

2-METHYL-6-PHYTYLQUINOL AND 2,3-DIMETHYL-5-PHYTYLQUINOL AS PRECURSORS OF TOCOPHEROL SYNTHESIS IN SPINACH CHLOROPLASTS

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Abstract—The incorporation of [Me-¹⁴C] from SAM-[Me-¹⁴C] into precursors indicates the following sequence of tocopherol synthesis in spinach: 2-methyl-6-phytylquinol (6-phytyltoluquinol) (**1a**) → 2,3-dimethyl-5-phytylquinol (phytylplastoquinol) (**2a**) → γ-tocopherol (**5a**) → α-tocopherol (**6**). **1a** is particularly preferred to 2-methyl-5-phytylquinol (**1b**) and 2-methyl-3-phytylquinol (**1c**). **1a** only forms **2a**. **2a** is converted to **6** via **5a** and, to a lesser extent, 2,5-dimethyl-6-phytylquinol (**2b**) to **6** via β-tocopherol (**5b**). Trimethylphytylquinol (**3**) is not an intermediate in the formation of **6**. All reactions are independent of light.

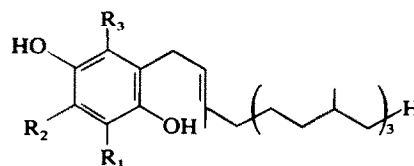
INTRODUCTION

Threlfall in 1971 [1, 2] proposed two equally plausible pathways for the biosynthesis of tocopherols. Thus the methylation might occur on the chromanol stage (**1a** → **4** → **5a** → **6** [1]) or on the phytylquinol and chromanol stage (**1a** → **2a** → **5a** → **6** [2]). In contrast, Janiszowska and Pennock in 1976 [3] concluded from their studies on whole plants of *Phaseolus* and *Tropaeolum* that methylation might occur more or less unspecifically yielding all isomers of phytylquinols and tocopherols. The paper presented here describes studies on tocopherol synthesis in spinach chloroplasts. The results essentially confirm the hypothesis in ref. [2].

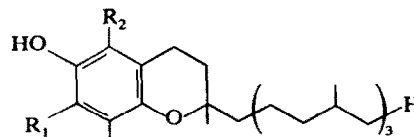
RESULTS

Optimization for tocopherol synthesis in chloroplasts. The need for SAM instead of methionine plus ATP

Only chloroplasts of non-differentiated leaves (1-2 weeks old) are capable of tocopherol synthesis. In these optimization experiments using very young leaves no substrate was added because the leaves contain relatively large amounts of prenylquinols as endogenous substrates (on average 1 nmol **2a** and nmol **1a** per mg chlorophyll). No C-methylation occurs if Met+ATP instead of SAM is applied. Obviously chloroplasts are not able to form SAM under these conditions. Chloroplasts osmotically shocked for a few minutes (in suspensions with more than 1 mg chlorophyll/ml) show nearly the same rates as more or less intact ones (suspensions with 60-80% intact chloroplasts). Optimal rates are found at pH 7.6 in



	R ₁	R ₂	R ₃
1a	Me	H	H
1b	H	Me	H
1c	H	H	Me
2a	Me	Me	H
2b	Me	H	Me
3	Me	Me	Me



	R ₁	R ₂
4	H	H
5a	Me	H
5b	H	Me
6	Me	Me

medium C according to Jensen and Bassham [4] containing 10 mM NaHCO₃. At pH 6.7 the rate is 85%, and at pH 6.1 55% of the optimum.

6-[¹⁴C] formed by the reaction with SAM-[Me-¹⁴C] in chloroplasts was examined for constancy of specific activity (sp. act. (dpm/nmol) after the addition of carrier-**6**: 1st TLC on system II 9.84; 2nd TLC on system IV 9.32; 3rd TLC on system III 8.48).

Steps of biosynthesis

The incorporation of $[Me-^{14}C]$ from SAM- $[Me-^{14}C]$ into **1a** yields labelled **2a** and the quinone of **2a**, **5a** and **6** in decreasing amounts (Table 1). Among the possible isomers of dimethyl compounds only **5a** could be detected after the formation of nitroso-derivatives (see Experimental). This indicates that only the 2,3-dimethyl compounds are formed from **1a**.

Furthermore, $[Me-^{14}C]$ from SAM- $[Me-^{14}C]$ is incorporated into **2a** yielding **6**- $[^{14}C]$ (Table 1). Intermediate formation of **3** could be detected neither with **2a** nor with **1a** as substrate (Fig. 1). It was not possible to show the predictable formation of **5a** by the cyclization of **2a** in experiments with SAM- $[Me-^{14}C]$, but it may be deduced undoubtedly from the following experiments. **5a**- $[^{14}C]$ was produced in experiments with **1a** and SAM- $[Me-^{14}C]$ in amounts much larger than in experiments with **4** and SAM- $[Me-^{14}C]$ (Table 1). Moreover, C-methylation of **5a** yielding **6**- $[^{14}C]$ was a reliable reaction for the standardization in the experiments in Table 1.

The C-methylation is strictly enzymic; if chloroplasts were omitted, no reaction occurred. Activity with endogenous substrates was negligible in *ca* 2-week-old leaves used here. Hence the following sequence in tocopherol synthesis can be derived: **1a** → **2a** → **5a** → **6** (**2a** → **3**). Considering the results mentioned above the other possible sequence **1a** → **4** → **5a** → **6** is only an unspecific pathway in spinach.

Substrate specificity

In spinach the pathway outlined above seems to be fairly specific. In the first step **1a** is the favoured substrate for the incorporation of a methyl group from SAM; the isomers **1b** and **1c** are both converted at

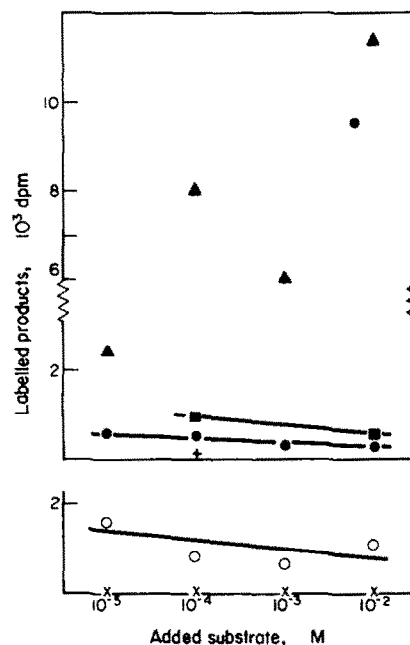


Fig. 1. ^{14}C -Incorporation from SAM- $[Me-^{14}C]$ into substrates of different concentrations: upper diagram—products from **1a** as substrate: **2a** (▲), **3** (+), **5a** (■), **6** (●); lower diagram—products from **2a** as substrate: **3** (x); **6** (○).

very low rates (^{14}C -labels in corresponding dimethyl compounds 100:10:5). For the second step the substrate of cyclization is **2a** and also **2b**; both are cyclized and methylated to **6** at comparable rates. In the third step **5a** is preferred to **5b** in the synthesis of **6** (^{14}C -labels in **6** 100:37).

Table 1. ^{14}C -Incorporation from SAM- $[Me-^{14}C]$ into substrates of tocopherol synthesis in chloroplasts suspensions

Substrate*	Product	^{14}C -Incorporation in % of added SAM- $[Me-^{14}C]$
+ 1a	2a	1.500
	Quinone of 2a †	0.545
	5a	0.628
	6	0.250
+ Quinone of 1a	2a	0.005
	Quinone of 2a †	0.570
	5a	0.310
	6	0.033
+ 4	2a ‡	0.029
	Quinone of 2a †‡	0.068
	5a	0.072
	6	0.019
+ 2a	6	0.107
+ Quinone of 2a	6 ‡	0.003
+ 5a	2a ‡	0.005
	Quinone of 2a †‡	0.007
	5a ‡	0.026
	6	0.230

* 2×10^{-3} , light, for further details see Experimental.

†Formed partially by oxidation during isolation procedure.

‡Formed presumably by methylation of endogenous substrates by SAM- $[Me-^{14}C]$.

Quinones instead of quinols as substrates

In any case quinones are methylated by SAM less well than the corresponding quinols. The quinone of **1a** was converted, at low rates, to the quinone of **2a**, **5a**, **6** and tracers of **2a**. The quinone of **2a** as a substrate, however, was converted to **6** in negligible amounts (Table 1).

Rates in the light compared to those in the dark

None of the steps show a significant difference between light and dark. As the quinol stage is better preserved in the dark, the rate of methylation seems somewhat higher in the dark than in the light.

Effect of substrate concentration

Using **1a** or **2a** as substrate at a concentration of 10^{-5} ; 10^{-4} , 10^{-3} and 10^{-2} M, the optimal substrate concentrations were tested. These are ca 10^{-2} M **1a** for the formation of **2a**, 10^{-4} – 10^{-3} M **1a** for **5a**, and 10^{-4} – 10^{-5} M **2a** for the formation of **6** (Fig. 1).

DISCUSSION

Phytolquinols serve as precursors in the biosynthesis of tocopherols. Firstly, the electrophilic substitution by SAM requires a substance present in the reduced stage. Secondly, a quinol structure is a prerequisite in the formation of the chromanol ring. In our experiments quinones seem to be reduced by factors formed during photosynthesis. One problem is why the two methylation steps take place at different stages, the first one at the phytolquinol stage yielding **2a** from **1a**, and the second one at the tocopherol stage yielding **6** from **5a**. Presumably this is caused by different compartmentation of the enzymes responsible for the methyl transfer at the monomethyl or dimethyl stage.

As demonstrated by Janiszowska and Pennock [3], the corresponding methyltransferases exhibit no substrate specificity in *Phaseolus*. Through isomers of dimethylphytylquinols and to a very small extent, α monomethylphytylquinols react with SAM, the pathway is channelled of the methylphytylquinol stage in spinach: firstly, only the 6-isomer is formed from homogentisate and prenyl-PP (Threlfall, D. R., personal communication) and, secondly, this isomer is the particularly favoured substrate in the methylation reaction yielding the 2,3-dimethyl compound **2a**. From there, the pathway is directed to **5a** and **6**. Modifications may be supposed only in species containing **5b** and **5a**, e.g. *Hordeum*, *Avena*, *Triticum*, etc. [5]. Furthermore the fact that **5a** is temporally preferred to **6** in time course studies on maize and lettuce (Schultz and Keppler, unpublished results) might be deduced from its sequence in synthesis and is not a regulation phenomenon (Fig. 2).

Since the side reaction from **1a** to **4** seems to be absent in spinach and the reaction of **4** to **5a** is only poor, the absence of **4** is explicable. Likewise in *Hedera* [6], *Hevea* [7, 8] and *Ficus* [9], **4** seems to be a product of a side reaction [1, 2, 6] rather than an intermediate in the formation of dimethyl and trimethyl tocopherols [8].

Though the above 'tocopherol pathway' [3] generally occurs in higher plants, it might be somewhat modified, especially in species containing tocotrienols

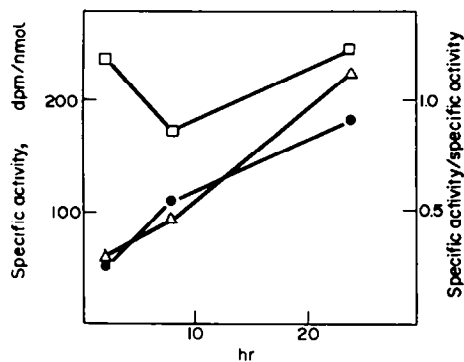


Fig. 2. Time course of ^{14}C -incorporation from tyrosine- $[\beta\text{-}^{14}\text{C}]$ in **5a** and **6** of illuminated maize shoots. The sp. act. of **5a**(\square) and **6**(Δ), respectively, and the ratio of sp. act. of **6**/sp. act. of **5a**(\bullet) are shown.

[5, 7–9]. In *Hevea* [7, 8] and *Ficus* [9] they are formed in tissues different from leaves. There the 'tocotrienol pathway' [3] is localized in the latex whereas the 'tocopherol pathway' is more or less restricted to the leaves [8]. Moreover, in *Hevea* a portion of tocotrienols is esterified with palmitate [5]. The hydrogenation of tocotrienols may be a principal reaction for converting them into tocopherols as was inferred from the incorporation of tritium from 'A' tritio-NADPH-(4R-diastereoisomer) into tocopherols in *Ficus* [9] or it may be a side reaction from α -tocopherol to **6** as in *Hevea* latex [3, 8].

EXPERIMENTAL

Radiochemicals. SAM-[Me- ^{14}C] (sp. act. 59 mCi/mmol) and methionine-[Me- ^{14}C] [sp. act. 60 mCi/mmol] were obtained from Amersham-Buchler, Braunschweig; tyrosine- $[\beta\text{-}^{14}\text{C}]$ was purchased from NEN, Dreieichenhain and later from CIS, Gif-sur-Yvette, France.

Synthesis of cis, trans 2,3-dimethyl-5-phytyl-1,4-benzoquinone (2,5-cyclohexadiene-1,4-dione-2,3-dimethyl-5-(3, 7, 11, 15-tetramethyl-2-hexadecenyl) (quinone of 2a)) [10]. All methyl quinones were prepared from the phenols by use of Frey's salt [11, 12] and reduced according to ref. [13]. Freshly distilled BF_3 -etherate (0.3 ml in 1 ml THF) was added drop by drop by syringe to a soln of 150 mg 2,3-dimethylquinol, 200 mg Al_2O_3 and 300 mg isophytol in 2 ml dry THF. The mixture was stirred for 15 hr (N_2 , dark). The substance was hydrolysed with ice, extracted with Et_2O and dried. The Et_2O phase was evapd. The resulting **2a** [13] was oxidized by Ag_2O (400 mg) in dry Et_2O . Purification was affected by column chromatography (Si gel (Merck 60); system I). MS (20°): M^+ m/e : 414 (50%), 189 (100), 151 (50), 63.5 (54.5), 62 (40.9), 44 (68.8), 42 (52.2). IR cm^{-1} : alkyl-H 2950, 2925, 2867; C=O 1649; C=C 1615. $^1\text{H NMR}$ (60 MHz, CDCl_3): δ 0.91 (12 H, Me chain); 1.23 (19 H, chain); 1.62 (3H, C=C—Me); 2.01 (2H, CH_2 -allyl to C=C chain); 2.04 (6H, Me quinone); 3.1 (2H, CH_2 -allyl to C=C-quinone and chain); 5.1 (1H, HC=C-chain); 6.5 (1H, quinone).

Synthesis of cis, trans 2,5-dimethyl-6-phytyl-1,4-benzoquinone (quinone of 2b) was according to above procedure except that 2,5-dimethyl quinol [11, 12] was used.

Synthesis of cis, trans-2-methylphytyl-1,4-benzoquinones

(2,5-cyclohexadiene-1,4-diones-2-methyl-(3, 7, 11, 15-tetra-methyl-2-hexadecenyl) (quinones of **1a**, **1b** and **1c**). Synthesis was carried out by the procedure described above. The fractionation of the isomer mixture was made by multiple TLC on system I (see [3]).

Reduction of phytylquinones for studies on chloroplasts. A photometrically adjusted amount of quinone (for extinction coefficients compare [14]) was dissolved in 1 ml MeOH, reduced with solid NaBH₄ for 2 min, transferred to Et₂O and washed with H₂O. The Et₂O phase was evapd to dryness under N₂ in vials (10×1 cm) and could be stored at -20° in the dark for 1 to 2 hr.

Chloroplasts were isolated from spinach according to ref. [4] but in a modified way as described in ref. [15]. The percentage of intact chloroplasts was determined by the Hill reaction [16].

Broken chloroplasts were prepared as above except that the chloroplast pellet centrifuged in Medium B [4] was suspended in H₂O until a chlorophyll content of ca 2 mg/ml was obtained.

Application of substrates for tocopherol synthesis in chloroplasts. The following substances were added to the vials (10×1 cm) containing the substrate (10⁻⁵-10⁻⁸ mol suspended in 20 μl EtOH): 50 μl 0.2 M NaHCO₃, 2.5 μCi SAM (= 50 μl soln in 0.1 N H₂SO₄) and 1 ml chloroplast suspension in Medium C, pH 7.6 (at least 1 mg chlorophyll/ml). The suspension was illuminated by halogen lamps (0.1 J/cm² sec) and stirred slowly at 20±2° for 30 min. For broken chloroplasts, however, 0.5 ml of chloroplast suspension in H₂O (containing at least 2 mg chlorophyll/ml) plus 0.5 ml Medium C, pH 7.6 [4] were used. In the methionine-[Me-¹⁴C] expt., 4 μCi (ca 0.1 μmol) methionine-[Me-¹⁴C] plus 2 μmol ATP were used. Where rates are cited in Results, the data are from expts performed with the same chloroplast suspension.

Application of tyrosine-[β-¹⁴C] to maize shoots. Tyrosine-[β-¹⁴C] (0.5 μCi) in 50 μl 0.01 N NaHCO₃ was added to excised maize seedlings (in the primary leaf stage) inserted into small vessels in couples. 10 seedlings per time interval were used. They were illuminated by white light bulbs (3 klx) and H₂O was added in 50 μl portions if needed.

TLC systems. System I—Si gel with petrol (bp 60-80°)-Et₂O, 15:1; system II—the same but 10:1; system III—Si gel with C₆H₆-CHCl₃, 1:1; system IV—reversed-phase system (cellulose impregnated with 7% paraffin oil in petrol, bp 100-140°) with Me₂CO-MeOH, 2:1; system V—the same with Me₂CO-H₂O, 85:15; system VI—the same with 96% EtOH (all layers with fluorescent indicator F 254 on glass (Schleicher & Schüll)).

Isolation of phytylquinones, tocopherols and other isoprenoids from chloroplast suspensions. Me₂CO (30 ml) and 60 ml petrol (bp 40-60°) were added to a separatory funnel containing the treated chloroplast suspension. Carriers were provided at a range of 50-100 μg each. H₂O (500 ml×2) was continuously added without shaking. After discharging the water phase carefully, the petrol phase was evapd to dryness under N₂. The residue was dissolved in a minimum of Me₂CO and chromatographed by system II. Usually the quinols were oxidized by an air supply before TLC. After recovering the substances from system II they were applied to system V. All substances were characterized by co-

chromatography with markers.

In system II the sequence of substances in the prenylhydroquinone plus tocopherol zone is (beginning with the origin of chromatogram up to R_f ca 0.25): **1a** and isomers, plastoquinol, **2a** and isomers, **3**, **4**, **5a+5b**, **6** (all substances are detectable with the Emmerly-Engel reagent). For the polyprenylquinone zone the sequence is (beginning from R_f ca 0.7 to ca 0.9): quinone of **1a** and isomers, phyloquinone, plastoquinone, quinone of **2a** and isomers, quinone of **3** (all quinones) are detectable by quenching fluorescence at 254 nm). In system V the sequence is: lower part—plastoquinone, quinone of **3**, quinone of **2a** and isomers, quinone of **1a** and isomers; middle part—**6**, **5a+5b**; upper part—plastoquinol, **3**, **2a** and isomers, **1a** and isomers.

Nitroso-derivatives of 5a and 5b were formed according to ref. [17]. NO-**5a** (R_f ca 0.2) was separated from NO-**5b** (R_f ca 0.4) in system VI.

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