c. measurements.

Arrangement of isoprene units

Fig. 2 shows the [1]H-n.m.r. spectrum of dolichol-19 isolated by preparative h.p.l.c. The spectrum is essentially in agreement with that reported by Feeney & Hemming (1967). The observed relative intensities are in good agreement with theoretical values for dolichols -18 to -20 calculated on the assumption of the presence of two trans units in the molecule, as listed in Table 1. The accuracy of the [1]H-n.m.r. measurements is confirmed by comparison of relative intensities of the signals in solanesol, ficaprenol-11 (Tanaka & Takagi, 1979), and polyprenol-18 from Ginkgo biloba (Ibata et al., 1983a). These findings clearly show that dolichol from pig liver contains just two internal trans units, as proposed by Burgos et al. (1963).

The arrangement of the internal trans and cis units can be determined by the ^{13}C-n.m.r. method (Tanaka et al., 1982a; Tanaka & Takagi, 1979). Fig. 3 shows the oridinary ^{13}C-n.m.r. spectrum and the INEPT ^{13}C-n.m.r. spectrum (Doddrell & Pegg, 1980) of dolichol-19. The signals are assigned by the comparison of the chemical shifts with those of model compounds (Tanaka et al., 1982a) as well as information from the INEPT spectrum as listed in Table 2. As shown in Fig. 4 the C-1 methylene carbon atoms in the cis units exhibited two signals at 31.97 and 32.22 p.p.m. and those in the trans units at 39.72 p.p.m., where the carbon atoms are designated for each isoprene unit, including both ω- and α-terminal units, as follows:

$$\begin{array}{c} 5 \\ C \\ | \\ -C-C=C-C- \\ 1\ 2\ 3\ 4 \end{array}$$
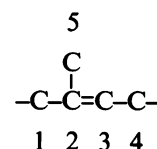
The signals at 32.22 and 31.97 p.p.m. are assigned to the C-1 methylene carbon atom of the cis units in cis–cis and trans–cis linkages, respectively. The signal at 39.72 p.p.m. is assigned to the C-1 methylene carbon

Table 1. Relative intensities of ^{1}H-n.m.r. signals in dolichols -18, -19 and -20

Theoretical values are in parentheses.

Chemical shift (p.p.m.)	Assignment	Relative intensity in dolichol:		
		18	19	20
0.82, 0.92	CH_3 α	0.83 (1)	0.92 (1)	1.00 (1)
1.60	CH_3 trans			
	ω (trans)	3.07 (3)	3.02 (3)	2.97 (3)
1.68	CH_3 cis α			
	ω (cis)	15.1 (15)	16.1 (16)	17.0 (17)
3.64, 3.67, 3.71	CH_2OH	1.06 (1)	1.08 (1)	1.10 (1)
5.12	$=CH$	16.9 (17)	17.9 (18)	18.9 (19)

atom of the *trans* units in the *trans–trans* and ω-*trans* linkages (Tanaka *et al.*, 1982*a*). If there is a *cis–trans* linkage, the C-1 methylene carbon in the *trans* unit is expected to resonate around 39.9 p.p.m. (Tanaka *et al.*, 1982*a*). The signal observed at 40.02 p.p.m. is ascribed to the C-3 methylene carbon atom in the α-terminal unit. The chemical shift of this particular carbon atom is estimated to resonate at 40.1 ± 1.5 p.p.m. by using an empirical calculated method (Bremser *et al*, 1982). This assignment was also confirmed by the comparison of the ^{13}C-n.m.r. spectrum of polyprenol-18 isoplated from *Ginkgo biloba*. The absence of the signal characteristic of the *cis–trans* linkage indicates that the *trans* units are in the ω-*trans–trans* sequence. The presence of the ω-*trans* linkage is further confirmed by the characteristic C-2 olefinic carbon signal of the ω-terminal unit resonating at 131.20 p.p.m. It has been established that the corresponding carbon atom in the ω-*trans* linkage shows a signal around 131.0–131.3 p.p.m., while that in the ω-*cis* linkage resonates around 131.5–131.6 p.p.m. (Tanaka *et al.*, 1982*a*; Tanaka, 1984).

The relative intensities of the signals reflecting the ω-*trans* + *trans–trans*, *trans–cis*, and *cis–cis* linkages were determined for dolichols -18, -19 and -20 together with those obtained by the gated decoupling measurement for dolichol-19, as listed in Table 3. Here, the spectra were obtained with multiple scans at a pulse

repetition time of 7 s or 15 s for a 45° pulse. These conditions are adequate for the quantitative measurement of the ^{13}C-n.m.r. spectrum by considering the spin-lattice relaxation times (T_1) of 1.44, 0.57 and 0.83 s for the *trans–trans* + ω-*trans*, *trans–cis* and *cis–cis* signals, respectively, and also the nuclear Overhauser values of 3.09, 2.91 and 2.72 for each signal. The observed intensity ratios are in good agreement with those expected for the arrangement of the isoprene units, i.e., the ω-terminal unit, two *trans* units and 15–17 *cis* units aligned in that order.

The presence of geometric isomers was suggested for polyprenol-11 isolated from the leaves of *Magnolia campbellii* by reversed-phase t.l.c. measurements (Sasak *et al.*, 1977). A similar method was applied to dolichol-19 in order to check the presence of geometric isomers.

Table 2. Assignment of ^{13}C-NMR signals in dolichols -18, -19 and -20

Chemical shift (p.p.m.)	Assignment
16.00	$-CH_3$ C-5 *trans*
17.67	$-CH_3$ C-5 ω(*trans*)
19.56	$-CH_3$ C-5 α
23.42	$-CH_3$ C-5 *cis*
25.31	$-CH_2$ C-4 *cis*-α
25.67	$-CH_3$ C-1 ω(*cis*)
26.44	$-CH_2$ C-4 *cis*
26.68	$-CH_2$ C-4 *trans*
26.82	$-CH_2$ C-4 ω
29.32	$-CH$ C-2 α
32.02	$-CH_2$ C-1 *trans–cis*
32.25	$-CH_2$ C-1 *cis–cis*
37.54	$-CH_2$ C-1 α
39.76	$-CH_2$ C-1 *trans–trans* ω-*trans*
40.02	$-CH_2$ C-3 α
61.21	$-CH_2OH$ C-4 α
124.21	$=CH$ C-5 *trans–cis*
124.27	$=CH$ C-3 *trans–trans*
124.45	$=CH$ C-3 ω-*trans*
125.07	$=CH$ C-3 *cis*
125.45	$=CH$ C-3 *cis*-α
131.20	$=C$ C-2 ω-*trans*
134.93	$=C$ C-2 *trans–cis*
135.00	$=C$ C-2 *cis*-α
135.22	$=C$ C-2 *cis*
135.35	$=C$ C-2 *trans–trans*
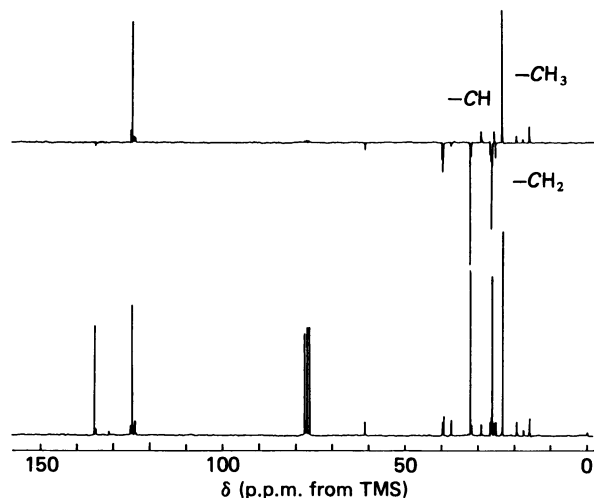
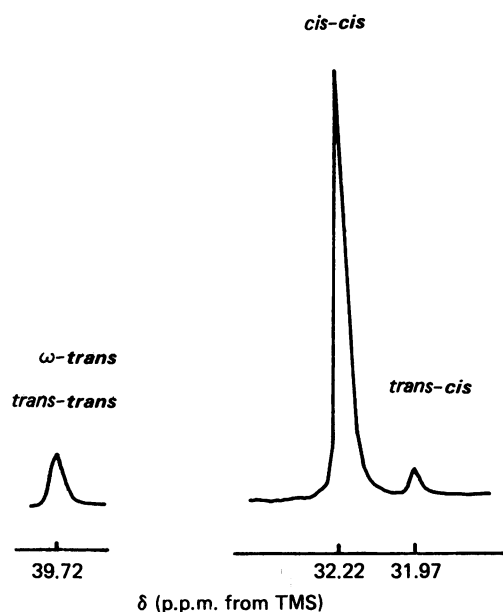**Fig. 3. Complete decoupling and INEPT ^{13}C-n.m.r. spectrum of pig liver dolichol-19**

Fig. 4. C-1 methylene signals of pig liver dolichol-19

Fig. 5. Complete decoupling ^{13}C-n.m.r. spectrum of synthetic dolichol-19

However, a distinct separation was not observed. The possibility of all-*trans* and all-*cis* isomers in dolichol was excluded by the absence of the characteristic ^{13}C-n.m.r. signals due to the *trans* α-terminal unit and the ω-terminal unit in the ω-*cis* linkage. These findings demonstrate that pig liver dolichol has the structure I as proposed by Burgos *et al.* (1963).

Small peaks were always observed as a shoulder or tailing of peak in the analytical and preparative h.p.l.c. of commercially obtained dolichol. Similar peaks were observed in the h.p.l.c. of pig liver dolichol (Pullarkat & Reha, 1982). The right-hand shoulder fraction of dolichol-19 was separated on preparative gel-permeation chromatography. The ^{13}C-n.m.r. spectrum of the fraction showed a small signal at 62.75, 36.53, 29.72, and 13.40 p.p.m. in addition to the signals due to dolichol. These signals are assigned to the CH_2OR, $CH_2C=O$, $(CH_2)_n$ and CH_3 carbons in an aliphatic ester. The ^{1}H-n.m.r. spectrum of the fraction showed the aliphatic methylene $(CH_2)_n$ signal at 1.25 p.p.m. This very small amount of the fatty acid ester is probably not an ester of dolichol, considering its elution volume in h.p.l.c. and also the low solubility of dolichol esters in ethanol. Dolichol may be contaminated by the fatty acid ester during the extraction process.
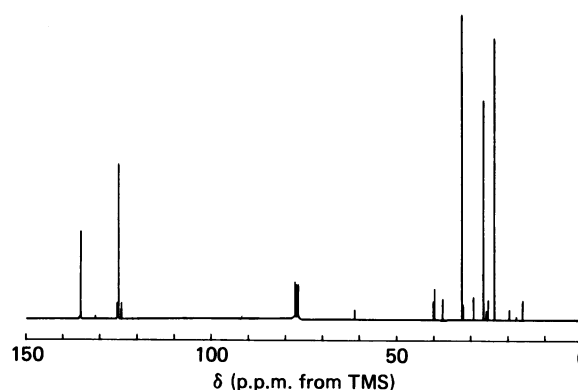
Comparison of natural and synthetic dolichols

The dolichol-19 isolated from synthetic dolichol by using reversed-phase h.p.l.c. exhibited an identical ^{13}C-n.m.r. spectrum as shown in Fig. 5. The synthesis of dolichol is achieved by the addition of the saturated α-terminal unit having the *S*-configuration to the polyprenyl acetate, using a Grignard coupling reaction. The starting polyprenol is isolated from the leaves of *Ginkgo biloba* (Suzuki *et al*, 1983). The structure of the polyprenol was found to be a typical two-*trans* polyprenol consisting of the ω-terminal unit, two *trans* units, 11–17 *cis* units and a *cis* α-terminal unit aligned in that order (Ibata *et al.*, 1983*a*). The agreement of the ^{13}C-n.m.r. chemical shifts and relative intensities clearly indicates that the synthetic and pig liver dolichols have an identical chemical structure.

REFERENCES

Adair, W. L., Jr. & Robertson, S. (1980) Biochem. J. **189**, 441–445

Bremser, W., Franke, B. & Wagner, H. (1982) Chemical Shift Ranges in Carbon-13 NMR Spectroscopy, Verlag Chemie, Weinheim

Burgos, J., Hemming, F. W., Pennock, J. F. & Morton, R. A. (1963) Biochem. J. **88**, 470–482

Butterworth, P. H. W. & Hemming, F. W. (1968) Arch. Biochem. Biophys. **125**, 503–508

Doddrell, D. M. & Pegg, D. T. (1980) J. Am. Chem. Soc. **102**, 6388–6390

Feeney, J. & Hemming, F. W. (1967) Anal. Biochem. **20**, 1–15

Gough, D. P. & Hemming, F. W. (1970) Biochem. J. **118**, 163–166

Table 3. Relative intensities of C_1 methylene signals in dolichols -18, -19 and -20

Chemical shift (p.p.m.)	Assignment	Relative intensity* in dolichols:				
		18†	19†	20†	19‡	19§
39.76	ω-*trans* + *trans–trans*	1.93 (2)	1.92 (2)	1.96 (2)	2.06 (2)	2.07 (2)
32.25	*cis–cis*	14.0 (14)	15.1 (15)	16.1 (16)	14.9 (15)	15.0 (15)
32.02	*trans–cis*	1.06 (1)	0.94 (1)	0.98 (1)	1.02 (1)	0.96 (1)

* Theoretical values are in parentheses.
† Complete decoupling measurement with pulse repetition time of 7 s.
‡ Complete decoupling measurement with pulse repetition time of 15 s.
§ Gated decoupling measurement with repetition time of 15 s.

Grange, D. K. & Adair, W. L., Jr. (1977) Biochem. Biophys. Res. Commun. **65**, 734–740

Hemming, F. W. (1979) MTP Int. Rev. Sci. Biochem. Ser. 1, **4**, 39–98

Hemming, F. W. (1983) in Biosynthesis of Isoprenoid Compounds (Porter, J. W. & Spurgeon, S. L., eds.), vol. 2, pp. 305–354, John Wiley, New York

Ibata, K., Mizuno, M., Takigawa, T. & Tanaka, Y. (1983*a*) Biochem. J. **213**, 305–311

Ibata, K., Kageyu, A., Takigawa, T., Okada, M., Nishida, T., Mizuno, M. & Tanaka, Y. (1983*b*) Phytochemistry **23**, 2517–2521

Ibata, K., Mizuno, M., Tanaka, Y. & Kageyu, A. (1984) Phytochemistry **23**, 783–786

Mańkowsky, T., Jankowski, W., Chojnacki, T. & Franke, P. (1976) Biochemistry **15**, 2125–2130

Martin, H. G. & Thorne, K. J. I. (1974) Biochem. J. **138**, 277–280

Pullarkat, R. K. & Reha, H. (1982) J. Biol. Chem. **257**, 5991–5993

Sasak, W., Mankowsky, T. & Chojnacki, T. (1977) Chem. Phys. Lipids **18**, 199–204

Suzuki, S., Mori, F., Takigawa, T., Ibata, K., Nishida, T., Mizuno, M. & Tanaka, Y. (1983) Tetrahedron Lett. **24**, 5103–5106

Tanaka, Y. (1984) in Recent Advances in High Resolution and Solid State Studies (Randall, J. C., ed.), pp. 233–244, American Chemical Society, Washington DC

Tanaka, Y. & Takagi, M. (1979) Biochem. J. **183**, 163–165

Tanaka, Y., Sato, H. & Kageyu, A. (1982*a*) Polymer **23**, 1087–1090

Tanaka, Y., Takeda, J. & Noguchi, K. (1982*b*) U. S. Patent, 4338404

Wong, T. K., Decker, G. L. & Lennarz, W. J. (1982) J. Biol. Chem. **257**, 6614–6618