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DEHYDROPHYLLOQUINONE, α-DEHYDROTOCOPHEROLQUINONE AND DEHYDROTOCOPHEROLS FROM ETIOLATED MAIZE AND BARLEY SHOOTS

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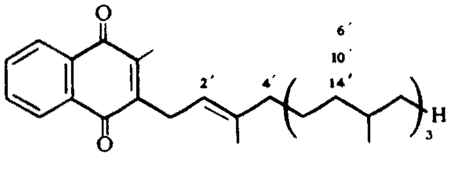
Key Word Index—*Zea mays*; *Hordeum vulgare*; Gramineae; *Spinacia oleracea*; Chenopodiaceae; *Phaseolus vulgaris*; Leguminosae; etiolated; dehydrophylloquinone; dehydrotocopherols; α-dehydrotocopherolquinone; UV; MS; TLC.

Abstract—Dehydrophylloquinone, α-dehydrotocopherolquinone, α-dehydrotocopherol and γ-dehydrotocopherol have been isolated from etiolated maize and barley shoots and excised etiolated maize shoots that have been exposed to light. They are not present, however, in green maize shoots, spinach leaves and etiolated bean leaves. Demethylphylloquinone was not detected in any of the tissues analysed.

INTRODUCTION

Phylloquinone (K; **1**), α-tocopherolquinone (α-TQ; **3**), α-tocopherol (α-T; **4a**) and γ-tocopherol (γ-T; **4d**), a group of quinones and chromanols which are related through the possession of a phytyl or phytyl-derived side chain, are normal constituents of the photosynthetic tissues of many higher plants [1]. The two quinones, the greater part of the α-T and some of the γ-T are present in the lamellae and osmiophilic globules of the chloroplasts [2–6]. The remainder of the α-T and γ-T, and when present δ-T, are to be found at sites outside the chloroplast [3, 6]. In addition to the above compounds some photosynthetic tissues contain β-, γ- and δ-TQs [7, 8] and spinach chloroplasts may also contain small amounts of either a naphthaquinone with the UV and TLC properties expected for 2-demethylphylloquinone (DK; **2**) [9] or a naphthaquinone which has a K-type of UV spectrum and is slightly less polar than K on TLC [10].

Etiolated (dark grown) shoots of barley (*Hordeum vulgare*) and maize (*Zea mays*) and etiolated leaves of French bean (*Phaseolus vulgaris*) contain K, α-T, γ-T (β-T in barley) and α-TQ, but the levels of these compounds in the etiolated tissues are much lower than those found in green tissues of the corresponding age [11, 12]. On illumination of etiolated tissues the levels of K, γ-T (only shown for maize), and α-T + α-TQ increase with time [11, 12]. The only related compounds that have been reported in etiolated tissues are DK and the K-type naphthaquinone found in spinach chloroplasts. Thus, Lichtenthaler and Becker [13] detected a naphthaquinone in etiolated barley shoots and etiolated *Raphanus sativus* which has the TLC properties expected for DK, and which has been referred to as 'vitamin K' and DK [13, 14], whilst Oku and Sughara [15] isolated a naphthaquinone from etiolated French bean leaves that is less polar than K on TLC and has a K-type of UV spectrum. The levels of both compounds were decreased on illumination of the etiolated tissue [13–15]. However, only in the cases of barley and radish were there concomitant increases in the levels of K [13, 14].



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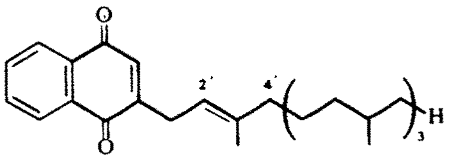

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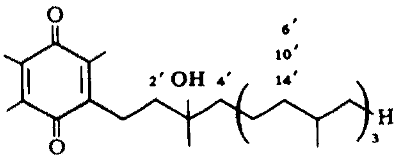

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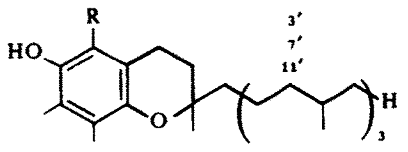

4a, R = Me
4b, R = H

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Table 1. Isolation of K-1, α-TQ-1, α-T-1 and γ-T-1 from etiolated maize and barley shoots and from etiolated maize shoots exposed to light

Compound	Etiolated maize shoots exposed to [G-^{14}C] shikimate and light* (μg/100 shoots)	Etiolated maize shoots exposed to [G-^{14}C] shikimate and light* Sp. act. (dpm/mg)	Etiolated maize shoots† (μg/100 shoots)	Etiolated barley shoots‡ (μg/1000 shoots)
PQ-9	510	11 200	299	1547
K	82	15 900	13	76
K-1	21	5000	10	30
α-TQ	64	4500	tr	60
α-TQ-1	16	ND	tr	50
α-T	142	4200	24	443
α-T-1	48	3800	32	400
γ-T	124	15 000	14	—
γ-T-1	45	1600	10	—

*600 etiolated 7-day-old maize shoots cv South African White Horse Tooth were excised and exposed with continuous illumination to 18μCi of D-[G-^{14}C] shikimate (1.9 μCi/μmol; New England Nuclear Corp.) for 24 hr.

†500 8-day-old maize shoots cv Kelvedon taken for analysis.

‡2000 7-day-old barley shoots cv Proctor taken for analysis.

In this communication we describe the isolation and partial characterization of monounsaturated (dehydro) forms of K, α-TQ, α-T and γ-T from etiolated maize and barley shoots and excised etiolated maize shoots that have been exposed to ^{14}C-substrates and light for periods of up to 24 hr. Throughout we use the terms dehydrophylloquinone (K-1), α-dehydrotocopherolquinone (α-TQ-1), α-dehydrotocopherol (α-T-1) and γ-dehydrotocopherol (γ-T-1) to mean either a mixture of the 3 biogenetically possible isomers of each compound or one of the biogenetically possible isomers of each compound.

RESULTS

Analysis of maize shoots

The samples of K-1, α-TQ-1, α-T-1 and γ-T-1 used for the characterisation studies were isolated either from batches of 6 to 8-day-old etiolated maize shoots that had been excised and exposed to radioactive substrates and light for periods of up to 24 hr or from batches of untreated 6 to 8-day-old etiolated maize shoots (Tables 1 and 4) by the standard procedures that we use for the purification of compounds of this type (see Experimental). In the adsorptive chromatographic systems used (alumina columns and TLC on Si gel) K-1, α-TQ-1, α-T-1 and γ-T-1 cochromatograph with K, α-TQ, α-T and γ-T respectively and are only resolved by reversed phase TLC (RPTLC) (Table 2). Although our standard purification procedures are quite adequate for isolating samples of α-TQ-1, α-T-1 and γ-T-1, they do not always give a satisfactory sample of K-1 for MS studies. A better procedure for the separation of K-1 and K is to take the mixture of quinones recovered from the first TLC step, remove any large amounts of PQ-9 by crystalization from EtOH and separate the K-1 from K by a modification of the HPLC procedure developed for the separation of K, PQ-9, α-T, α-TQ and ubiquinone by Lichtenthaler and Prenzel (personal communication). This procedure gives a pure sample of K-1 and, unlike TLC, it will resolve K-1 from DK. There was no indication in any of the chromatographic systems employed that the monosaturated samples were made up of mixtures of isomers. However, this does not exclude

Table 2. TLC systems used for the purification and characterization of K-1, α-TQ-1, α-T-1 and α-T-1

	R_f					
	Si gel G			Si gel G-Ag$^+$	Si gel G-paraffin	
Compound	C_6H_6-petrol (3:2)	Bu_2O–petrol (1.9)	C_6H_6–$CHCl_3$ (1:1)	MeCOEt–C_6H_{14} (3:17)	Aq. 90% Me_2CO	Aq. 85% Me_2CO
PQ-9	0.19	0.80		0.45	0.03	
K	0.24	0.72		0.64	0.14	
DK	0.24	0.66		0.59	0.19	
K-1	0.24	0.66		0.59	0.22	
α-T		0.35	0.51	0.30	0.71	0.42
α-T-1		0.35	0.51	0.26	0.79	0.54
			$CHCl_3$			
γ-T			0.57	0.22		0.57
γ-T-1			0.57	0.20		0.66
α-TQ			0.41	0.30		0.74
α-TQ-1			0.41	0.30		0.84

the possibility that we may be dealing with mixtures of positional isomers. The new compounds could not be isolated in any great quantity (Tables 1 and 4) and their characterizations are based on their UV, MS and TLC properties.

The UV spectra of K-1 (λ_{max}243.5, 248.5, 260, 290 and 326 nm in cyclohexane) and α-TQ-1 (λ_{max}261.5 and 270 nm in cyclohexane) in cyclohexane and NaOAc-buffered EtOH (both before and after reduction with $NaBH_4$) and of α-T-1 (λ_{max}292 and 299 nm) and γ-T-1 (λ_{max}295 and 302 nm) in cyclohexane are qualitatively superimposable on those of K, α-TQ, α-T and γ-T respectively. These spectral properties show that K-1 and α-TQ-1 have the same nuclear substitution pattern and side-chain up to at least C-4′ as K(**1**) and α-TQ(**3**) respectively, and that α-T-1 and γ-T-1 have the same chromanol nuclei as α-T(**4a**) and γ-T(**4d**) respectively. When set against these findings the TLC properties (RP, Ag^+ and absorptive) (Table 2) are consistent with K-1, α-TQ-1, α-T-1 and γ-T-1 being either monounsaturated (dehydro) forms or esterified monohydroxy (in chain) derivatives of K, α-TQ, α-T and γ-T. The possibility that α-T-1 and γ-T-1 are T-1 dimers or trimers can be discounted, since on the basis of the known TLC properties of tocopherol monomers, dimers and trimers, they would be expected to have either higher or lower R_f values than α-T (in the case of α-T-1) and γ-T (in the case of γ-T-1) [16].

The MS of each compound has an apparent M^+ that is 2 amu lower than that of its congener (i.e. K, α-TQ, α-T or γ-T) and major fragmentation ions that have *m/e* values identical to those of the congener (Table 3). The fact that the major fragmentation ions are identical to those of the congeners confirms that, as deduced from the UV spectra, K-1 and α-TQ-1 have the same nuclei and side chains up to the C-4′ position as K and α-TQ respectively, and α-T-1 and γ-T-1 have the same chromanol nuclei as α-T and γ-T respectively [17, 18]. The MS are consistent, therefore, with the compounds being monounsaturated forms of K, α-TQ, α-T and γ-T. However, the same spectra would also be obtained for the esterified monohydroxy (in chain) derivatives of K, α-TQ, α-T and γ-T and for the dimeric forms of α-T-1 and γ-T-1 [16]. As indicated already the likelihood that we had isolated dimeric or trimeric forms of α-T-1 and γ-T-1 can be discounted on the basis of the TLC properties of the compounds. The possibility that the compounds are esterified monohydroxy derivatives, in which case the M^+ peaks would be formed as a result of the facile loss of fatty acid (cf. misidentification of plastoquinones B (fatty acid esters of monohydroxy-plastoquinones-9) [19] as dehydroplastoquinones-9 [20] was eliminated with the demonstrations that neither treatment with sodium dihydro-*bis*-(2-methoxyethoxy) aluminate (SDA) nor saponification has any effect on the UV, TLC and MS properties of α-T-1 and γ-T-1 and that catalytic hydrogenation of K-1 and α-TQ-1 (these compounds are alkali labile and cannot be saponified) gives rise to compounds with the same UV, TLC and MS properties as 5,6,7,8,2′,3′-hexahydro K and α-TQ respectively.

The UV, MS and TLC properties are all consistent with the compounds being dehydro forms of K, α-TQ, α-T and γ-T, in which the unsaturation occurs beyond the C-4′ position in the case of the quinones and beyond the C-1′-position in the case of the chromanols. On biogenetic grounds the most obvious possibilities are that the new compounds are the 6′, 10′ or 14′ or mixtures of 6′, 10′ and 14′ dehydro forms of K and α-TQ and the 3′, 7′ or 11′ or mixtures of the 3′, 7′ and 11′ dehydro forms of α-T and γ-T.

Analysis of green maize shoots, spinach leaves, etiolated barley shoots and etiolated French bean leaves

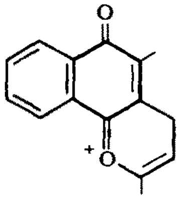
Each of these tissues was analysed for the presence of K-1 and α-T-1. Spinach, barley and French bean tissues were also examined for the various naphthaquinones that they had been reported to contain (see Introduction). Etiolated barley shoots were found to contain K, K-1, α-T and α-T-1, but no DK (Table 1). The other tissues were found to contain only K and α-T, despite the fact that in the cases of spinach and French beans leaves the scale of the analyses were such that the naphthoquinones isolated by other workers should have been easily detectable.

DISCUSSION

Etiolated maize shoots and excised etiolated maize shoots that have been exposed to light contain one or more isomers of dehydrophylloquinone (K-1), α-dehydrotocopherolquinone (α-TQ-1), α-dehydrotocopherol

Table 3. MS data for K-1, α-TQ-1, α-T-1 and γ-T-1

Compound	*m/e* (M+)	*m/e* (base peak)	Compound	*m/e* (M+)	*m/e* (base peak)
K	450(**1**)$^+$	225(**5**)	α-T	430(**4a**)$^+$	165(**7a**)
K-1	448	225(**5**)	α-T-1	428	165(**7a**)
α-TQ	446(**2**)$^+$	221(**6**)	γ-T	416(**4d**)$^+$	151(**7b**)
α-TQ-1	444	221(**6**)	γ-T-1	414	151(**7b**)



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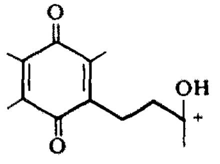

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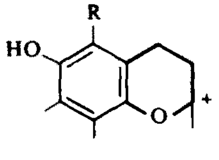

7a, R = Me
7b, R = H

(α-T-1) and γ-dehydrotocopherol (γ-T-1) (Table 1). The first three types of compounds are also present in etiolated barley shoots (Table 1) and it is to be expected that β-T-1 (β-T replaces γ-T in barley) will also be present. It is almost certain that K-1 is the naphthaquinone in barley referred to as 'vitamin K' and DK by Lichtenthaler and Becker [13], since its TLC properties (the only properties cited in ref. [13]) are such that it cannot be distinguished from DK. Indeed, the only effective way of separating DK and K-1 is by HPLC.

K-1, DK, α-T-1 and the polar naphthaquinones (see Introduction) were not detected in green maize shoots, spinach leaves or etiolated bean leaves. Our failure to confirm that spinach leaves contain DK and a non-polar naphthaquinone, may be due to the differences in the ages and physiological conditions of the tissues analysed by ourselves and by Crane *et al.* [9, 10]. However, our inability to find the non-polar naphthaquinone of etiolated bean leaves is most difficult to explain, especially in view of the fact that we found a substantial amount of K (but no other naphthoquinones in etiolated bean leaves) whereas Oku and Sugahara [15] found substantial amounts of a non-polar naphthaquinone but only traces of K.

It would appear from the analyses performed in this study that K-1, α-TQ-1, α-T-1 and γ-T-1 are products of some but not all etiolated tissues and that they are normally absent from green tissues (Tables 1 and text). In this respect it is of interest that a compound, presumably of dietary origin, with the TLC properties expected of 2-methyl-3-Δ^6-dehydrophytyl-1,4-naphthaquinone (i.e. a K-1) has been isolated from beef liver, and that an α-T-1 and two α-tocodienols have been isolated from palm seed oil (Dunphy, personal communication) (a plant product that also contains tocotrienols [22]).

On consideration of the TLC properties of the dehydrocompounds (Table 2), it is quite clear that in many studies involving etiolated tissues K and K-1, α-TQ and α-TQ-1, α-T and α-T-1 and γ-T and γ-T-1 have been co-estimated as K, α-TQ, α-T and γ-T respectively [11, 12, 23]. In our own studies, it is probable that the compounds were co-estimated when we investigated the effect of light on the quinone and chromanol levels in etiolated maize [11], although in subsequent biosynthetic studies when the purification procedures included RPTLC steps the various pairs of congeners would have been resolved from each other [1].

The reasons why the dehydro compounds should accumulate in substantial amounts in etiolated tissues are not clear. On biosynthetic grounds it would appear that, depending on whether in the biosynthesis of K, α-TQ, α-T and γ-T the side chains are transferred to the nuclei as phytyl- or geranylgeranyl- residues, the tissues either are using tetrahydrogeranylgeranylpyrophosphate(s) in place of phytylpyrophosphate or are unable to carry out the complete reduction of all the molecules of geranylgeranyl-substituted intermediates, so that mixtures of the dehydro and the normal products are formed. Lichtenthaler and Becker [13] and Lichtenthaler and Kleudgen [14] have proposed that on illumination of etiolated barley shoots the compound they refer to as DK, but which is almost certainly K-1 (see above), is converted to K. If this transformation takes place then it is reasonable to expect that it will also occur in etiolated maize shoots and that in addition α-TQ-1, α-T-1 and γ-T-1 (β-T-1 in barley) will be converted to α-TQ, α-T and γ-T respectively. In the course of our biosynthetic studies, we have found that the dehydro compounds isolated from etiolated maize shoots that have been exposed to ^{14}C-substrates and light always have lower sp. act. than their congeners (Tables 1 and 4). These observations would seem to exclude the dehydro compounds as normal intermediates in the biosynthesis of K, α-TQ and tocopherols. However, the true biosynthetic relationships between these compounds will only be established by experiments addressed to this problem.

EXPERIMENTAL

Biological material. Etiolated seedlings of *Z. mays* cvs Rhodesian White Horse Tooth, South African White Horse Tooth and Kelvedon, *H. vulgare* cv Proctor and *P. vulgaris* cv Masterpiece were grown under our standard conditions [26]. *S. oleracea* cv Prickly Standwell was grown at the Botanic Gardens, University of Hull.

Exposure of maize seedlings to ^{14}C substrates. Details are given in the papers cited in the text.

Extraction and preliminary fractionation of lipids. The lipids were extracted from shoots and leaves by our routine Procedure B [26]. They were then resolved into various isoprenoid quinone- and chromanol-containing fractions by chromatograpny on columns of acid-washed Al_2O_3 (Brockmann III) (Woelm; anionotropic) developed by stepwise elution with 0.25, 1, 3, 5, 8, 12 and 20% Et_2O–petrol (40–60°) [26]. The grade of the columns was adjusted such that PQ-9, K and K-1, α-T and α-T-1, γ-T

Table 4. Incorporation of ^{14}C-substrates into K and K-1 by etiolated 7-day-old maize shoots exposed to light

Incubation conditions					K		K-1	
Shoots								
(Cultivatar)	(Nos)	Substrate	Dose (μCi)	Period of illumination (hr)	(μg)	dpm/mg)	(μg)	(dpm/mg)
RWHT*	300	DL-MVA[2-^{14}C] (10.9μCi/μmol)	40	24	138	39 700	32	30 000
RWHT*	600	DL-Shikimate[1,2-^{14}C] (8.7μCi/μmol)	50	24	491	10 800	78†	2500†
SAWHT*	120	*o*-Succinyl benzoate [2,3-^{14}C] (2.5 μCi/μmol)	3.3	18	216	1 340 000	76‡	622 000‡

*RWHT, Rhodesian White Horse Tooth; SAWHT, South African White Horse Tooth.
†Values obtained in the course of the expts described in [24].
‡Values obtained in the course of the expts described in [25].

and γ-T-1, and α-TQ and α-TQ-1 were eluted by 1, 3, 5 and 20% Et_2O–petrol respectively.

Purification of quinones and chromanols. The various quinones and chromanols present in the fractions obtained from column chromatography of the lipid extracts were purified by combinations of TLC on Si gel G impregnated with Rhodamine 6G and RPTLC on Si gel impregnated with paraffin (Table 2) [26]. After development, and spraying with 0.05% Rhodamine 6G in Me_2CO in the case of RP chromatograms, the compounds were located by examination of the plate under UV light (254 nm) and recovered from the gel by elution with Et_2O [26]. The sequence used for all compounds was (i) adsorptive TLC, (ii) RPTL and (iii) adsorptive TLC to remove paraffin from (ii). The solvent systems used for adsorptive TLC were C_6H_6–petrol (2:3) (PQ-9, K and K-1), C_6H_6–$CHCl_3$ (1:1) (α-T and α-T-1), $CHCl_3$ (γ-T and γ-T-1, and α-TQ and α-TQ-1) and for RPTLC were aq. 90% Me_2CO (PQ-9, K and K-1, and α-T and α-T-1) and aq. 85% Me_2CO (γ-T and γ-T-1, and α-TQ and α-TQ-1) (see Table 2 for R_f values).

HPLC. In the part of the investigation concerned with the nature of the quinones and chromanols present in etiolated maize and barley shoots, etiolated bean leaves and green spinach leaves, the K-1 was separated from K and PQ-9 by HPLC. The 1% Et_2O–petrol fraction from column chromatography was subjected to quantitative TLC on Si gel G developed with C_6H_6–petrol (2:3). The PQ-9, K and K-1 recovered from the plate was taken up in small vol. of hot EtOH (1–2 ml) and the PQ-9 allowed to crystallise out at −20°. The K and K-1 present in the mother liquors were then subjected to quantitative HPLC in a Jobling System 1 HPL chromatograph fitted with a 450 × 2 mm (i.d.) glass column packed with LiChrosorb SI 60 10 μm (Merck). The column was developed at 50 bar with 0.25% dioxane–isooctane (0.3 ml/min) and the effluent monitored at 254 nm. The R_t of K, K-1 synthetic DK and PQ-9 were 19, 22, 25 and 30 min respectively.

Catalytic hydrogenation of K-1. Catalytic reduction (PtO_2/H_2) of K-1 was carried out under standard conditions [27]. The octahydro derivative (λ_{max} 243.5 and 248.5 nm in cyclohexane) was purified by HPLC (R_t, 12 min) under the conditions described above.

*SDA (sodium dihydro-*bis*(2-methoxyethoxy) aluminate), treatment and attempted saponification of α-T-1 and γ-T-1.* α-T-1 or γ-T-1 were taken up in dry Et_2O and treated with SDA. After 15 min the reaction mixture was treated with 10% HCl and the γ-T-1 or γ-T-1 recovered by TLC. The saponification of α-T-1 or γ-T-1 was carried out by the procedure given in the A.M.C. Report [28].

Spectrophotometric assays. K, PQ-9, α-TQ, α-T and γ-T were assayed by our standard spectrophotometric procedures [26, 29]. K-1, α-TQ-1, α-T-1 and γ-T-1 were assayed in cyclohexane by using the same $E^{1\%}_{1cm}$ factors that were used for the assays of K, α-TQ, α-T and γ-T respectively.

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