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Intermediates of tocopherol biosynthesis in the unicellular alga *Scenedesmus obliquus*

The presence of three isomeric methylphytylbenzoquinones

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Three isomers of methylphytylbenzoquinone have been isolated from lipids of the unicellular alga *Scenedesmus obliquus*, the most abundant being 2-methyl-6-phytylbenzoquinone (65% of the total). The 2-methyl-3-phytyl and 2-methyl-5-phytyl isomers amounted to 8 and 27% respectively. Previously problems have been encountered in the separation of the 3-phytyl and the 6-phytyl isomers, but in the present study it was found that they separated readily as quinols. Phytyl plastoquinone was also found and the relevance of these compounds to the biosynthesis of α-tocopherol is discussed. As well as phylloquinone, a hydroxyphylloquinone was detected, and studies indicated that it is the 5′ carbon atom to which the hydroxy group is attached. Such a compound has been found by workers using other unicellular algae.

INTRODUCTION

The biosynthesis of α-tocopherol involves condensation of homogentisate and phytyl pyrophosphate to form a methylphytylbenzoquinol which can, by cyclization and subsequent methylations, yield α-tocopherol. Threlfall *et al.* (1971) described the isolation and characterization of 2-methyl-6-phytyl-1,4-benzoquinone† from *Euglena gracilis*, strain Z. Although in the cell this quinone is likely to be present as the quinol, it is oxidized to the quinone during lipid extraction. If 2-methyl-6-phytylbenzoquinol were cyclized, it would yield δ-tocopherol (8-methyltocol), which in turn could be methylated via *S*-adenosylmethionine to produce β-tocopherol (5,8-dimethyltocol) or γ-tocopherol (7,8-dimethyltocol) and finally α-tocopherol (5,7,8-trimethyltocol). *E. gracilis* was also found to contain 2,3-dimethyl-5-phytyl-1,4-benzoquinone, which is the phytyl analogue of plastoquinone and hitherto has been known as 'phytyl plastoquinone' (Whistance & Threlfall, 1970*a*). This compound has since been detected in several plant tissues and, in studies with radiochemical precursors of α-tocopherol, it has been found to be closely associated with the tocopherol-biosynthetic pathway (Janiszowska & Pennock, 1976; Soll & Schultz, 1980; Hutson & Threlfall, 1980). Soll & Schultz (1980) proposed that 2-methyl-6-phytylbenzoquinol was methylated to yield phytyl plastoquinol, which could then be cyclized to yield γ-tocopherol and, after another methylation, α-tocopherol. Initially those authors suggested that cyclization of 2-methyl-6-phytylbenzoquinol to δ-tocopherol was a small side reaction, but more recently Schultz *et al.* (1985) showed tocopherol biosynthesis to proceed by two pathways with either δ-tocopherol or phytyl plastoquinol as intermediates.

In 1976, Janiszowska & Pennock suggested that a 'biosynthetic grid' may be involved in α-tocopherol biosynthesis, since radiolabelled 2-methyl-6-phytylbenzoquinone and 2-methyl-5-phytylbenzoquinone were converted by young *Phaseolus vulgaris* (French bean) seedlings into δ-tocopherol (8-methyltocol) and 7-methyltocol respectively, and radioactivity from both precursors was also found in phytyl plastoquinone. Furthermore it was shown that not only δ-tocopherol, but also 7-methyltocol and 5-methyltocol, could be incorporated into α-tocopherol by *P. vulgaris* seedlings. Therefore it was suggested that condensation of phytyl pyrophosphate and homogentisate gave rise to both 2-methyl-5-phytylbenzoquinol and 2-methyl-6-phytylbenzoquinol and perhaps even 2-methyl-3-phytylbenzoquinol, and these intermediates could be used for tocopherol synthesis.

Chemically, when toluquinol is condensed with either isophytol or phytol, the preferred substitution position for the side chain is *para* to the methyl group of toluquinol. Thus with BF_3 as catalyst, Janiszowska & Pennock (1976) found 2-methyl-5-phytylbenzoquinol to be the main product and, when the reaction was carried out in formic acid, 7-methyltocol was the main product (Marcinkiewicz *et al.*, 1959).

Although the studies of Janiszowska & Pennock (1976) implicated 2-methyl-5-phytylbenzoquinone as well as 2-methyl-6-phytylbenzoquinone in tocopherol biosynthesis, work by Threlfall's group in Hull (Thomas & Threlfall, 1975; Hutson & Threlfall, 1980; Marshall *et al.*, 1985) and Schultz's group in Hanover (Soll & Schultz, 1980; Schultz *et al.*, 1985) consistently showed that radiolabelled homogentisate was only incorporated into 2-methyl-6-phytylbenzoquinone by chloroplasts or chloroplast fragments and no other isomer was detected. Certainly separation and identification of the three methylphytylbenzoquinone isomers after synthesis from toluquinol and isophytol is difficult because, in addition to the three positional isomers, there are 2′-*cis* and 2′-*trans* isomers in each case. Janiszowska & Pennock

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† This compound has been referred to as '2-demethylphytylplastoquinone', but in the present paper we describe the isolation of two other isomers of this compound, one of which cannot be biosynthetically related to phytyl plastoquinone; the generic name is therefore preferred.

(1976) were able to show the presence of all three positional isomers in a synthetic mixture by reductive cyclization to the related tocols, but could not identify 2-methyl-3-phytylbenzoquinone by t.l.c. properties, and similarly Hutson & Threlfall (1980) were unable to identify this isomer in a synthetic preparation. It was finally realized that 2-methyl-3-phytylbenzoquinone co-chromatographs on most t.l.c. systems with 2-methyl-6-phytylbenzoquinone (Pennock & Threlfall, 1983). Recently Marshall *et al.* (1985) have separated the 3-phytyl from the 6-phytyl isomer by triple development on t.l.c. and reported the chromatographic properties of all three isomers. These workers confirmed earlier work by their group that 2-methyl-6-phytylbenzoquinone was the only isomer formed by chloroplast preparations from homogentisate and phytyl pyrophosphate.

In the present paper we report a simple t.l.c. method for separating the three isomers and show the presence of all three isomers in heterotrophically grown *Scenedesmus obliquus*.

EXPERIMENTAL AND RESULTS

Synthesis of methylphytylbenzoquinones

Toluquinol (0.4 g) and isophytol (1 g) were dissolved in dry dioxan (10 ml) and 0.1 ml of BF_3 etherate (BF_3–diethyl ether complex) was added. The mixture was maintained at 50 °C for 1 h with N_2 bubbling through the reaction flask. The mixture was extracted with light petroleum (b.p. 40–60 °C)/diethyl ether (1:1, v/v) and the extract washed several times with water to remove unused toluquinol. Evaporation of the solvent yielded the crude reaction product containing a mixture of methylphytylbenzoquinols.

At this stage the reaction mixture was either separated into the various methylphytylbenzoquinols by t.l.c. or was oxidized to yield the related quinones by addition of a small amount of Ag_2O (0.1 g) to the reaction product dissolved in diethyl ether (5 ml) for 1 h. Removal of the Ag_2O by filtration gave the methylphytylbenzoquinone mixture.

Separation of methylphytylbenzoquinones

The synthesis of methylphytylbenzoquinones as described above gives six isomers, namely the 2′-*cis* and 2′-*trans* isomers of 2-methyl-3-phytylbenzoquinone, 2-methyl-5-phytylbenzoquinone and 2-methyl-6-phytylbenzoquinone. The isomers are relatively difficult to separate because of the chromatographic similarity of the 3-phytyl and 6-phytyl isomers.

Isolation of the six isomers was achieved by an initial separation of the methylphytylbenzoquinol mixture into two bands on t.l.c. (see Table 1), and oxidation of these bands followed by further t.l.c. yielded two quinones in one case and four quinones in the other. The more polar band gave the 2′-*cis* and 2′-*trans* isomers of 2-methyl-

Table 1. Chromatographic and spectrophotometric properties of terpenoid quinones and related compounds

Notes: *a*, silica-gel GF_{254}; diethyl ether/light petroleum (b.p. 40–60 °C), 3:47 (v/v); *b*, silica-gel GF_{254}; diethyl ether/light petroleum (b.p. 40–60 °C), 3:7 (v/v); *c*, colour produced on t.l.c. with Fast Blue B reagent (Jatzkewitz & Lenz, 1956). Quinols will produce colours with this reagent, but these were not recorded; *d*, u.v. absorption maxima were determined in hexane; *e*, Spherisorb 5 μ ODS; dichloromethane/methanol, 1:4 (v/v), 1 ml/min, detection at 255 nm; *f*, Partisil 5 μ Si; methyl-t-butyl ether/hexane, 1:99 (v/v), 1 ml/min, detection at 255 nm; *g*, Partisil 5 μ Si; methyl-t-butyl ether/hexane, 1:19 (v/v), 1 ml/min, detection at 295 nm for tocols and 330 nm for tocochromenols; *h*, Lichrosorb 5 μ Si; dioxan/hexane, 1:999 (v/v), 1 ml/min, detection at 252 nm.

Quinone or related compound	Note ...	R_F on t.l.c.		Colour with Fast Blue	Absorption maximum (nm)	H.p.l.c. elution vol. (ml)			
		a	*b*	*c*	*d*	*e*	*f*	*g*	*h*
Plastoquinone-9		0.62	0.87	–	255	28.5	17.0	–	–
2,3-Dimethyl-5-phytylbenzoquinone		0.48	0.85	–	254	8.0	14.2	–	–
2,6-Dimethyl-5-phytylbenzoquinone		0.49	0.85	–	254	8.0	20.0	–	–
3,6-Dimethyl-5-phytylbenzoquinone		0.50	0.85	–	254	8.0	19.0	–	–
2′-*cis*-2-Methyl-3-phytylbenzoquinone		0.39	0.74	–	249	7.0	25.6	–	27.2
2′-*trans*-2-Methyl-3-phytylbenzoquinone		0.35	0.69	–	249	7.0	28.0	–	28.3
2′-*cis*-2-Methyl-5-phytylbenzoquinone		0.45	0.79	–	251	7.0	19.2	–	21.5
2′-*trans*-2-Methyl-5-phytylbenzoquinone		0.42	0.74	–	251	7.0	22.5	–	24.3
2′-*cis*-2-Methyl-6-phytylbenzoquinone		0.39	0.74	–	252	7.0	25.6	–	27.2
2′-*trans*-2-Methyl-6-phytylbenzoquinone		0.35	0.69	–	252	7.0	28.0	–	29.7
2′-*cis*/*trans*-2-Methyl-3-phytylbenzoquinol		–	0.22	–	291	–	–	–	–
2′-*cis*/*trans*-2-Methyl-5-phytylbenzoquinol		–	0.38	–	291	–	–	–	–
2′-*cis*/*trans*-2-Methyl-6-phytylbenzoquinol		–	0.38	–	291	–	–	–	
Phylloquinone		0.45	0.85	–	249	10.0	14.5	–	–
Hydroxyphylloquinone		–	0.35	–	249	–	–	–	–
α-Tocopherol		–	0.57	White	298	–	–	5.2	–
α-Tocopherolquinone		–	0.15	–	262	–	–	–	–
5-Methyltocochromenol		–	0.50	Brick red	268	–	–	9.2	–
7-Methyltocochromenol		–	0.46	Light brown	265	–	–	10.8	–
8-Methyltocochromenol		–	0.41	Dark brown	265	–	–	11.8	–
5-Methyltocol		–	0.51	Orange	296	–	–	8.1	–
7-Methyltocol		–	0.45	Slate grey	298	–	–	9.5	–
8-Methyltocol (δ-tocopherol)		–	0.42	Purple	298	–	–	10.6	–

3-phytylbenzoquinone, whereas the other band gave 2′-*cis* and 2′-*trans* isomers of both 2-methyl-5-phytylbenzoquinone and 2-methyl-6-phytylbenzoquinone. As Table 1 shows, 2′-*trans*-2-methyl-3-phytylbenzoquinone migrates with 2′-*trans*-2-methyl-6-phytylbenzoquinone on t.l.c., but they can be separated easily as quinols.

When the methods described above were used, the synthetic mixture was found to consist of 2-methyl-3-phytylbenzoquinone (16.5%), 2-methyl-5-phytylbenzoquinone (46%) and 2-methyl-6-phytylbenzoquinone (37.5%). In each case about 25% of the quinone was present as the 2′-*cis* isomer. Marshall *et al.* (1985) found approximately equal amounts of the 5-phytyl and the 6-phytyl isomers, but the 3-phytyl isomer constituted only 8% of the total. Marcinkiewicz *et al.* (1959) condensed phytol with toluquinol in formic acid and produced 5-, 7- and 8-methyltocol in an approximate ratio of 5:9:6, suggesting that the side chain is inserted *ortho* to the methyl group (i.e. 5-methyltocol or 2-methyl-3-phytylbenzoquinone) at a rate almost equivalent to that *meta* to the methyl group (i.e. 8-methyltocol or 2-methyl-6-phytylbenzoquinone). The low level of 2-methyl-3-phytylbenzoquinone in synthetic preparations may well be caused by the relative instability of this isomer. We have prepared mixtures of the three isomers in equal amounts, but after a few weeks storage the level of the 3-phytyl isomer had fallen substantially.

Characterization of the methylphytylbenzoquinones

The methylphytylbenzoquinones were chromatographed on several t.l.c. and h.p.l.c. systems (see Table 1), and in particular it was found that all isomers were eluted at the same position on the reversed-phase h.p.l.c. system but could be separated on a normal-phase h.p.l.c. system (0.1% dioxan in hexane as solvent) as shown by Marshall *et al.* (1985). Conditions for the complete separation of all six isomers by this system were critical, optimal separation being obtained with a column recently re-activated. Usually 2′-*cis* isomers of 2-methyl-3-phytylbenzoquinone and 2-methyl-6-phytylbenzoquinone were the most difficult to distinguish, and chromatograms were routinely carried out without this separation.

The u.v. absorption spectrum of each sample was recorded in hexane (Table 1) and the spectra were similar to those quoted by Marshall *et al.* (1985), but absorption maxima were usually about 2 nm lower in the present study. Marshall *et al.* (1985) used cyclohexane as solvent and this may be responsible for the discrepancy. Each sample showed an absorption maximum near 250 nm, with a shoulder near 257 nm. In the case of 2-methyl-5-phytylbenzoquinone the shoulder was quite prominent. Three isomeric dimethyl-*p*-benzoquinones were available as model compounds for comparison.The model compounds have two methyl groups in the benzoquinone ring compared with a methyl and a phytyl group in the methylphytylbenzoquinones. The spectra of the model compounds were as follows: 2,3-dimethylbenzoquinone (kindly provided by Mr. A. M. Senaidy and Dr. M. M. Barnes, of this Department), $\lambda_{max.}$ 246 nm, shoulder at 253 nm; 2,5-dimethylbenzoquinone (Eastman–Kodak Co.), $\lambda_{max.}$ 249 nm and $\lambda_{max.}$ 257 nm; and 2,6-dimethylbenzoquinone (Aldrich Chemical Co.), $\lambda_{max.}$ 250 nm, with a shoulder at 256 nm. These spectra are 2–3 nm lower in each case than the three related isomeric methylphytylbenzoquinones and in particular it was interesting that the 2,5-dimethylbenzoquinone showed a second maximum at 257 nm, indicating that the *para*-substituted quinones show greater absorption in this region.

Final confirmation of the identities of the methylphytylbenzoquinone isomers was carried out by cyclization of each quinone to a tocochromenol by refluxing for 2 h in pyridine (McHale & Green, 1962) and subsequent reduction of the chromenol in ethanol with hydrogen in the presence of PtO catalyst, yielding the related tocol. Thus, for example, 2-methyl-3-phytylbenzoquinone was cyclized to 3,4-dehydro-5-methyltocol (5-methyltocochromenol), which was reduced to 5-methyltocol, and by a similar procedure 2-methyl-5-phytylbenzoquinone and 2-methyl-6-phytylbenzoquinone gave 7-methyltocol and 8-methyltocol (δ-tocopherol) respectively.

The products of these reactions were compared with authentic samples of 5-, 7- and 8-methyltocol prepared by the method of Marcinkiewicz *et al.* (1959). R_F values on t.l.c., colours with the Fast Blue B reagent (Jatzkewitz & Lenz, 1956) and elution volumes on h.p.l.c. are given in Table 1. In all cases the materials produced by reductive cyclization of methylphytylbenzoquinone isomers were identical with the authentic tocols.

Growth of *Scenedesmus obliquus* and lipid extraction

Scenedesmus obliquus was cultured heterotrophically in the dark at 30 °C on a nitrate medium, supplemented with 0.5% glucose and 0.25% yeast extract (Kessler *et al.*, 1957). The alga was harvested in the late-exponential phase of growth. Lipids were extracted with hot methanol and transferred to diethyl ether (Powls & Britton, 1977). Total chlorophyll was estimated by the method of Arnon (1949).

Isolation and characterization of terpenoid quinones and tocopherols in *S. obliquus*

Total lipids from *S. obliquus* were fractionated on t.l.c. [silica-gel G; diethyl ether/light petroleum (3:7, v/v)] and three bands were eluted: (*a*) a quinone band containing plastoquinone, phytyl plastoquinone, phylloquinone and methylphytylbenzoquinones; (*b*) a tocopherol band containing mainly α-tocopherol; and (*c*) a hydroxyquinone band containing both α-tocopherolquinone and hyroxyphylloquinone. The quinone band was subjected to reversed-phase h.p.l.c. [Spherisorb 5 μ ODS; dichloromethane/methanol, 1:4 (v/v)], which separated plastoquinone, phylloquinone, phytyl plastoquinone and methylphytylbenzoquinones (see Table 1). Isomers are not normally separable on reversed-phase chromatography and therefore the phytyl plastoquinone and methylphytylbenzoquinone fractions from reversed-phase h.p.l.c. were subjected to further analysis on normal-phase h.p.l.c. (see Table 1). The tocopherol fraction was applied to normal-phase h.p.l.c. [Partisil 5 μ Si; methyl t-butyl ether/hexane 1:19 (v/v)]. The hydroxyquinone fraction contained some chlorophyll, which was removed by additional chromatography on t.l.c. [silica-gel G; diethyl ether/light petroleum, 3:7 (v/v)] and α-tocopherolquinone was separated from hydroxyphylloquinone.

The compounds were estimated spectrophotometrically from the various $\lambda_{max.}$ values in hexane as follows: plastoquinone [$\lambda_{max.}$ 255 nm, ϵ ($M^{-1} \cdot cm^{-1}$) 18030]; phytyl plastoquinone ($\lambda_{max.}$ 255 nm, ϵ 17800); methylphytylbenzoquinone (all isomers) ($\lambda_{max.}$ 249–252 nm,

ϵ 16800); phylloquinone and hydroxyphylloquinone ($\lambda_{max.}$ 249 nm, ϵ 18000); α-tocopherol ($\lambda_{max.}$ 292 nm, ϵ 3650) and α-tocopherolquinone ($\lambda_{max.}$ 262 nm, ϵ 19040) (Table 1).

Vitamins K

Bishop & Wong (1971, 1974) reported finding very low levels of phylloquinone in *S. obliquus*, and their findings are confirmed in the present study. The identity of the phylloquinone was established by u.v. spectroscopy, co-chromatography with authentic marker on t.l.c. and h.p.l.c. and mass spectrometry. A quinone with the properties of a hydroxylated phylloquinone was also found in *S. obliquus*. 5′-Hydroxyphylloquinone has been identified in *Euglena gracilis* strain Z (Law *et al.*, 1973) and a similar, and possibly identical, compound has been found in *Anacystis nidulans* and *Chlorella pyrenoidosa* (Henninger *et al.*, 1965; Allen *et al.*, 1967; Whistance & Threlfall, 1970*b*). The compound found in *S. obliquus* had chromatographic properties similar to those quoted by Law *et al.* (1973), a u.v. spectrum characteristic of a vitamin K containing a 2′,3′ double bond, and mass spectrometry showed a molecular ion (M^+) at 466, indicating addition of oxygen to phylloquinone (M_r 450). A peak at m/z 448 indicated loss of water, and both phylloquinone and hydroxyphylloquinone showed a base peak at m/z 225 characteristic of the methylnaphthoquinone ring with a four-carbon side chain (Law *et al.*, 1973). Fragment ions at m/z 252 and 265 in the spectrum of hydroxyphylloquinone were present at an increased intensity compared with those in the spectrum of phylloquinone, indicating that the hydroxy group could have been present at position 5′ and, following loss of water, a double bond would be introduced at 4′,5′ or 5′,6′. Such a bond could result in the fragment ions of increased intensity at m/z 252 and 265.

Thus the evidence available suggests that the hydroxylated phylloquinone in *S. obliquus* is 5′-hydroxyphylloquinone as established in other algae (Law *et al.*, 1973).

Plastoquinones

Plastoquinone is well established in *S. obliquus* (Bishop, 1971; Bishop & Wong, 1971), and in the present study as well as plastoquinone-9, small quantities of phytyl plastoquinone were found (see Table 2). Hutson & Threlfall (1980) and Etman-Gervais *et al.* (1977) found plastoquinone-8 as well as plastoquinone-9 in higher-plant tissues, but there was no evidence of the octaprenyl isoprenologue in *S. obliquus*. Theoretically, three dimethyl phytylbenzoquinones are possible, i.e. 2,3-dimethyl-5-phytylbenzoquinone (phytyl plastoquinone), 2,6-dimethyl-5-phytylbenzoquinone and 3,6-dimethyl-5-phytylbenzoquinone. In *S. obliquus*, only the 2,3-dimethyl isomer, i.e. phytyl plastoquinone, was detected.

Methylphytylbenzoquinones

The material eluted from reversed-phase h.p.l.c. at the elution volume of authentic methylphytylbenzoquinones had a u.v. spectrum with a maximum near 252 nm clearly different from plastoquinone and phytyl plastoquinone. An aliquot was examined by normal-phase h.p.l.c. [Lichrosorb 5 μSi; dioxan/hexane (1:999, v/v)] and showed a major peak corresponding to 2′-*trans*-2-methyl-6-phytylbenzoquinone and a second peak corresponding with 2′-*trans*-2-methyl-5-phytylbenzoquinone (Fig. 1). Related *cis* isomers were present at lower concentrations than were found in the synthetic mixture. A very small peak which was eluted with authentic 2′-*trans*-2-methyl-3-phytylbenzoquinone was present.

Table 2. Terpenoid quinones and tocopherols in *Scenedesmus obliquus*

Scenedesmus obliquus cells were grown heterotrophically in the dark and the following compounds were estimated spectrophotometrically after fractionation of the total lipid by t.l.c. and h.p.l.c.

Compound	Amount (μg/mg of chlorophyll)*
Plastoquinone	670
Phytyl plastoquinone	5.20
2-Methyl-3-phytylbenzoquinone	0.04
2-Methyl-5-phytylbenzoquinone	0.15
2-Methyl-6-phytylbenzoquinone	0.36
Phylloquinone	41.0
5′-Hydroxyphylloquinone	227
α-Tocopherol	1160

* Chlorophyll amounted to about 35 μg/g wet weight.

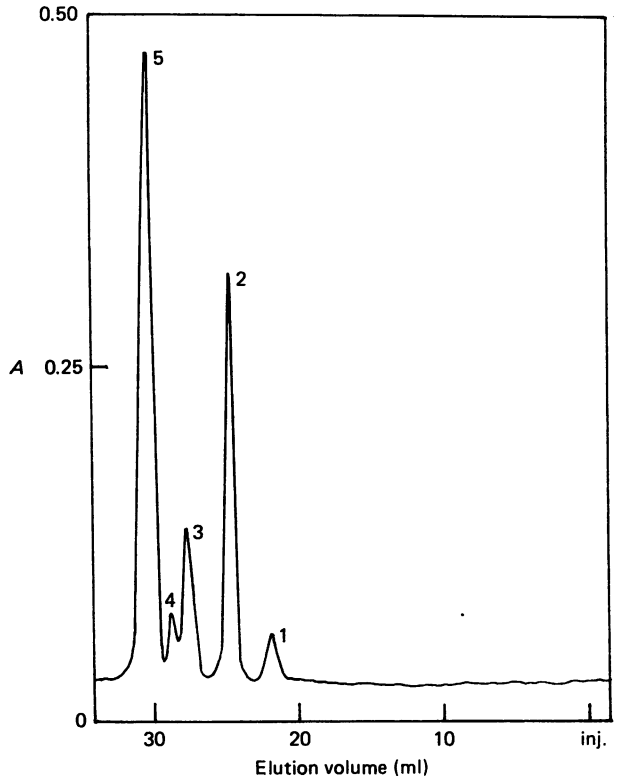


Fig. 1. H.p.l.c. trace of methylphytylbenzoquinones isolated from *S. obliquus*

H.p.l.c. details: adsorbent, Lichrosorb 5μ Si; solvent, dioxan/hexane, 1:999 (v/v); flow rate, 1 ml/min; wavelength of detection, 252 nm. Key to compounds: 1, 2′-*cis*-2-methyl-6-phytylbenzoquinone; 2, 2′-*trans*-2-methyl-5-phytylbenzoquinone; 3, 2′-*cis*-2-methyl-3-phytylbenzoquinone and 2′-*cis*-2-methyl-6-phytylbenzoquinone; 4, 2′-*trans*-2-methyl-3-phytylbenzoquinone; 5, 2′-*trans*-2-methyl-6-phytylbenzoquinone. Abbreviation: inj., injection.

The u.v. spectrum and chromatographic properties of each sample agreed with the related authentic quinone.

Another aliquot was cyclized with pyridine (as described above) and the resulting tocochromenols migrated on normal-phase h.p.l.c. with authentic 5-methyltocochromenol, 7-methyltocochromenol and 8-methyltocochromenol. The proportions present were approximately equivalent to those of the related quinones. The tocochromenols were reduced to tocols with hydrogen in the presence of PtO and yielded the corresponding tocols. The tocochromenols and tocols formed from the methylphytylbenzoquinone fraction were chromatographed on t.l.c. and in each case migrated with the authentic material and gave the characteristic colour with Fast Blue B salt.

Tocopherols and tocopherolquinone

The tocopherol fraction, when examined on normal-phase h.p.l.c., showed only one main peak: α-tocopherol. No β-, γ- or δ-tocopherols were found, but very small amounts of α-tocotrienol and α-tocomonoenol were detected by both t.l.c. and h.p.l.c. α-Tocopherolquinone was present in different preparations to various extents, and when extractions were carried out in the dark, the level of α-tocopherolquinone was very low, suggesting that it is an extraction artefact. No evidence for the presence of α-tocopherolquinol in the total lipid was obtained.

DISCUSSION

Three isomeric methylphytylbenzoquinones have been identified in heterotrophically grown *S. obliquus* cells. For the first time the identity of endogenous methylphytylbenzoquinones has been confirmed by h.p.l.c. and by h.p.l.c. of their cyclized derivatives. The presence of 2-methyl-3-phytylbenzoquinone is reported for the first time in a plant tissue, but the amount detected is quite small and would probably have been overlooked by t.l.c. The percentage composition of the methylphytylbenzoquinone mixture varied with different batches of *S. obliquus*, but 2-methyl-6-phytylbenzoquinone was always predominant, amounting to 60–70% of the total, 2-methyl-5-phytylbenzoquinone was 20–35% and 2-methyl-3-phytylbenzoquinone was 5–10% of the total.

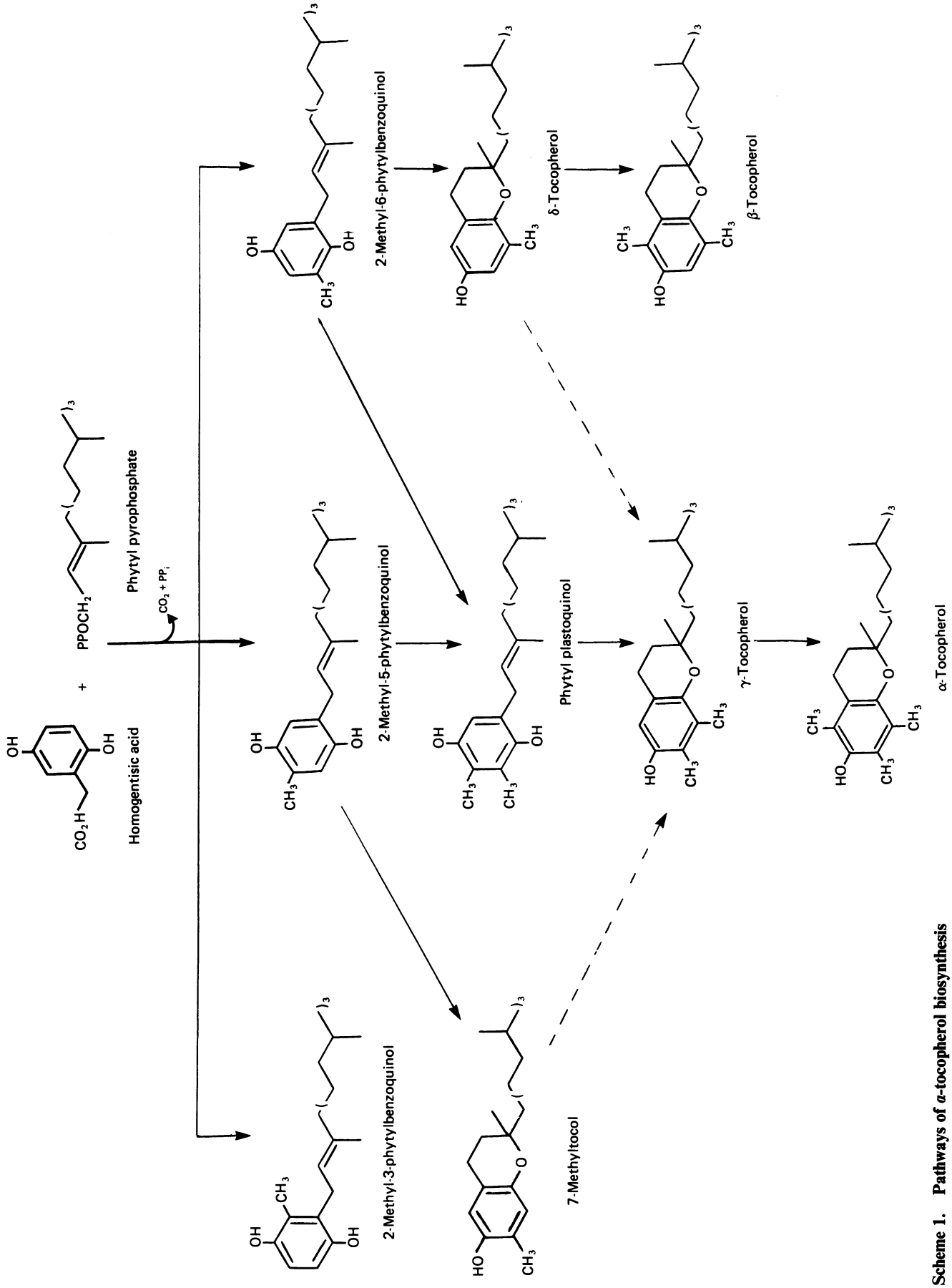
No evidence was found for the presence of these compounds as quinols, which would normally be their functional form in the chloroplast. However, *S. obliquus* cells are very difficult to extract, and the lipid extraction procedure with hot methanol probably results in oxidation of any quinols present to quinones.

The presence of these isomers reinforces the view put forward by Janiszowska & Pennock (1976) that α-tocopherol biosynthesis proceeds via several alternative pathways (see Scheme 1). The basic principles of tocopherol biosynthesis are well established. Homogentisate, derived from tyrosine, provides the aromatic ring, together with one of the ring methyl groups, and phytyl pyrophosphate is the precursor of the side chain and the heterocyclic ring. However, it is clear that, in some tissues and under certain conditions, geranylgeranyl pyrophosphate may be utilized in place of phytyl pyrophosphate, so producing the tocotrienols. Tocotrienols are found in cereals, seed oils and the latex of the rubber tree, *Hevea brasiliensis*, but it is not clear whether tocotrienols are formed because there is an absence of phytyl pyrophosphate and neither is it clear whether the tocotrienols are precursors of the low levels of tocopherols detected in these tissues. The 'tocotrienol pathway' may be located in a different cell compartment to the 'tocopherol pathway' and may involve separate enzymes.

What is clear from both biosynthetic and distribution studies is that 2-methyl-6-phytylbenzoquinol is formed from homogentisate and phytyl pyrophosphate. Furthermore, it is clear that 2-methyl-6-phytylbenzoquinol can undergo two possible transformations. δ-Tocopherol (8-methyltocol) is found in many plant tissues, particularly in seed oils and the older leaves of higher plants (Dicks, 1965), and 2-methyl-6-phytylbenzoquinol is the obvious precursor of this tocopherol, although a concerted condensation and cyclization reaction between homogentisate and phytyl pyrophosphate could result in the formation of δ-tocopherol without the release of the quinol intermediate. 2-Methyl-6-phytylbenzoquinol can also be methylated via *S*-adenosylmethionine to form phytyl plastoquinol (Hutson & Threlfall, 1980; Soll & Schultz, 1980), and it has been proposed that phytyl plastoquinol is a key intermediate in α-tocopherol formation, being cyclized to γ-tocopherol. What then is the role of δ-tocopherol if any? Initially Soll & Schultz (1980) thought that the formation of δ-tocopherol was a small side reaction, but more recently Schultz *et al.* (1985) see α-tocopherol being formed either by cyclization of phytyl plastoquinone or by methylation of δ-tocopherol.

β-Tocopherol can be found in some plants, particularly in cereal oils and in the leaves of mature plants (Dicks, 1965) and also in the blue–green alga *Anabaena variabilis* (Powls & Redfearn, 1967). β-Tocopherol clearly cannot be formed by cyclization of phytyl plastoquinol, and there is no evidence for the occurrence of 2,5-dimethyl-6-phytylbenzoquinol. Therefore methylation of δ-tocopherol at the 5-position appears to be the most likely route for β-tocopherol formation. This shows something of the variable nature of tocopherol biosynthesis: γ-tocopherol may well be produced mainly by cyclization of phytyl plastoquinol, whereas β-tocopherol is more likely to be formed by methylation of δ-tocopherol. To explain this phenomenon one would have to suggest that the methylation enzymes are specific for certain positions in the aromatic ring. Thus 2-methyl-6-phytylbenzoquinol may be methylated *ortho* to the ring methyl group giving phytyl plastoquinol, whereas 8-methyltocol (δ-tocopherol) can be methylated *para* to the ring methyl group to form 5,8-dimethyltocol (β-tocopherol).

Further complications are raised by this and previous publications (Janiszowska & Pennock, 1976; Janiszowska & Rygier, 1985), showing that 2-methyl-6-phytylbenzoquinol is not the only product of homogentisate and phytyl pyrophosphate condensation. Janiszowska & Rygier (1985) found 2-methyl-5-phytylbenzoquinol and 7-methyltocol (its cyclized derivative) in young seedlings of *Calendula officinalis* (marigold) as well as 2-methyl-6-phytylbenzoquinone and δ-tocopherol. Janiszowska & Pennock (1976) found both the 5-phytyl and 6-phytyl isomers of methylphytylbenzoquinone in *Phaseolus vulgaris* and showed them to be incorporated into both phytyl plastoquinone and tocopherols. In *S. obliquus* the presence of all three methylphytylbenzoquinone isomers has been established by a variety of methods, including h.p.l.c., and the importance of these isomers in



Scheme 1. Pathways of α-tocopherol biosynthesis

tocopherol biosynthesis requires further investigation. The biosynthesis of α-tocopherol is patently not random, since although all three possible isomers of methylphytylbenzoquinone have been found in *S. obliquus*, only one of the three possible dimethylphytylbenzoquinone isomers, i.e. phytyl plastoquinone, has been found. Furthermore, there are seven possible methylated derivatives of tocol, but only four well-documented tocopherols are found in Nature.

As described in the Introduction, Marshall *et al.* (1985) and Schultz *et al.* (1985) find only one methylphytylbenzoquinone isomer, namely 2-methyl-6-phytylbenzoquinone. How can these findings be reconciled with the finding of three isomers in *S. obliquus* and two isomers in *Calendula* and *Phaseolus*? It may be significant that both Threlfall's group and Schultz's group work with either chloroplasts or chloroplast fragments, whereas in the present study and in those of Janiszowska & Rygier (1985) whole cells or leaves were used. There is plenty of evidence to show that tocopherols are not confined to the chloroplast, and indeed whereas most α-tocopherol is found in the chloroplast, most non-α-tocopherol is extrachloroplastidic (Newton & Pennock, 1971). The available evidence also suggests that γ-tocopherol is not only found, but is also made, extrachloroplastidically (Janiszowska & Pennock, 1976; Hutson & Threlfall, 1980). Therefore it is likely that there are two distinct tocopherol-biosynthetic pathways, one chloroplastidic and one extrachloroplastidic and possibly these two pathways are not identical. The chloroplastidic pathway is mainly concerned with α-tocopherol formation, whereas the extrachloroplastidic pathway results in the formation of β-, γ-, and δ-tocopherols. Hutson & Threlfall (1980) found that after administration of radiolabelled homogentisate to leaves, phytyl plastoquinone was labelled equally in both chloroplast and extrachloroplastidic fractions. This adds to the contention that two distinct pathways exist, and although the essential components of each pathway are similar, it is possible that a difference in the proportions of methylase and cyclase enzymes could result in the two pathways having separate predominating routes. Thus whereas 2-methyl-6-phytylbenzoquinol might be utilized for phytyl plastoquinol formation in chloroplasts, the overall enzyme specificity extrachloroplastidically may be more inducive to the intermediacy of 2-methyl-5-phytylbenzoquinol or even 2-methyl-3-phytylbenzoquinol. The pathway in chloroplasts has been well studied (Hutson & Threlfall, 1980; Soll & Schultz, 1980), although the preparations used by these two groups are characterized by the low level, if not absence, of the cyclase enzyme(s). The extrachloroplastidic pathway has not been studied with isolated systems, and this remains a major approach to be developed.

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