Tetrahydrogeranylgeraniol, a Precursor of Phytol in the Biosynthesis of Chlorophyll a — Localization of the Double Bonds

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Dedicated to Prof. Dr. A. Butenandt *on the Occasion of His 75. Birthday*

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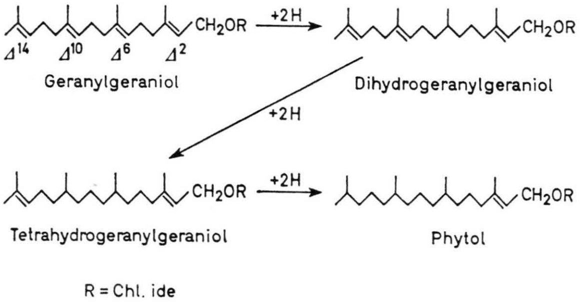
Pheophytins esterified with phytol and tetrahydrogeranylgeraniol are isolated from etiolated oat seedlings after short (1 min) exposure to light and a subsequent dark period of 15 to 20 min. After saponification of the pheophytins, a mixture of the alcohols was isolated. The structure of tetrahydrogeranylgeraniol was established as 3,7,11,15-tetramethyl-$\Delta^{2,14}$ hexadecadiene-1-ol (**3a**). The implications for chlorophyll biosynthesis are discussed.

Introduction

During our research on the last steps of chlorophyll biosynthesis, we could isolate pheophytins which are esterified with not only phytol (P), but also with tetrahydrogeranylgeraniol (THGG), dihydrogeranylgeraniol (DHGG) and geranylgeraniol (GG) [1].

Based on these findings, a biosynthetic sequence has been proposed, in which chlorophyllide a is first esterified to Chl_{GG}. This pigment should then hydrogenated successively to Chl_{DHGG}, Chl_{THGG} and finally to Chl_{P} [1]. The activated alcohol substrate for the esterification is probably geranylgeraniol-pyrophosphate [2].

Scheme 1



The number of double bonds in the alcohols have been established from the molecular ions in their mass spectra [3, 4]. The heaviest alcohol is P (**4**), the alcohols THGG (**3**), DHGG (**2**) and GG (**1**) are smaller by 2, 4 and 6 mass units respectively.

To further evaluate this sequence it is necessary to establish the order of hydrogenation of the three double bonds Δ-6, Δ-10, Δ-14 when transforming GG to P.

As the pigments were available only in small amounts (5–10 nmol/g fresh weight), the alcohols were identified and the double bonds localized by gc-ms technique combined with microchemical procedures. By this means, and by chemical correlations the structure **3a** of tetrahydrogeranylgeraniol was identified as presented in this paper.

Results and Discussion

Isolation and enrichment of THGG: In 7½ days old etiolated oat seedlings, the kinetic of esterification of Chl.id after the photohydrogenation of PChl(id) has been studied [1]. The amount of THGG reaches after 30–40 min a maximum (1.5 nmol/g fresh weight). At this time, 70–80% of total Chl.id is esterified and the stepwise hydrogenation GG to PP has proceeded to such an extent, that the ratio of $Chl_{P} : Chl_{THGG}$ is about 5 : 1.

To reduce the amount of Chl_{P}, the seedlings were harvested already after 15–20 min. The amount of THGG then was only about 1 nmol/g fresh weight, but the ratio $Chl_{P} : Chl_{THGG}$ was decreased to 3 : 1 [1]. The chlorophylls were extracted and demetalated. The mixture of pheophytins was separated from other pigments by tlc [3].

Abbreviations: Chl, Chlorophyll a; Chl. ide, Chlorophyllide a; Pchl(ide), Protochlorophyll(ide); Phe, Pheophytin a; GG, Geranylgeraniol; DHGG, Dihydrogeranylgeraniol; THGG, Tetrahydrogeranylgeraniol, P, Phytol. Chl_{GG}, Chl_{DHGG}, Chl_{THGG}, Chl_{P}, chlorophyllide a, esterified with the respective alcohol.

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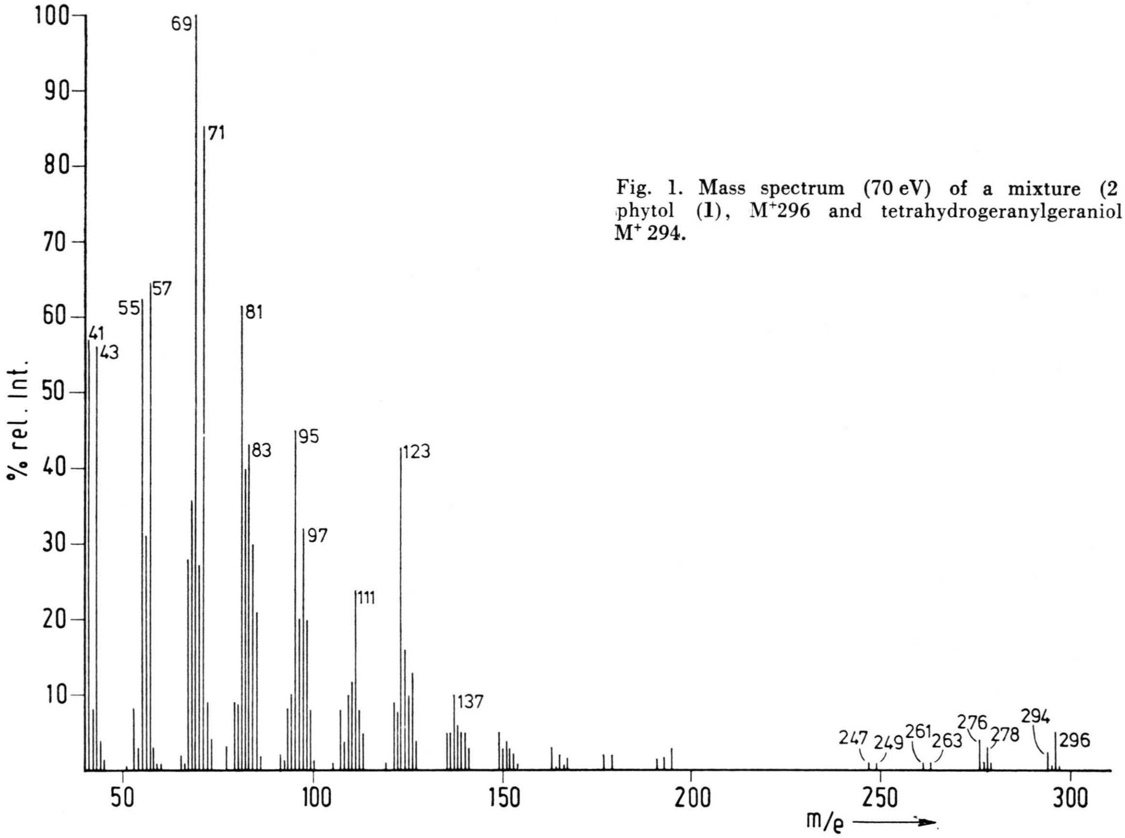


Fig. 1. Mass spectrum (70 eV) of a mixture (2 : 1) of phytol (1), M^+296 and tetrahydrogeranylgeraniol (3 a), M^+ 294.

After saponification [4], the alcohols were then separated into three zones on tlc-plates previously buffered and treated with silvernitrate. The two slower zones contained GG and DHGG. From the fast zone, P and THGG were eluted together. By repeated chromatography, THGG could be enriched to a ratio of P : THGG = 2 : 1. The mixture of these two alcohols was used for structure elucidation. The results of gaschromatographic separation of these alcohols have been published previously [1].

Structure elucidation: The mass spectrum of a mixture of P and THGG (Fig. 1) is analogous to the spectra of P [4], GG and DHGG [3].

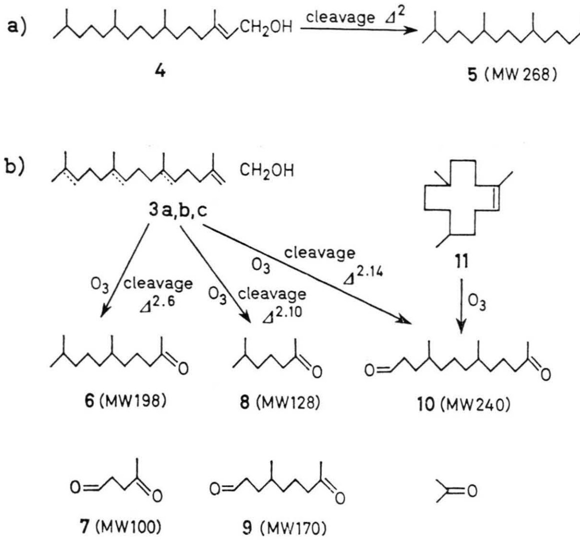
Significant are the regions of the molecular ions, which differ by two mass units each, and of the ions (M-18) derived by loss of water from the molecular ions. Rüdiger *et al.* have already pointed out that *m/e* 69 is the base peak in the spectra of GG and DHGG but *m/e* 71 in the spectrum of P [3]. This was taken to be an indication for the Δ-14-double bond in the former two. In the mass spectrum of the mixture of P and THGG we found both *m/e* 69 and *m/e* 71, and we consider this an indication of the Δ-14-double bond in THGG too. As the position of double bonds in unsaturated hydrocarbons cannot be established unequivocally by using mass spectrometry alone, the alcohols were degraded by microozonolysis according to Beroza [5], the resulting ketones/aldehydes separated by gaschromatography and their structures established from the ms-fragmentation..

First phytol was studied. This alcohol yields 6,10,14-trimethyl-2-oxopentadecane (**5**) on ozonolysis and reductive workup (scheme 2a).

The structure was deduced by its ms-fragmentation (see mass spectra). The molecular ion can only be observed by overloading the column (injection of about 5 μg). Under normal gc/ms-conditions, *m/e* 250 ($M - H_2O$) is the heaviest ion.

Phytol is the final product of the hydrogenation sequence in the plant material. Therefore the Δ-2-double bond should be present in all four alcohols. Dependent on the position of the second double bond in THGG, different ketones/aldehydes are to be expected from the ozonolysis of the structural

Scheme 2a/2b



isomers of THGG (**3a**, **3b**, and **3c**) (scheme 2b): 3,7,11,15-tetramethyl-$\Delta^{2,6}$-hexadecadiene-1-ol (**3c**) is degraded to 6,10-dimethyl-2-oxoundecane (**6**) and 1,4-dioxopentane (**7**). 3,7,11,15-tetramethyl-$\Delta^{2,10}$-hexadecadiene-1-ol (**3b**) should yield 6-methyl-2-oxoheptane (**8**) and 6-methyl-1,8-dioxononane (**9**). From degradation of 3,7,11,15-tetramethyl-$\Delta^{2,14}$-hexadecadiene-1-ol (**3a**) one would expect 4,8-dimethyl-1,12-dioxotridecane (**10**) and acetone. Glycolaldehyde is a reaction product in any case.

From the ozonolysis of the mixture P – THGG = 2 : 1 the ketone 5 was obtained as main product with a retention time in gc of 10.5 min. For the second peak with a retention time of 21.5 min the structure 4,8-dimethyl-1,12-dioxotridecane (**10**, MW 240) was deduced from the ms-fragmentation. The heaviest ion at *m/e* 222 is derived by loss of water from the molecular ion. Independently **10** was obtained from the ozonolysis of 1,5,9-trimethylcyclododecene (**11**) [6] as well as from a mixture of **11** with phytol. The retention times and the mass spectra respectively of the ketoaldehydes (**10**) from both origins are in agreement with one another.

In addition, the ketones/aldehydes derived from the ozonolysis of the P-THGG mixture were converted to their dinitrophenylhydrazones. They were separated by tlc, and their mass spectra recorded. Reaction of dinitrophenylhydrazine with the products of ozonolysis of phytol (**4**) and trimethylcyclododecene (**11**) yielded the reference substances. According to R_F-values, their ratio and to their mass spectra products received from the alcohol mixture are identical with these references. Thus, the two double bonds in THGG are located at position Δ-2 and Δ-14 (structure **3a**).

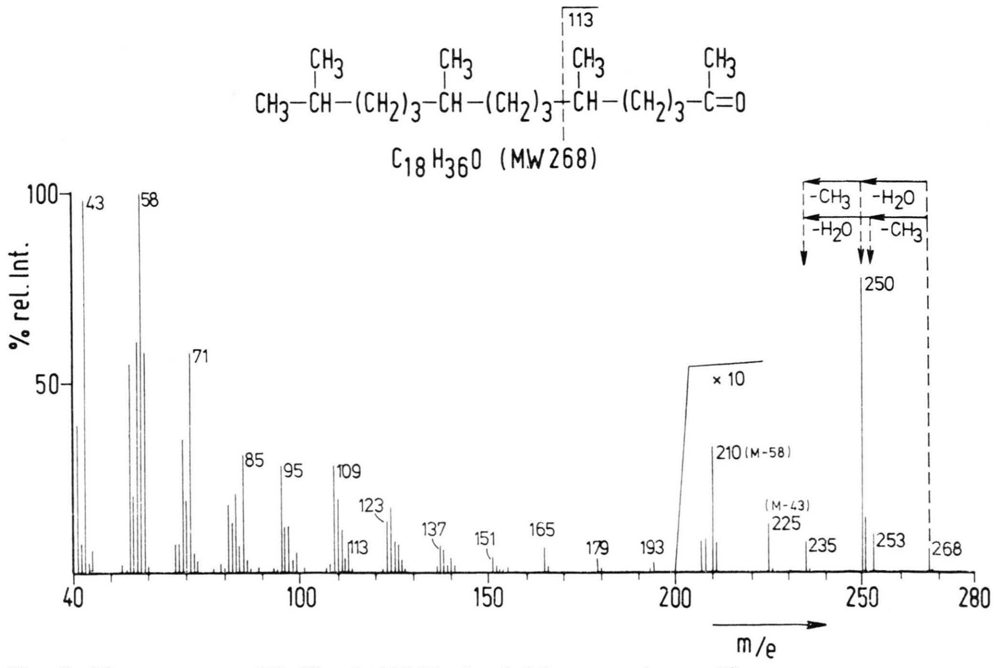


Fig. 2. Mass spectrum (70 eV) of 6,10,14-trimethyl-2-oxopentadecane (**5**).

Mass spectra: The mass spectra of the ketones and their DNP-derivatives confirm the structures discussed above.

6,10,14-Trimethyl-2-oxopentadecane (**6**): The molecular ion *m/e* 268 of this ketone is of very low intensity and eliminates water (→ *m/e* 250) by a thermal or an electron impact process. The elimination of water following the loss of a methyl radical (*m/e* 253 → *m/e* 235) is known from long chain methylketones [7, 8]. The α-cleavage producing the ions *m/e* 43 and *m/e* 225 are characteristic for methylketones. The ion *m/e* 58 arising from the Mc Lafferty rearrangement and its complement *m/e* 210 confirm the structure. The ion *m/e* 95 could be interpreted as $(113 - H_2O)$.

4,8-Dimethyl-1,12-dioxotridecane (**10**): The molecular ion of this ketoaldehyde is not observable but the two oxygen containing functional groups are indicated by water eliminations from the ions *m/e* 197 (α-cleavage, → *m/e* 179) and *m/e* 182 (McLafferty rearrangement, → *m/e* 164).

Tetrahydrogeranylgeraniol, as shown above, is 3,7,11,15-tetramethyl$^{\Delta 2,14}$-hexadecadiene-1-ol (**3a**). In the oat seedlings hydrogenation starts with *all-trans*-geranylgeraniol (**1**) bound to chlorophyll. From phytol it is known that it has *trans* configuration [9]. As in GG and phytol the double bonds are *trans*, we postulate this also for the intermediate THGG. As the two double bonds in THGG are located on both ends of the molecule and as the Δ-2 double bond is available in all for isoprenoid alcohols we assume that hydrogenation starts with the Δ-6-double bond (→ Chl$_{DHGG}$). Hence structure **2** is deduced for DHGG. This was already assumed from the mass spectrum of the compound itself [3]. It is not possible to distinguish by ozonolysis whether the double bonds in DHGG are in 2,6,14- or 2,10,14-position because both isomers were to yield the same ketoaldehyde. From **2** hydrogenation proceeds to saturate the Δ-10-double bond to form THGG (**3a**). The last step in the biosynthesis of Chl is the hydrogenation of the Δ-14-double bond to form Chl$_P$. It seems necessary for the enzymatic hydrogenation system to detect the double bond unequivocally, so that the hydrogenation can pass off in the order shown from the structure of the different acohols. The esterified pigment is probably sterically fixed in such a manner that the Δ-6-double bond is accessible for the active center of the enzyme system.

Experimental

All solvents were distilled before use. Ether was filtered over aluminum-oxid to eliminate peroxides.

For gaschromatography a Carlo Erba Fractovap 2101, equipped with a 50 m FFAP glascapillary with an internal diameter 0.3 mm was used. Injection temperature was 250 °C. The injection was splitless at 150 °C oven temperature, and the temperature raised with 2 °C/min to 180 °C. Carrier gas was 1.6 ml Helium/min. The gaschromatograph was coupled to the Varian CH7a/SS 100 mass spec-

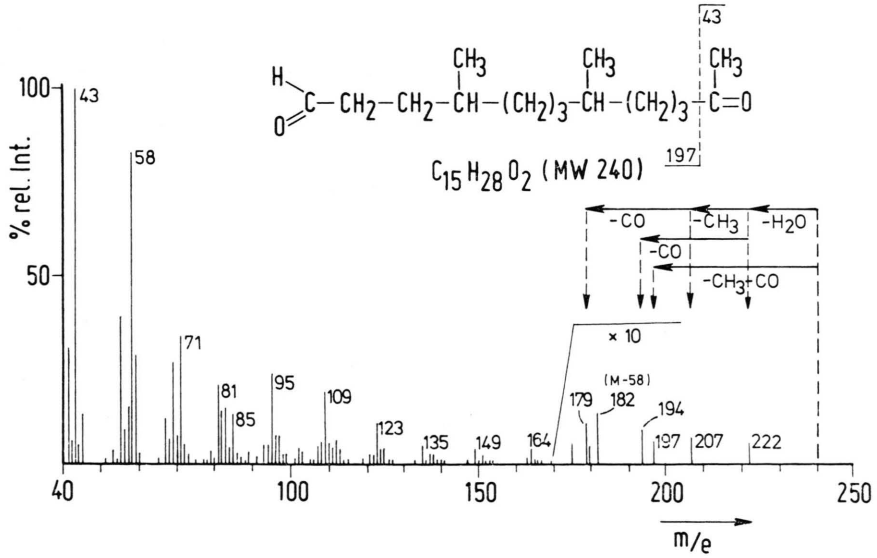


Fig. 3. Mass spectrum (70 eV) of 4,8-dimethyl-1,12-dioxotridecane (**10**).

trometer-datasystem by glt-capillary 0.3 mm internal diameter from SGE, Melbourne. MS-conditions were electron energy 70 eV, emission 1 mA.

Isolation and enrichment of THGG (**3a**): Oat seedlings (*Avena sativa* L., Bayerische Futter und Saatbau GmbH, München) were grown on moist vermiculite at 27 °C in darkness. $7^{1}/_{2}$ days old seedlings were harvested at green safe-light and the leaves irradiated with white fluorescent light (2000 lx) for 1 min After a dark period of 15 – 20 min (22 – 24 °C) the leaves were dipped in liquid nitrogen and ground in a warring blendor with acetone. Centrifugation (G50K, WKF) at $12000 \times g$ (4 °C) and filtration gives a clear solution of the extract in acetone which was kept cold in an ice bath. After addition of water, the pigments were extracted from acetone into ether and demetalated by treatment with 10% HCl for 30 min. The resulting pheophytins were then separated from carotenoids and unesterified pheophorbides by tlc on silicagel using petrolether/diethylether/acetic acid = 70/24/2 for development. The esterified pigments were eluted from the scraped-off green zone with acetone. They were further separated into two zones on silicagel tlc plates buffered with (0.03 M phosphate buffer pH 7.5) and treated with 5% silver nitrate during preparation. After development with carbon tetrachloride/acetone = 88/12 a slower zone containing pheophytin esterified with GG and DHGG and a faster zone containing pheophytin esterified with P and THGG were eluted with acetone. The solvent was evaporated and the pigments were then saponified with methanol/potassiumhydroxide [4]. A further enrichment of THGG was achieved by chromatography on buffered and silver nitrate treated tlc-plates. The plates were first developed in carbon tetrachloride/acetone (90/9) for 4 – 6 cm, dried and then developed with petrolether/chloroform/acetone = 50/10/15. The main zone localized by spraying a strip of the plate with potassium permanganat-solution was eluted with acetone, diluted with water and extracted with petrolether. The organic phase was washed thoroughly with water to eliminate silver-nitrate. After drying with sodium sulfate, the ratio P/THGG = 2 : 1 in hexane was estimated by gc.

Ozonolysis: About 20 – 25 μg phytol + THGG (2 : 1) in hexane (50 μl) were ozonised according to Beroza's technique [5]. After reductive workup using triphenylphosphine, the mixture was immediately analyzed by gc/ms. 1,5,9-Trimethylcyclododecene was ozonised in carbondisulfid, the degradation is uncomplete in hexane.

Dinitrophenylhydrazones: The ketones/aldehydes from the microozonolysis were treated over night with 100 μl dinitrophenylhydrazine solution (200 mg DNP in 1 ml conc. sulfuric acid, diluted with 1.5 ml water and 5 ml ethanol). 200 μl chloroform were added, the organic solvent was washed with water and dried with magnesium sulfate. The components were separated on a silicagel tlc-plate (benzene/ethylacetate = 10/1) which was purified before development with acetone. The components were eluted with acetone. Mass spectra (direct inlet system) were recorded using a programmed evaporation combined with a cyclic scan. MS (DNP-derivative of **5**) 70 eV, *m/e* (rel. int.): 448 $[M^+]$ (22), 433 $[M^+ - CH_3]$ (8), 431 $[M^+ - H_2O]$ (6), 413 $[M^+ - (OH + H_2O)]$ (19) 238 (29), 178 (55) [10, 11]. MS (DNP-derivative of **10**) 70 eV, *m/e* (rel. int.): 600 $[M^+]$ (9), 570 $[M^+ - NO]$ (5), 565 $[M^+ - (OH + H_2O)]$ (10), 238 (52), 178 (100).

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