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ISOLATION OF PHYTYLPLASTOQUINONE AND A PHYTYLPLASTOHYDROQUINONE MONOMETHYL ETHER FROM *EUGLENA GRACILIS*

G. R. WHISTANCE and D. R. THRELFALL

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth

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Abstract—The isolation and characterization of phytylplastoquinone (2,3-dimethyl-5-phytyl-1,4-benzoquinone) and a phytylplastohydroquinone monomethyl ether (2,3-dimethyl-4-methoxy-5 (or 6) phytylphenol) from cells of *Euglena gracilis* strain Z, *E. gracilis* var. *saccarophila* and *E. gracilis* strain 1224/5 g is described.

INTRODUCTION

THE PAST decade has witnessed the isolation from oxygen-producing photosynthetic tissues of a number of 2,3-dimethyl-5-polyprenyl-1,4-benzoquinones which have been given the trivial name plastoquinone.[1,2] These quinones differ from each other only in the nature of their polyprenyl side-chains, which may be either unsubstituted (plastoquinones), monohydroxylated (plastoquinones C), monoacylated (plastoquinones B) or both monohydroxylated and monoacylated (plastoquinones Z).

The majority of the plastoquinones possess nonaprenyl side-chains [e.g., plastoquinone-9 (where the numeral indicates the number of prenyl units in the side chain), plastoquinones-B, -C and -Z], with plastoquinone-9 being the most widely distributed.[1,2] Indeed, this quinone has been detected in the photosynthetic regions of all plant species so far examined. Plastoquinones-B, -C and -Z, on the other hand, appear to be mainly confined to higher plant tissues. In addition to the above quinones two other plastoquinones have been isolated: these are plastoquinone-3, isolated from spinach chloroplasts,[3] and plastoquinone-4, isolated from leaves of horse chestnut.[4]

In the present paper we report the isolation from photosynthetically grown cells of *Euglena gracilis* of 2,3-dimethyl-5-phytyl-1,4-benzoquinone [phytylplastoquinone (II)]. In addition, we present evidence for the presence in *E. gracilis* of a monomethyl ether of the reduced form of this quinone, i.e. 2,3-dimethyl-4-methoxy-5 (or 6)-phytylphenol [phytylplastohydroquinone monomethyl ether (I)].

RESULTS

Whistance,[5] whilst investigating the biosynthesis of plastoquinone-9 by *Euglena gracilis* strain Z, isolated from this organism a plastoquinone with chromatographic properties which differed from those of all the known naturally occurring plastoquinones. This quinone was

[1] J. F. PENNOCK, *Vitamins and Hormones* **24**, 307 (1966).
[2] J. C. WALLWORK and J. F. PENNOCK, *Chem. Ind.* 1571 (1968).
[3] D. MISITI, H. W. MOORE and K. FOLKERS, *J. Am. Chem. Soc.* **87**, 1402 (1965).
[4] H. ECK and A. TREBST, *Z. Naturforsch.* **186**, 446 (1963).
[5] G. R. WHISTANCE, Ph.D. Thesis, University College of Wales, Aberystwyth (1969).

eluted from an alumina column by 0·25% E/P* which meant that it was not polar enough to be a member of the plastoquinone-10 to -1 series (eluted by 1% E/P), a monohydroxyplastoquinone (eluted by 20% E/P), or a monohydroxy monoacylplastoquinone. Furthermore, on treatment with $LiAlH_4$ under the conditions described by Griffiths,[6] the quinone was unaltered, showing that it was not a new monoacyl form of plastoquinone, i.e. a new plastoquinone B. The finding that the quinone was eluted from an alumina column in the fraction immediately preceding the one containing plastoquinone-9 was reminiscent of the behaviour of phylloquinone [2-methyl-3-phytyl-1,4-naphthaquinone (eluted by 0·25% E/P)] and menaquinone-9 [2-methyl-3-nonaprenyl-1,4-naphthaquinone (eluted by 1% E/P)] and suggested that quinone might be phytylplastoquinone. To investigate this further autotrophically grown cells (63 g wet wt. from 50 l. of medium) of *E. gracilis* strain Z were extracted and 440 μg of quinone obtained. Whilst purifying the quinone by adsorptive and reversed phase TLC, 405 μg of a second compound, compound E, was obtained whose u.v. spectral properties were typical of a *p*-benzohydroquinone or benzochromanol.

Characterization of Phytylplastoquinone [2,3-*Dimethyl*-5-*phytyl*-1,4-*benzoquinone* (*II*)]

The identity of phytylplastoquinone was established as follows. (1) Its u.v. spectra in cyclohexane ($\lambda\lambda_{max}$ 254 and 261 nm) and ethanol (λ_{max} 255 nm, changing to 291 nm after treatment with $NaBH_4$) were qualitatively identical to those of phytylplastoquinone. (2) It reacted with cysteine (followed spectrophotometrically), indicating the presence of an unsubstituted position in the ring.[7] (3) On catalytic hydrogenation the wavelengths of maximal u.v. absorption in cyclohexane moved from 254 nm and 261 nm to 257 nm and 264 nm, a shift which can be attributed to saturation of the double bond in the isoprene unit adjacent to the nucleus.[3] (4) Its TLC properties (adsorptive, reversed-phase and silver ion) were identical to those of authentic phytylplastoquinone (Table 1). (5) Its mass spectrum was

TABLE 1. TLC SYSTEMS USED FOR THE PURIFICATION AND CHARACTERIZATION OF PHYTYLPLASTOQUINONE AND COMPOUND E (PHYTYLPLASTOHYDROQUINONE MONOMETHYL ETHER)

Compound	R_f values		
	System I. Adsorptive benzene–light petroleum (2:3, v/v)	System II. Silver ion acetone–butan-2-one (1:19, v/v)	System III. Reversed phase aq. 95% acetone (v/v)
Phytylplastoquinone	0·40	0·75	0·52
Phytylplastohydroquinone	0·22	—	0·86
Phylloquinone	0·40	0·75	0·52
Compound E	0·45	—	0·81
Plastoquinone-9	0·40	0·58	0·25

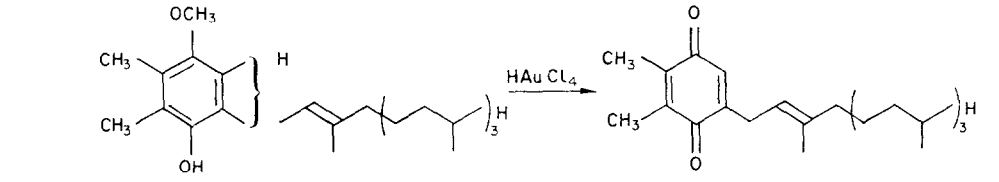
* Abbreviation: E/P, solution of diethyl ether in light petroleum (b.p. 40–60°).

[6] W. T. GRIFFITHS, *Biochem. Biophys. Res. Commun.* **25**, 596 (1966).

[7] E. R. REDFEARN, in *Biochemistry of Quinones* (Edited by R. A. MORTON), p. 149, Academic Press, London (1965).

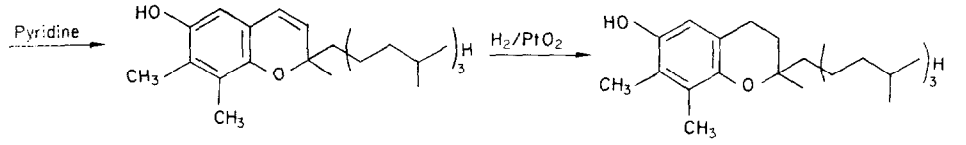
[8] W. T. GRIFFITHS, D. R. THRELFALL and T. W. GOODWIN, *Europe. J. Biochem.* **5**, 124 (1968).

identical to that of phytylplastoquinone, showing peaks at $m/e=416$ (M+2; due to a dis mutation reaction in the spectrometer resulting in the formation of the ions of the corresponding hydroquinones[9]), $m/e=414$ (M) and 189 (base peak). [The latter is observed in all



I, Phytylplastohydroquinone monomethyl ether

II, Phytylplastoquinone



III, Phytylplastochromenol

IV, Phytylplastochromanol (γ-Tocopherol)

SCHEME I. CHEMICAL SYNTHESIS OF PHYTYLPLASTOCHROMANOL (γ-TOCOPHEROL) FROM PHYTYLPLASTOQUINONE OR PHYTYLPLASTOHYDROQUINONE MONOMETHYL ETHER.

TABLE 2. COMPARISON OF U.V. SPECTROSCOPIC AND TLC PROPERTIES OF PHYTYLPLASTOCHROMANOL WITH THOSE OF DIMETHYLTOCOLS

Compound	$\lambda\lambda_{max}$ in cyclohexane (nm)	TLC properties on Kieselgel G: System IV. Benzene–chloroform (1:1, v/v) (R_f)	TLC properties on Kieselgel G: System V. Diisopropyl ether–light petroleum (1:5, v/v) R_f	TLC properties on Kieselgel G: System V. Diisopropyl ether–light petroleum (1:5, v/v) Colour produced with diazotized *o*-dianasidine[10]
Phytylplastochromanol	295, 302	0·29	0·20	Dark blue
γ-Tocopherol (7,8-dimethyltocol)	295, 302	0·29	0·20	Dark blue
β-Tocopherol (5,8-dimethyltocol)	293, 298	0·29	0·25	Pale brown
ζ-Tocopherol (5,7-dimethyltocol)	293, 298	0·39	0·22	None

plastoquinone spectra and proves that they contain the same 2,3-dimethyl-5-monoprenyl-1,4-benzoquinone unit (or an isomer of it[9]).] (6) Its dimethyltocol (IV), synthesized by the sequence of reactions outlined in Scheme I, was found to have u.v. spectral and TLC properties identical to those of γ-tocopherol [(7,8-dimethyltocol) Table 2].

[9] B. C. DAS, M. LOUNASMAA, C. TENDILE and E. LEDERER, *Biochem. Biophys. Res. Commun.* **21**, 318 (1965).
[10] Analytical Methods Committee, Vitamin E Panel, *Analyst* **84**, 356 (1959).

Characterization of Compound E [Phytylplastohydroquinone Monomethyl Ether (I)]

The u.v. spectrum (λ_{max} 287 nm with a shoulder at 295 nm in cyclohexane) of compound E and its reaction with Emmerie–Engel reagent suggested that it was either an isoprenoid *p*-benzohydroquinone or benzochromanol. To investigate this a sample was treated with gold chloride, since this would oxidize a hydroquinone to its corresponding quinone and a chromanol to its corresponding γ-hydroxyquinone. The product obtained by this procedure, on the basis of the criteria outlined in the previous section, was shown to be phytylplastoquinone. This result eliminated the possibility that compound E was a chromanol. Further, the chromatographic properties of compound E differed from those of phytylplastohydroquinone (Table 1). The low resolution mass spectrum of compound E showed a mass peak at $m/e = 430$ and had no readily interpretable fragmentation pattern. High resolution mass spectrometry gave the accurate mass value as 430·3808, which corresponds to a molecular formula of $C_{29}H_{50}O_2$ (430·381061). This result, coupled with the previous observations, led us to conclude that compound E was a phytylplastohydroquinone monomethyl ether (I). Support for this comes from (*a*) the knowledge that on mild oxidation hydroquinone monomethyl ethers are converted to their corresponding quinones, and (*b*) the finding that on Zeisel degradation of compound E which had been biogenetically labelled from L-[*Me*-^{14}C]methionine 65 per cent of the radioactivity was recovered in the tetramethylammonium iodide (see Experimental section for details). However, because of the small amounts of phytylplastohydroquinone monomethyl ether which were available and the lack of information about compounds of this type, it was not possible to determine which oxygen atom of phytylplastohydroquinone is involved in ether formation.

Distribution of Phytylplastoquinone and Phytylplastohydroquinone Monomethyl Ether in Plants

It was shown that, in addition to *E. gracilis* strain Z, these compounds are also present in *E. gracilis* var. *saccarophila* and *E. gracilis* strain 1224/5 g. The amounts found, together with the amounts of phylloquinone and plastoquinone-9 present in these organisms, are given in Table 3.

Griffiths,[11] whilst investigating the nature of the isoprenoid quinones present in the green alga, *Tribonema*, isolated a compound which, although it had the same TLC properties as phylloquinone, differed markedly from this quinone in its u.v. spectroscopic properties.

TABLE 3. LEVELS OF PHYTYLPLASTOQUINONE, COMPOUND E (PHYTYLPLASTOHYDROQUINONE MONOMETHYL ETHER), PLASTOQUINONE-9 AND PHYLLOQUINONE IN VARIOUS STRAINS OF *E. gracilis*

Strain	Dry weight of cells analysed (g)	Concentration (μg/g dry wt.)			
		Phytylplasto-quinone	Compound E	Plastoquinone-9	Phylloquinone
Strain Z	11·0	40	37	2000	Trace
Var. *saccarophila*	3·5	17	17	300	17
Strain 1224/5 g	1·0	11	10	810	Trace

[11] W. T. GRIFFITHS, Ph.D. Thesis, University College of Wales, Aberystwyth (1965).

From an examination of the published u.v. spectrum it is clear that this compound was a mixture of phytylplastoquinone and phylloquinone, in which the former was the major component.

In our studies with higher plant tissues we have never detected these compounds, although in the case of phytylplastoquinone this is perhaps understandable in view of the relatively small amounts of tissue analysed and the difficulty involved in detecting small amounts of this compound in the presence of phylloquinone.

DISCUSSION

Large-scale extraction of photosynthetically grown cells of *Euglena gracilis* strain Z has allowed the isolation and characterization of a new naturally occurring plastoquinone, phytylplastoquinone, and one of its hydroquinone monomethyl ethers. Two other strains of *E. gracilis* (var. *saccarophila* and 1224/5 g) were also found to contain these compounds, and it is almost certain that the peculiar phylloquinone isolated from the alga, *Tribonema*, by Griffiths[11] was a mixture of phylloquinone and phytylplastoquinone. However, as yet, we have been unable to detect these compounds in higher plant tissues, although this might be attributable to the relatively small amounts of material taken for analysis.

In biosynthetic terms the occurrence in nature of phytylplastoquinone is of obvious interest, since structurally it can be envisaged as a precursor of γ-tocopherol. By analogy with the established chemistry of compounds of this type, the conversion could proceed via the chromenol or the hydroquinone, i.e. phytylplastoquinone $\rightarrow$ phytylplastochromenol $\rightarrow$ γ-tocopherol (cf. Scheme I) or phytylplastoquinone $\leftrightharpoons$ phytylplastohydroquinone $\rightarrow$ γ-tocopherol. At present there is no evidence to support either of these biosynthetic sequences, and it is just as plausible that phytylplastoquinone is a terminal product of biosynthesis. There would, however, seem to be little doubt that phytylplastohydroquinone monomethyl ether is formed from phytylplastohydroquinone by an *O*-methylation reaction involving S-adenosylmethionine as methyl donor.

EXPERIMENTAL

Biological Material

Euglena gracilis strain Z, *E. gracilis* var. *saccarophila* and *E. gracilis* strain 1224/5 g were obtained from The Culture Collection of Algae and Protozoa, Botany School, Cambridge. Photosynthetic cells of the organisms were obtained by growing them in the light on a medium containing 0·5% proteose peptone (Oxoid) and 0·2% yeast extract (Difco) in tap water under conditions similar to those described previously.[12]

Incubation of E. gracilis *strain Z with* L-[Me-^{14}C]*Methionine*

Cells from 10 l. of a 7-day-old culture were harvested by centrifugation, washed with 0·05 M potassium phosphate buffer (pH 7·0) then resuspended in 100 ml of 0·05 M potassium phosphate buffer (pH 7·0) containing 50 μc of L-[*Me*-^{14}C]methionine (56·3 mc/m-mole). The suspension, in a 500 ml conical flask, was continually gassed by bubbling a 5%-CO_2–air mixture through a coil of perforated polythene lying at the bottom of the flask. The suspension was incubated in the light for 17 hr at 28°. At the end of this time [^{14}C] Compound E (8600 dpm) was isolated and subjected to Zeisel degradation (see below).

Extraction of the Lipid Fraction

Cells were harvested from the culture media by centrifugation and the lipid extracted by a routine procedure.[12]

[12] D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* **103**, 573 (1967).

Isolation and Purification of Phytylplastoquinone, Compound E (Phytylplastohydroquinone Monomethyl Ether) and other Isoprenoid Quinones and Phenols from the Lipid Fraction

The lipid fraction was resolved into various terpenoid-containing fractions by chromatography on a column of Brockmann grade III acid-washed alumina (Woelm; anionotropic) developed by stepwise elution with light petroleum (b.p. 40–60°) containing increasing amounts of peroxide-free diethyl ether.[12] Phytylplastoquinone, compound E and phylloquinone, together with relatively large amounts of a transparent oil, were eluted by 0·25% E/P.

The isoprenoid quinones and phenols under investigation were purified by a combination of adsorptive TLC on Kieselgel G impregnated with Rhodamine 6G and reversed-phase (RP) TLC on Kieselgel G impregnated with paraffin. Details of the detection and recovery of compounds from these systems have been reported previously.[13]

Phytylplastoquinone, compound E and phylloquinone were separated from contaminating lipids, but not resolved from each other, by adsorptive TLC in System I (Table 1). It is possible to separate phytylplastoquinone and phylloquinone from compound E in this system (Table 1); however, very low plate loadings are required. In the experiments with *E. gracilis* strain Z and *E. gracilis* strain 1224/5 g the recovered mixture of compounds had a u.v. spectrum which was qualitatively identical to that of phytylplastoquinone, which accounts for the ease with which it was detected by Whistance.[5] In *E. gracilis* var. *saccarophila* on the other hand, the u.v. spectra were similar to composite spectra of phylloquinone and phytylplastoquinone, with the spectral properties of phylloquinone predominant. The compounds were then subjected to RP-TLC in System III (Table 1), when compound E was well resolved from both phytylplastoquinone and phylloquinone which once again co-chromatographed. The compounds were recovered and separated from contaminating paraffin by adsorptive TLC in system I. The mixture of phytylplastoquinone and phylloquinone was treated with ethanolic $NaBH_4$ (0·2 ml of ethanol containing a small crystal of $NaBH_4$) and immediately subjected to RP-TLC in system III. Phytylplastohydroquinone was recovered and separated from paraffin by TLC in system I. During this procedure spontaneous oxidation of the hydroquinone to the *p*-benzoquinone took place. Phylloquinol was rarely recovered from the RP-TL system; this is probably because it was destroyed by the alkali produced in the $NaBH_4$-reduction step.

The procedures used for the purification of the other isoprenoid compounds examined were similar to those which have been described previously.[13]

Chloro-Auric Acid Oxidation of Compound E

The sample (0·25 μmole) was dissolved in 5 ml of ethanol and 0·2 ml of aq. 20% (w/v) chloro-auric acid added. After leaving to stand in the dark for 25 min, the reaction mixture was poured into a separating funnel containing 10 ml of light petroleum (b.p. 40–60°) and 10 ml of water. The mixture was then swirled gently, after which the petroleum layer, containing phytylplastoquinone, was removed and evaporated to dryness.

Reaction of Phytylplastoquinone with Cysteine

A purified sample of phytylplastoquinone (about 0·15 μmole) was dissolved in 3 ml of ethanol, then 10 μl of 0·2 M-cysteine in 0·1 M sodium phosphate buffer (pH 7·6) was added to both the sample and reference cell, and the u.v. spectra determined after 0, 5, 10 and 20 min. The changes observed were similar to those described by Redfearn[7] for the reaction of plastoquinone with cysteine.

Catalytic Hydrogenation of Phytylplastoquinone

Phytylplastoquinone (0·15 μmole) was hydrogenated with shaking for 1 hr in 3 ml of cyclohexane–ethanol (1:1, v/v) in the presence of PtO_2 (5 mg) until no more H_2 was absorbed. After removal of the catalyst by filtration, the solvent was evaporated and the perhydrophytylplastohydroquinone oxidized to perhydrophytylplastoquinone by chloroauric acid. The conditions employed were identical to those described above for the oxidation of compound E. The product, after purification by adsorptive TLC in system I, showed $\lambda\lambda_{max}$ 257 and 264 nm in cyclohexane.

Chemical Conversion of Phytylplastoquinone to γ-Tocopherol (Scheme I)

Phytylplastoquinone (0·35 μmole) was refluxed for 1 hr in freshly redistilled pyridine (1 ml), after which the mixture was diluted with saturated NaCl solution and extracted with light petroleum (b.p. 40–60°). After adsorptive TLC in System IV (Table 2), a compound (R_f 0·29) was obtained which had u.v. spectrum expected of phytylplastochromenol (III), i.e., $\lambda\lambda_{max}$ 234, 268 and 333 nm with a shoulder at 276 nm. The phytylplastochromenol was dissolved in 3 ml of cyclohexane–ethanol (1:1, v/v) and hydrogenated under conditions identical to those used for the hydrogenation of phytylplastoquinone (see above). The product [phytylplastochromanol (IV)] was purified by adsorptive TLC in system IV (Table 2), and shown to have the same u.v. spectroscopic and TLC properties as authentic γ-tocopherol (Table 2).

[13] G. R. WHISTANCE, D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* **105**, 145 (1967).

Zeisel Degradation of [^{14}C]*Compound E*

^{14}C radioactivity in the methoxy group of compound E was determined as described by Threlfall, Whistance and Goodwin.[13]

Quantitative Assay

Phylloquinone, plastoquinone, γ-tocopherol, α-tocopherol, α-tocopherolquinone and ubiquinone were assayed by the procedures of Threlfall and Goodwin[12] and Whistance *et al.*[13] Phytylplastoquinone was assayed in ethanolic solution by reduction with $NaBH_4$. The $\Delta E^{1\%}_{1cm}$ (255 nm) of authentic phytylplastoquinone was found to be 380. Compound E was estimated from the amount of phytylplastoquinone formed when it was oxidized with chloro-auric acid.

Radioassay

Details of scintillation counting and proportional counting were as described by Whistance *et al.*[13] All counts were corrected for background and instrument efficiency.

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