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Chlorophylls. VI.* Epimerization and Enolization of Chlorophyll *a* and Its Magnesium-free Derivatives

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The properties of chlorophyll *a'* (Chl *a'*) were investigated in detail by visible, IR and ^{1}H NMR spectroscopy. A new method for the preparation of Chl *a'* was developed which permits the isolation of this pigment in a yield of 40 % and a high degree of purity. The visible and IR spectra of Chl *a'* were found virtually identical with those of Chl *a*. The whole ^{1}H NMR spectrum of Chl *a'* was measured in acetone-d_6, THF-d_8 and benzene-d_6 and compared with that of Chl *a*. The NMR spectra of these two compounds were found slightly different in the positions of the methine, C10,C10b methyl and C5a methyl proton resonances. The $t_{\frac{1}{2}}$ of the interconversion between Chl *a'* and Chl *a* is at room temperature *ca.* 1 h, 24 h and several days, in acetone, THF and benzene, respectively. Experimental evidence for the aggregation of Chl *a'* in benzene-d_6 at a concentration $\geq 10^{-2}$ M was obtained by NMR spectroscopy. Careful survey of the NMR spectra at low field did not reveal any resonance peak attributable to an enolic proton, in spite of the fact that the underlying mechanism for the interconversion between Chl *a* and Chl *a'* is keto-enol tautomerism. These observations confirm the earlier results supporting the epimer nature of Chl *a'*. The higher solubility of Chl *a'* in nonpolar solvents, its greater tendency to pheophytinize and slight spectroscopic difference, in comparison to Chl *a*, were interpreted on the basis of the conformational alterations resulting from the stereochemical change at C10 of ring V. More definitive evidence for these conclusions was obtained by trapping the enols of Chl *a*, pheophytin *a* and methylpheophorbide *a* as trimethylsilyl ethers. The electronic absorption spectrum of the silylated enol of Chl *a* was found essentially different in the Soret band region but quite similar in the red band region, to that of Chl *a*.

* Part V, See Ref. 1.

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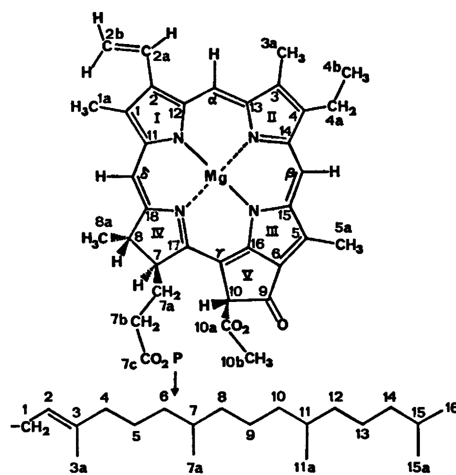


Fig. 1. Structure and proton numbering system of chlorophyll *a*.

Besides the central Mg atom, the cyclopentanone or isocyclic ring V is the most reactive part of the chlorophyll (Chl) molecule (Fig. 1). Owing to the activation of the C10 atom by the methoxycarbonyl group, the C10 hydrogen is enolizable.[2–4] The enolization of the β-keto ester system is in a key position in several modification reactions [3–21] of chlorophyll. The participation of the enol forms of Chl in the primary events of photosynthesis has long been a matter of speculation.[22–30]

In 1968, strong evidence, in support of the concept [5] that Chl *a'* is identical with the C10 epimer of Chl *a*, was presented on the basis of ^{1}H NMR spectroscopy.[4,6,31] The chelated enol of Chl *a* was suggested as an alternative structure for Chl *a'* by Hynninen [32] on the basis of

the following experimental evidence: (i) The solubility of Chl *a′* in nonpolar hydrocarbons was found to be higher than that of Chl *a*, *e.g.*, Chl *a′* did not "crystallize" like Chl *a* on washing the light petroleum solution of the pigment with cold distilled water; (ii) the electronic absorption spectrum of Chl *a′* was found to be somewhat different from that of Chl *a* (the differences found are admittedly small); (iii) Chl *a′* had a greater tendency to pheophytinize than Chl *a*. This was indicated by the following observations:[33] (a) Prolonged shaking of the light petroleum solution of Chl *a′* with water resulted into a pheophytin *a*-like pigment. Similar treatment of Chl *a* did not give pheophytin *a*. (b) When Chl *a′* and Chl *a* were dissolved separately in acetylacetone which is a good complexing agent for Mg, and the solutions were permitted to stand overnight, Chl *a′* yielded more of a Mg-free compound than Chl *a* as indicated by the visible absorption (VIS) spectra; (iv) Chl *a′* could not be separated from Chl *a* by multiple liquid-liquid partition.

Owing to these inconsistencies, a detailed investigation on the physical and chemical properties of Chl *a′* seemed desirable. Furthermore, to obtain a more definitive solution to the problem, the "trapping", *viz.* the preparation of relatively stable organic derivatives of the enol forms of Chl *a* and its Mg-free pigments, was undertaken. The properties of Chl *a′* as well as the preparation and spectro-

Table 1. Electronic absorption spectra of chlorophyll derivatives.

	Compound	Solvent	Peak positions in nm, peak ratios (R)[a] and halfwidths of the red absorption band and the Soret band, $w_{I\frac{1}{2}}$ and $w_{S\frac{1}{2}}$, in nm.
a	Chlorophyll *a*	TEA[b]	660.5(1.32), 615(7.78), 578(14.6), 537(21.9), 502(36.8), 429.0(1.00), 408(1.38), 380(2.11), $w_{I\frac{1}{2}}$ 17.2, $w_{S\frac{1}{2}}$ 43.5
b	Chlorophyll *a*	EE	660.0(1.29), 613(8.59), 573(19.9), 532(44.2), 494(106.0), 428.0(1.00), 409(1.62), 380(2.69), 326(5.13), $w_{I\frac{1}{2}}$ 16.8, $w_{S\frac{1}{2}}$ 37.7
c	Chlorophyll *a′*	EE	660.0(1.26), 613(7.89), 574(16.5), 533(33.8), 494(71.0), 427.5(1.00), 410(1.48), 380(2.60), 325(5.00), $w_{I\frac{1}{2}}$ 16.8, $w_{S\frac{1}{2}}$ 37.2
d	TMS ether of the enol of chlorophyll *a*	0.5 % PrOH in LP	662.0(2.07), 611(12.4), 435.0(1.00), 363(1.09), 315(2.47), $w_{I\frac{1}{2}}$ 25.5, $w_{S\frac{1}{2}}$ 119.8
e	Pheophytin *a*	0.5 % PrOH in LP	668.0(1.86), 608(14.3), 560(41.5), 532(11.7), 504(9.49), 470(30.8), 408.0(1.00), 320(5.91), $w_{I\frac{1}{2}}$ 15.6, $w_{S\frac{1}{2}}$ 51.1
f	TMS ether of the enol of pheophytin *a*	2.0 % THF in LP	750(29.1), 645.0(8.08), 603(29.4), 551(145.5), 503(16.5), 420.0(1.00), 350(1.12), $w_{I\frac{1}{2}}$ 28.8, $w_{S\frac{1}{2}}$ 129.4
g	TMS ether of the enol of pheophytin *a*	EE + traces of HCl	666.0(2.61), 608(12.5), 558(21.6), 532(11.0), 502(7.94), 463(13.4), 406(1.00), $w_{I\frac{1}{2}}$ 22.7, $w_{S\frac{1}{2}}$ 56.5
h	Methyl pheophorbide *a*	EE	666.0(2.16), 608(15.5), 558(73.8), 532(12.0), 503(10.1), 470(32.0), 408(1.00), 320(5.58), $w_{I\frac{1}{2}}$ 18.0, $w_{S\frac{1}{2}}$ 53.8
i	TMS ether of the enol of Me-pheophorbide *a*	2.0 % THF in LP	750(17.9), 645.0(7.27), 606(22.9), 550(122.6), 503(14.8), 418.0(1.00), 348(1.07), $w_{I\frac{1}{2}}$ 30.6, $w_{S\frac{1}{2}}$ 130.6
j	Phase-test intermediate of pheophytin *a*	THF	712(23.5), 666.0(3.96), 610(24.7), 522(1.47), 375.0(1.00), $w_{I\frac{1}{2}}$ 23.3, $w_{S\frac{1}{2}}$ 85.1
k	Silylated 9-hydroxy-9-desoxo deriv. of Chl *a*	0.5 % PrOH	638.0(3.21), 592(28.7), 519(40.2), 412.0(1.00), $w_{I\frac{1}{2}}$ 15.9, $w_{S\frac{1}{2}}$ 18.6
l	9-hydroxy-9-desoxo deriv. of pheophytin *a*	EE	655.0(2.80), 600(32.5), 550(67.1), 500(9.24), 397.0(1.00), $w_{I\frac{1}{2}}$ 14.6, $w_{S\frac{1}{2}}$ 36.1
m	Mg-purpurin 7-methyl-ethylphytyl ester	EE	669.0(2.54), 572(11.4), 525(26.5), 495(39.8), 422.0(1.00), $w_{I\frac{1}{2}}$ 47.6, $w_{S\frac{1}{2}}$ 56.5
n	Mg-unstable chlorin 7-methylphytyl ester	EE	653.0(2.01), 608(9.43), 565(15.2), 522(17.2), 418.0(1.00), $w_{I\frac{1}{2}}$ 21.2, $w_{S\frac{1}{2}}$ 38.8
o	Chlorin e_6 $Me_{3(2)}$ ester	EE	665.0(2.91), 609(31.8), 557(181.4), 530(34.3), 499(12.2), 399.0(1.00), $w_{I\frac{1}{2}}$ 18.2, $w_{S\frac{1}{2}}$ 34.4

[a] R = absorbance at Soret band divided by absorbance at wavelength indicated. [b] TEA = triethylamine, EE = ethyl ether, PrOH = 1-propanol, LP = light petroleum (b.p. 20 – 40 °C), THF = tetrahydrofuran.

scopic characterization of the trimethylsilyl (TMS) ethers of the enols of Chl *a*, pheophytin *a* and methyl pheophorbide *a* are described in the present communication.

RESULTS AND DISCUSSION

Properties of Chlorophyll a′. The VIS spectrum of Chl *a′* was re-examined (Table 1; c). It was found virtually identical to that of Chl *a* (Table 1; a,b). Differences were even smaller than those reported previously.[32]

The NMR spectrum (B) of Fig. 2 shows that Chls *a* and *a′* differ from each other by the positions of the methine and the C10 proton resonances. Also the chemical shifts (δ) of 10b and 5a methyl groups are slightly different. The differences of the chemical shifts ($\Delta\delta$) are 0.02, 0.03, 0.04, 0.12, 0.03, and 0.03 ppm for α, β, δ, 10, 10b, and 5a protons, respectively. These values show that the closer we come to the point where the structural change takes place, the greater is $\Delta\delta$. The epi-C10 proton resonance peak can be observed at 5.99 ppm between the double doublets of the vinyl-2b group. This peak cannot arise from an enolic hydroxyl group since a slow exchange rate is observed for it.[6] On the basis of the areas of the δ proton peaks, it was estimated that the mixture contained *ca.* 60 % of Chl *a* and 40 % of Chl *a′*. The positions of both C10 and epi-C10 proton peaks depend on the concentration of the pigment and the nature of solvent. Thus, in benzene-d_6 the chemical shift of the epi-C10 proton is 5.76 ppm at a concentration of 5×10^{-3} M (Fig. 1; C) while at a concentration of 10^{-2} M in the same solvent, a value of 5.51 ppm has been observed for this proton.

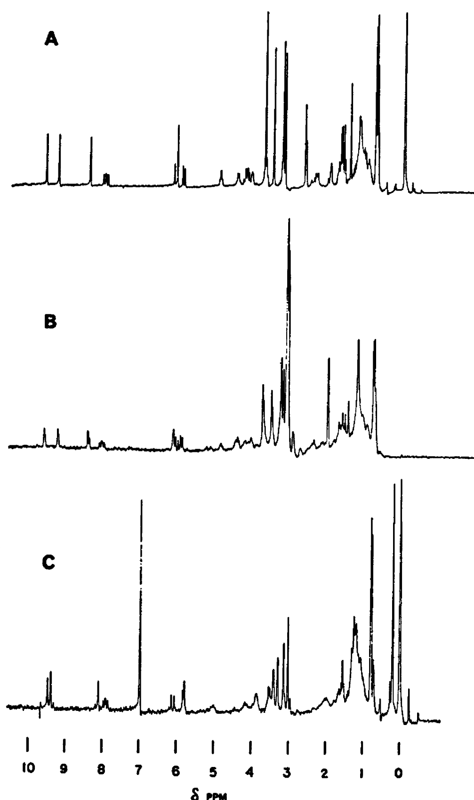


Fig. 2. ^{1}H NMR spectra of (A) chlorophyll *a* in acetone-d_6; (B) a mixture of Chl *a* and Chl *a′* in acetone-d_6; and (C) Chl *a′* in benzene-d_6. $c\simeq5\times10^{-3}$ M. For the assignment of the signals, see Table 2 and Ref. 53.

In acetone-d_6, the interconversion between Chl *a* and *a′* was so rapid that it was impossible to obtain an NMR spectrum of pure Chl *a′* even at −20 °C. In THF-d_8, the interconversion was much slower. At −18 °C, *ca.* 100 % of Chl *a′* was still present. After 5, 7 and 24 h standing at room temperature, 90, 85, and 60 % of Chl *a′* was found to be present in the mixture, respectively. This interconversion rate in THF-d_8 is slower than that reported previously.[6] It seems likely that this difference arises from different purity grades of the Chl *a′* preparations. Even small amounts of impurities, particularly bases, are expected to affect the interconversion rate to a considerable extent. In benzene-d_6 Chl *a′* appeared to be quite stable even at room temperature. Standing overnight is required to show some reduction in the intensity of the epi-C10 proton peak.

At a concentration of *ca.* 5×10^{-3} M in benzene-d_6, Chl *a′* showed no clear indication of aggregation (the slight lowering of the intensities of the resonance peaks corresponding to methyl groups 10b, 5a, and 1a, may be interpreted to result from aggregation). The first experimental evidence for the effect that Chl *a′* really aggregates in nonpolar solvents was obtained when ^{1}H NMR spectra of Chl a′ were measured in benzene-d_6 at concentrations $\geq10^{-2}$ M. At these concentrations, the methyl

resonance peaks were collected together and the spectra resembled that previously reported [34] for aggregated Chl *a* in $CDCl_3$. Further evidence for the aggregation of Chl *a'* in nonpolar solvents has been obtained recently by visible and IR spectra.[35] Therefore, the observed higher solubility of Chl *a'* in nonpolar solvents is best interpreted in terms of the unfavorable stereochemical properties of the primed derivative which prevent it from forming Chl-water adducts.[1,6,32,36,37] In Chl *a'* the methoxycarbonyl group is on the same side of the ring plane as the phytylpropionic acid residue at C7. Since there is not enough room for both, the methoxycarbonyl group is likely to become pushed closer to the C9=O group. Owing to the resulting steric hindrance, Chl *a'* seems to have reduced coordination tendency in comparison to that of Chl *a*. Evidence for this effect is provided by the comparative sodium borohydride reduction experiments of Chls *a* and *a'* which show that Chl *a* is reduced more readily than Chl *a'*.[38]

A minimal requirement for the change of stereochemical configuration at C10, is the abstraction of the C10 proton, which yields the enolate ion. In spite of the fact that the underlying mechanism for the epimerization undoubtedly is keto-enol tautomerism, the concentration of the enol form(s) of Chl *a* in acetone, THF, or benzene must be very low, since none of the ^1H NMR spectra of Chl *a'* or the equilibrium mixtures of Chl *a* and Chl *a'* gave any indication of a resonance peak at lower field (10–20 ppm) corresponding to a hydrogen-bonded hydroxyl group.[39] These observations confirm the earlier results [6] indicating the epimer nature of Chl *a'*.

Table 2. Comparison of ^1H NMR chemical shifts [a] (δ [ppm]) from internal HMS) of chlorophyll *a* and some derivatives.

	Chl *a*	Pheophy *a*	$\Delta\delta$	Mg-Purpurin 7-MeEtPhy ester	$\Delta\delta$	Multiplicity
β-H	9.57	9.52	0.05	9.37	0.20	s
α-H	9.27	9.22	0.05	9.09	0.18	s
δ-H	8.40	8.74	−0.34	8.22	0.18	s
2a-H_X	8.04	8.03	0.01	7.93	0.11	dd, J=12,18
2b-H_B	6.14	6.25	−0.11	6.06	0.08	dd, J=1,18
2b-H_A	5.92	6.08	−0.16	5.86	0.06	dd, J=1,12
10-H	6.06–6.11	6.21	−0.15(−0.10)	–	–	s
P-2-H	4.90	4.80	0.10	5.04	−0.14	t
P-1-CH_2	4.46	4.55	−0.09	4.54	−0.08	q, J=7
8,7-H	4.18,4.06	4.25,4.14	−0.07,−0.08	4.32,4.15	−0.14,−0.09	q,q, J=7,7
10b-CH_3	3.72	3.77	−0.05	3.73	−0.01	s
4a-CH_2	3.67	3.50	0.17	3.62	0.05	q, J=7
5a-CH_3	3.49	3.53	−0.04	3.38	0.11	s
1a-CH_3	3.24	3.33	−0.09	3.12	0.12	s
3a-CH_3	3.18	3.04	0.14	3.09	0.09	s
7a,7b-CH_2	2.37–1.96	2.25–1.97	0.12(−0.01)	2.20–1.96	0.17–0.00	m
P-4-CH_2	1.76	1.69	0.07	1.82	−0.06	t, J=7
8a-CH_3	1.66	1.77	−0.11	1.65	0.01	d, J=7
4b-CH_3	1.61	1.54	0.07	1.56	0.05	t, J=7
P-3a-CH_3	1.44	1.40	0.04	1.49	−0.05	s
P-7,P-11,P-15-H	1.21	1.20	0.01	1.20	0.01	s
P-5,P-6,P-8,P-9, P-10,P-12, P-13,P-14-CH_2	1.18–0.960	1.10–0.860	0.08–0.10	1.18–0.960	0.00–0.00	m
P-7a,P-11a,P-15a, P-16-CH_3	0.785,0.756	0.749,0.720	0.036,0.036	0.792,0.763	−0.007,−0.007,	s,s
	0.723,0.694	0.668,0.641	0.055,0.053	0.733,0.700	−0.010,−0.006	s,s
9a-CH_2	–	–	–	4.90	–	q, J=16

[a] In acetone-d_6.

The observed higher concentration Chl *a′* in the equilibrium mixture in triethylamine (40 %) as compared to that in pyridine (25 %) or THF (20 %) correlates with the higher basicity of TEA over that of pyridine or THF. In TEA the real thermodynamic equilibrium is attained rapidly whereas in pyridine or THF its attainment may require a long time. A higher concentration of the enol is also possible in TEA, since this solvent can form stronger hydrogen bonds with the enol than pyridine or THF. However, the concentration of the enol even in TEA must be rather low, since the visible absorption spectrum of Chl *a*, allowed to stand overnight in TEA, did not indicate the presence of the enol form (Table 1; a).

The single stereochemical change at C10 of the Chl molecule also results in other kinds of alterations in the properties of the molecule. As noted above, the positions of the methine proton resonance peaks are slightly different in Chl *a* and Chl *a′*. This shows that the epimerization affects the ring current, *viz.* the delocalized π electron system of the chlorin macrocycle. This effect seems to be somewhat embarrassing at first sight, since such alterations as the abstraction of Mg or oxidative cleavage of ring V cause only relatively small changes in the NMR chemical shifts (Table 2). The effect probably results from the conformational alterations caused by the stereochemical change at C10. There is chemical strain already in ring V of Chl *a*, which is indicated among other things by the unusually long distance of 1.57 Å between C9 and C10.[40] The stereochemical change at C10 is likely to cause more strain in ring V, which results in conformational alterations (puckering) in the whole molecule. The observed higher tendency of Chl *a′* to pheophytinize, can be explained on this basis. The conformational alterations presumably affect also the bonds between the pyrrole N atoms and the central Mg atom. This reflects the sensitivity of the bonds formed by the Mg atom in the chlorophyll molecule.

The IR spectra in the carbonyl region of Chl *a* (A) and Chl *a′* (B), both measured in THF, are presented in Fig. 3. The similarity of the IR spectra of the two compounds is evident. Both spectra show a band at *ca.* 1700 cm^{-1} caused by C9=O stretching vibrations, and another at *ca.* 1740 cm^{-1} which arises from C10a and C7c ester carbonyl vibrations.[7] A band at *ca.* 1650 cm^{-1} had been previously [33] observed in the IR spectrum of Chl *a′*. However, it seems now likely that the Chl *a′* preparation used for the measurement contained some residual propanol after the effluent (0.5 % PrOH in light petroleum) solution of Chl *a′* was evaporated to "dryness" at reduced temperature. The propanol presumably forms hydrogen bonds to the C9=O group, which results in the appearance of the band at 1650 cm^{-1} in the IR spectrum.

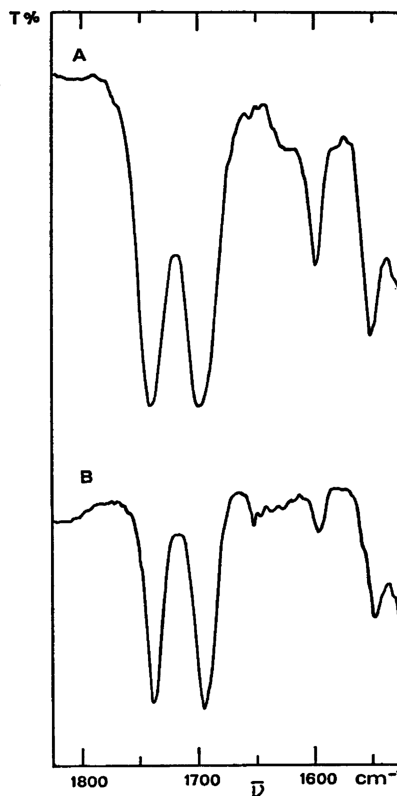


Fig. 3. IR spectra in the carbonyl stretch region for (A) Chl *a* and (B) Chl *a′*, both in THF solution. Light-path = 0.2 mm. $c \simeq 10^{-2}$ M.

Trapping of the enol of chlorophyll a *and its Mg-free derivatives.* Attempts to methylate or acetylate the enol of Chl *a* were unsuccessful. Diazomethane in diethyl ether, methanol–diethyl ether (1:10, v/v) or pyridine–diethyl ether (1:1, v/v) yielded only Chl *a′* and Chl *a* after standing for 2 h at 0 °C. After standing

for about 24 h, a small amount of 10-hydroxy-chlorophyll *a* (VIS spectrum similar to that of Chl *a*) could be separated on a sucrose column in addition to Chl *a′* and Chl *a*.

When Chl *a′* (5 mg) and Chl *a* (5 mg) were heated separately in acetic anhydride–pyridine, 3:10, v/v (10 ml) at 80 °C for 1 h, both samples yielded *ca.* 25 % of Chl *a′* and 75 % of Chl *a* (visual estimation) as evidenced by the chromatographic separations on two sucrose columns at 0 °C. No acetylated derivatives or allomerization products could be detected in either case.

These trapping experiments provide further evidence for the fact that the concentration of the enol is very small in solvents like diethyl ether or pyridine, thus indicating the instability of the enol form of Chl. Its lifetime may be only a few seconds.

The attempts to trap the enol of Chl *a* or pheophytin *a* by silylation with ethyl trimethylsilylacetate (ETSA) and tetrapropylammonium fluoride (TPAF) in THF [41] were also unsuccessful. When larger amounts of ETSA and TPFA (see EXPERIMENTAL) were used and the reaction was performed under an N_2 atmosphere, a blue compound, spectroscopically similar to the sodium borohydride reduction products of Chl *a*,[38,48] was separated on the sucrose column as a product from Chl *a*. Since this blue compound migrated more rapidly than Chl *a′* and since all of the sodium borohydride reduction products of Chl *a*[38] move slower than Chl *a* under the same experimental conditions, it seems likely that the blue compound was a silylated derivative of 9-desoxo-9-hydroxy-Chl *a* produced by reductive silylation.

When ETSA and TPAF were used in smaller amounts and O_2 was not carefully excluded from the reaction mixture, the principal components were, in the order of their migration rates on the sucrose column: Chl *a′*, Mg-purpurin 7-methylethylphytyl ester (Table 1; m, and Table 2), Chl *a*, and presumably Mg-unstable chlorin 7-methylphytyl ester (Table 1; n).[12] It seems likely that both the Mg-purpurin 7-MeEtPhy ester and the Mg-unstable chlorin 7-MePhy ester were derived from the same precursor, the Mg-purpurin 7-MePhy ester with a free carboxyl group at C6.[12] Owing to the instability of this compound, it was presumably partially transesterified with ETSA to produce Mg-purpurin 7-MeEtPhy ester which is more stable than the corresponding free carboxyl derivative; partially it was solvated by water and lactonized to yield Mg unstable chlorin 7-MePhy ester (a 10-hydroxy-lactone derivative).[12] These results indicate that the oxidation of ring V of the enolate ion by O_2 (singlet oxygen?) [21] is the principal reaction under the above silylation conditions.

Although ETSA is reported to be a new efficient silylating agent,[43] the results described above suggest that it is not applicable to the chlorophyll *β*-keto ester. Furthermore, nothing is known about its applicability to the silylation of *β*-keto esters in general.[41]

A silylation method suitable for trapping the enols of Chl *a*, pheophytin *a* and methylpheophorbide *a* was developed by combining TPAF with the conventional silylating agent,[44] chlorotrimethylsilane (CTMS) in triethylamine. In THF the reaction takes place smoothly and is almost completed in 1 h. In the case of Chl *a* the color of the reaction mixture changes from blue-green to yellow-green and in the case of pheophytin *a* or Me-pheophorbide *a*, from brown to yellow-green. No transient yellow or red color of the phase-test intermediate (Table 1; j) can be observed during the course of the reaction, and very little oxidation products are produced even under conditions where O_2 is not strictly excluded. This indicates that CTMS traps the enol efficiently. The reaction can be followed by visible absorption spectroscopy and thin-layer chromatography (TLC) on cellulose [45] using pyridine–light petroleum (1:15, v/v) as a solvent system. In the TLC, the silylated derivative migrates most rapidly as a yellow-green spot which is nonfluorescent under UV light.

Although the silylated enols were easily formed, their isolation from reaction mixtures proved to be a difficult task. The silylated enols of Me-pheophorbide *a* and pheophytin *a* were stable enough to permit their purification on a sucrose column using 2 % (v/v) THF in light petroleum as a solvent system. The silylated enol of Chl *a* was more labile. It went quite easily back to the original Chl *a* or was converted to the silylated enol of pheophytin *a*. Its partial purification succeeded on a sucrose column using 0.5 % 1-propanol in light petroleum as a solvent system and a temperature of 0 °C.

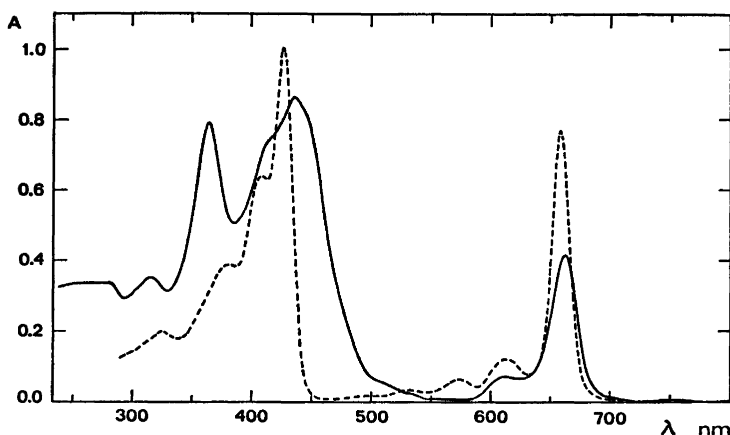


Fig. 4. VIS spectra of Chl *a* (– – –) and the silylated enol ether of Chl *a* (———), both in light petroleum containing 0.5 % 1-propanol.

Spectroscopic properties of the silylated enols of chlorophyll a, *pheophytin* a *and methyl pheophorbide* a. The VIS spectra of Chl *a* and its silylated enol are shown in Fig. 4. The silylated enol of Chl *a* has a red band at approximately the same position as Chl *a*. The principal differences between the two compounds exist in the Soret band region. The Soret band of the enol is divided into two peaks of almost equal intensity. The ratio, A_{435}/A_{662}, attains a value of 2.97 (Table 1; d). Since there is virtually no absorption at 570 nm, only a small amount of Chl *a* can be present in the enol preparation. On comparing the VIS spectrum of the silylated enol of Chl *a* with that of the cyclochlorophyll *a*-enol derivative prepared by Eschenmoser *et al.* (see Fig. 2 in Ref. 46), it can be seen that the Soret band regions of the two enols are quite similar; the main difference is in the positions of the red bands (662 nm in the silylated enol and 688 nm in the cyclochlorophyll *a*-enol derivative).

Fig. 5 presents the VIS spectra of Me-pheophorbide *a* (pheophytin *a*) and its silylated enol. The enol shows a red band at 645 nm, and a divided Soret band at 418 and 350 nm; the ratio, A_{418}/A_{465}, is 7–8. The spectrum of the enol also shows a broad band of low intensity at 750 nm and there is still slight absorption beyond 800 nm. The broad band at 750 nm is

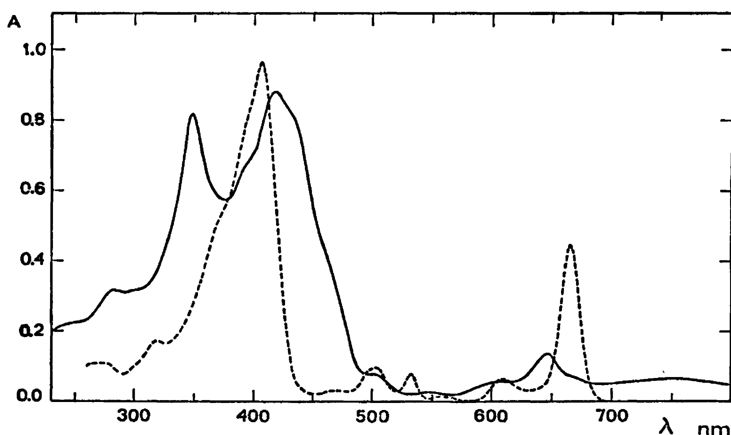


Fig. 5. VIS spectra of methyl pheophorbide *a* (– – –) and the silylated enol ether of methyl pheophorbide *a* (———), both in light petroleum containing 2 % THF.

Table 3. ^1H NMR chemical shifts (δ [ppm]) of methylpheophorbide *a* and the trimethylsilyl ether of the enol of methylpheophorbide *a* in benzene-d_6 solution.

	Methyl-pheo-phorbide a^a	TMS ether of^b the enol	$\Delta\delta$	Multi-plicity
β-H	9.33	8.24	1.09	s
α-H	9.25	8.19	1.06	s
δ-H	8.18	7.03	1.05	s
2a-H_X	7.71	7.19	0.52	dd, J=12,18
2b-H_B	5.98	5.64	0.34	dd, J=1,18
2b-H_A	5.78	5.46	0.32	dd, J=1,12
10-H	6.20	–	–	s
7-H	4.16	4.66	−0.50	q, J=9
8-H	3.95	3.22	0.73	q, J=9
10b-CH_3	3.37	3.60	−0.23	s
7d-CH_3	3.26	3.07	0.19	s
5a-CH_3	3.18	2.73	0.45	s
4a-CH_2	3.13	2.98	0.15	q, J=9
1a-CH_3	2.91	2.46	0.45	s
3a-CH_3	2.90	2.37	0.53	s
7a-CH_2	2.29	1.81	0.48	m
7b-CH_2	2.06	1.73	0.33	m
8a-CH_3	1.51	1.27	0.24	d, J=8
4b-CH_3	1.42	1.22	0.20	t, J=7
NH	0.79	2.26	−1.47	s
	−1.38	2.14	−3.52	s

$^a\delta$ relative to internal hexamethyldisiloxane. $^b\delta$ relative to the trimethylsilyl group.

missing almost completely from the VIS spectrum of the silylated enol of Chl *a*. The silylated enol of Me-pheophorbide *a* differs by its VIS spectrum from the "peripheral Mg-complex" of Scheer and Katz [17] and the "cyclopheophorbide *a*-enol derivative" of Eschenmoser *et al.*[30] principally in the region 600–800 nm. The two last-mentioned enol derivatives have a red band at *ca.* 690 nm whereas the silylated enol's most intensive red band is at 645 nm. By comparing the enol spectra in Figs. 4 and 5, it can be observed that the spectrum of the metal-free compound is considerably different from that of the corresponding Mg-derivative in the region 600–800 nm. This is an essential difference when compared with the enols of the cycloderivatives of Eschenmoser *et al.*[30,46] In the latter case, the VIS spectrum of the cyclopheophorbide *a*-enol derivative is virtually identical with that of the cyclochlorophyll *a*-enol derivative.

The ^1H NMR chemical shifts of Me-pheophorbide *a* and its silylated enol in benzene-d_6 are compared in Table 3. $\Delta\delta$ obtains the largest values (*ca.* 1.1) in the case of β, α, and δ methine protons, which implies a large effect on the ring current. This can be expected, since in the enol, ring V becomes included into the delocalized π electron macrocycle of the porphyrin ring. The ^1H NMR spectrum of the silylated enol of Me-pheophorbide *a* compares fairly well with that of the cyclopheophorbide *a*-enol derivative.[30]

Fig. 6 shows the IR spectra of Me-pheophorbide *a* and the silylated enol of pheophytin *a* (Me-pheophorbide *a*) in CCl_4. The enol has a broad band at *ca.* 1740 cm^{-1} which arises from 10a and 7c ester carbonyl vibrations. The

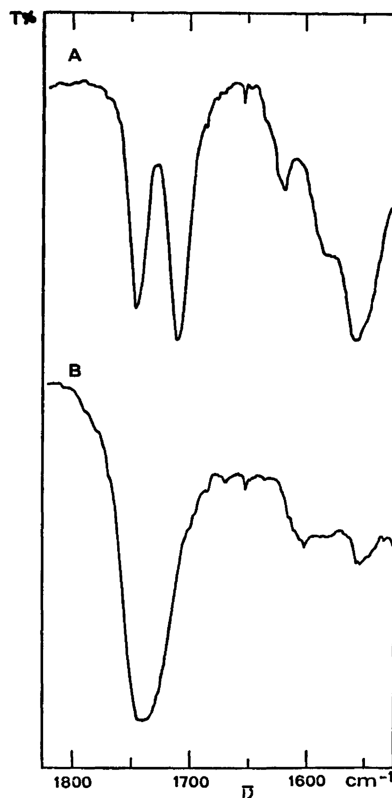
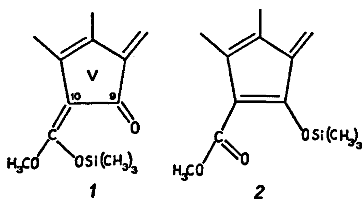


Fig. 6. IR spectra in the carbonyl stretch region of (A) methyl pheophorbide *a* and (B) the silylated enol of pheophytin *a* (methyl pheophorbide *a*), both in CCl_4 solution. Light-path = 0.2 mm. $c \simeq 3\times10^{-2}$ M (A) and 6×10^{-3} M (B).

C9=O band which is at 1710 cm^{-1} in the spectrum of Me-pheophorbide *a*, is missing from the spectrum of the enol. This eliminates structure *1* and suggests structure *2* for silylated enols.



EXPERIMENTAL

Isolation of pheophytin a *(a crude preparation).* Chloroplast pigments were extracted with acetone from dehydrated alfalfa (*Mediocago sativa*) (Western Alfalfa Corp., Shawnee Mission, Kansas, USA) in a Soxhlet extractor. The extract was evaporated to dryness and the pigments were dissolved in 95 % ethanol. After addition of hydrochloric acid and water, pheophytins *a* and *b* precipitated in the cold.[48] Pheophytin *a* was isolated from the pheophytin mixture by preparative high-pressure liquid chromatography.[48]

Pheophorbide a was prepared from the crude pheophytin *a* preparation by shaking its diethyl ether solution (*ca.* 300 ml) with 30 % (w/w) hydrochloric acid for 30 min. The small amount of pigments remaining in the ether layer after this period was discarded. The pigments were transferred to fresh diethyl ether by dilution of the lower phase. After washing, the diethyl ether extract was evaporated to dryness. Two hundred mg of crude pheophytin *a* (which presumably contained some solvent in addition to other impurities) yielded 60 mg of pheophorbide *a*. The VIS spectrum revealed no impurities. TLC on cellulose [45] showed the presence of a trace amount of pheophytin *a*.

Methylpheophorbide a was prepared by methylation of pheophorbide *a* with diazomethane.[20] An ethyl ether solution of diazomethane was prepared by saponification of nitrosomethylurea [49] with sodium hydroxide (5 N) in a simple device described by Fales *et al.*[50] 60 mg of pheophorbide *a* yielded 58 mg of methylpheophorbide *a*. The VIS spectrum (Table 1; h) and TLC revealed no impurities. ^1H NMR spectrum: Table 3. The method yielded a small amount of by-products which seemed to consist of chlorin e_6 methyl esters on the basis of the VIS spectrum (Table 1; o) and TLC.[45]

Isolation of chlorophyll a, *chlorophyll* b *and pheophytin* a. Chloroplast pigments were extracted from freeze-dried *Chlorella vulgaris* with methanol–light petroleum (b.p. 20–40 °C) according to Strain and Svec.[51] The extract was evaporated to dryness in a rotatory evaporator and the residue (1 g) was dissolved in 500 ml of light petroleum (b.p. 20–40 °C). Xanthophylls were extracted from the light petroleum solution of the pigments with 70 % (w/w) aqueous methanol (8 × 300 ml). The light petroleum solution was washed with distilled water (4 × 500 ml). Chlorophyll *a* and *b* were isolated from this solution by precipitation and centrifugation.[1] The precipitate was washed with cold light petroleum and dissolved in 10 ml of diethyl ether. Chromatography on a sugar-column [51] showed that the precipitate consisted principally of chlorophylls *a* and *b*; no carotenoids and only trace amounts of chlorophylls *a′* and *b′* as well as pheophytin *a* could be detected on the sugar column. Chlorophylls *a* and *b* were eluted from the column (A) and were collected separately in the effluent. The pigments remaining in the supernatant after the centrifugation were separated on another sugar column (B). The separation revealed that the principal pigments in this fraction were: β-carotene, pheophytin *a*, Chl *a′* and Chl *b′*. Small amounts of Chl *a*, Chl *b*, and oxidation products of the chlorophylls and pheophytin *a* were also detected in this separation. The pheophytin *a* and Chl *a′* zones were eluted and collected together in the effluent. The effluent solution of the pigments was evaporated to dryness and the residue was dissolved in ethyl ether. This solution was shaken with 13 % (w/w) hydrochloric acid to remove magnesium from Chl *a′*. The VIS spectrum and TLC revealed no impurities in the pheophytin *a* obtained.

Preparation and isolation of chlorophyll a′. Purified Chl *a* (*e.g.* 2.5 mg) was dissolved in 2.0 ml of triethylamine (TEA) and the solution was permitted to stand for 12 h at room temperature in the dark. After this period, the color of the solution was still blue and its VIS spectrum (Table 1; a) was closely similar to that of chlorophyll *a*. The pigments formed in TEA were separated on a sucrose column employing 0.5 % 1-propanol as the eluent. The glass column used had an inside diameter of 3.0 cm and a height of 50 cm and was provided with an ice-water jacket. The column was packed by the slurry method ($h = 20$ cm).[1] The icing sugar (Finnish Sugar Co., Helsinki, Finland) was passed through a 60 mesh sieve before use. The sample for the separation was prepared by evaporating the TEA solution to dryness and dissolving the residue in 2.0 ml of the eluent. The separation revealed the presence of only two components: the faster migrating Chl *a′* and the slower migrating Chl *a*. Both components were eluted from the column and were collected into two separate flasks kept on a dry-ice bath. The amounts were determined spectrophotometrically: Chl *a′* 1.0 mg (40 %), Chl *a* 1.5 mg (60 %). Chl *a* was also partially converted into Chl *a′* on standing overnight in pyridine or in 0.5 % 1-propanol in light petroleum, on heating in 1-propanol at 50 °C for 15

min or on equilibrating the light petroleum solution of Chl *a* with 70 % aqueous methanol for 1 h at r.t. In these latter cases, however the yield of Chl *a'* was lower (15 – 25 %). IR spectrum of Chl *a'*: Fig. 3. ^{1}H NMR spectrum: Fig. 2.

Silylation experiments with ETSA[52] *and TPAF.*[41] To Chl *a* (18 mg), as a dry film at the bottom of a 25 ml volumetric flask provided with a glass stopper, were added TPAF (20 mg), ETSA (200 μl), and tetrahydrofuran (THF, 1 ml) under N_2 and on a dry-ice bath (-78 °C). When the color changed to red, another 100 μl of ETSA was added; the color changed back to green. Immediately after this, 5 ml of the eluent (0.5 % 1-propanol in light petroleum, b.p. 30 – 60 °C) were added and the mixture was separated on a sucrose column prepared as described in the isolation of Chl *a'* ($h = 49.0$ cm). At least 8 different components were separated on the column. Five of the more rapidly migrating components were eluted off the column and collected separately in the effluent and characterized by their VIS spectra. The most rapidly migrating blue component was probably a TMS ether of a 9-desoxo-9-hydroxy-derivative of Chl *a*, since its visible absorption spectrum (Table 1; k) was similar to that of 9-desoxo-9-hydroxy-Chl *a*;[42] also the spectrum of the Mg-free pigment (Table 1; l) matched that of 9-desoxo-9-hydroxy-pheophytin *a*.[42] The other components eluted in the effluent were probably: pheophytin *a*, Chl *a'*, Chl *a* and 10-hydroxy-Chl *a*. The components remaining at the upper part of the column were presumably allomerization (oxidation) products of chlorophyll *a*. The uppermost of them was characterized as Mg-unstable chlorin 7-methylphytyl ester on the basis of its VIS spectrum (Table 1; n) and the corresponding Mg-free pigment.

When the reaction was performed in a serum bottle with smaller amounts of TPAF (2 mg) and ETSA (20 μl) and under conditions where O_2 was not carefully excluded and during a longer time (12 h), no blue rapidly migrating component could be detected on the sucrose column. Instead, a new, bright-green component appeared between Chl *a'* and Chl *a*. The bright-green component was collected separately in the effluent and was spectroscopically (VIS spectrum: Table 1; m. ^{1}H NMR spectrum: Table 2) characterized as an Mg-purpurin 7-methylethylphytyl ester. Another principal oxidation product under these conditions appeared to be Mg-unstable chlorin 7-methylphytyl ester (Table 1; n).

Silylation trials of pheophytin *a* using TPAF and ETSA were unsuccessful. In this case, besides unchanged pheophytin *a*, a purple pigment which moved very slowly on the sucrose column, could be separated. The VIS spectrum of this pigment resembled that of phase-test intermediate of pheophytin *a*. On the addition of a small amount of HCl, the color of the pigment changed to blue-green.

Silylation with chlorotrimethyl silane (CTMS) and TPAF. Preparation and isolation of the silylated enol of methylpheophorbide a *(pheophytin* a*).* 58 mg of Me-pheophorbide *a* were dissolved in acetone (10 ml) in a 25 ml volumetric flask provided with a glass stopper. The solution was evaporated to dryness under an N_2 stream. 50 mg of TPAF were added (the flask was immediately closed after weighing of TPAF). Then, while in a dry-ice bath and under an N_2 stream, 5.0 ml of THF, 4.0 ml of triethylamine (TEA) and 2.0 ml of CTMS were added to the reaction mixture. After this, the flask was sealed with a stopper and Parafilm and the reagents were mixed at room temperature for 5 to 10 min. During this time, the grey-brown color of the mixture changed to yellow-green. The mixture was permitted to stand at -78 °C for 1 h, whereafter it was allowed to warm up to room temperature (5 min) and was poured into 800 ml of light petroleum (PE), b.p. 20 – 40 °C. The yellow-green suspension was allowed to stand at room temperature until the precipitate collected at the bottom of the flask; the clear solution was carefully decanted off and filtered twice; and the clear filtrate was evaporated to dryness under an N_2 stream. The residue was dissolved in 5 ml of the eluent (2.0 %, v/v, THF in light petroleum, b.p. 20 – 40 °C) and the solution obtained was introduced with a pipette to the top of a sucrose column, newly packed ($h = 35$ cm) as described in the isolation of Chl *a'*. On elution, a yellow-green, fast-moving zone separated from a grey slower moving one. Both components were eluted from the column and collected separately in the effluent, and characterized spectroscopically. The visible absorption spectrum of the yellow-green component was consistent with the formation of a new compound while the grey-green component, was identified as unchanged methylpheophorbide *a*. The yellow-green component was refractionated on a sucrose column; only a trace amount of a grey component (presumably a new amount of Me-pheophorbide *a* formed from the silylated enol) separated from the faster moving yellow-green component. The latter was collected from the effluent and characterized spectroscopically as the trimethylsilyl ether of the enol of Me-pheophorbide *a*. VIS spectrum: Table 1; h and f, Fig. 5. ^{1}H NMR spectrum: Table 3. IR spectrum: Fig. 6.

Preparation and isolation of the silylated enol of chlorophyll a. The preparation of the silylated enol of Chl *a* was performed essentially under the same reaction conditions at that of Me-pheophorbide *a*. The isolation of the enol of Chl *a*, however, differed from the above procedure and was performed as follows. After standing for 1 h at -78 °C, the reaction mixture was directly introduced to the top of a newly prepared sucrose column ($h = 35$ cm). After allowing the solution to absorb into the sugar

layer, the column was eluted with 0.5 % 1-propanol in light petroleum, b.p. 20 – 40 °C. Only one yellow-green, fast-migrating zone was observed on the column. The component was eluted from the column and collected in the effluent and characterized spectroscopically. VIS spectrum: Table 1; d and Fig. 4. The peaks in the ^{1}H NMR spectrum were not adequately resolved to allow assignment.

If a longer sugar column ($h = 45$ cm) was used in the isolation, a small amount of a green pigment was observed to separate from the yellow-green principal zone at the lower end of the sugar layer. The green pigment appeared to be identical with Chl *a* on the basis of its VIS spectrum. If the procedure used in the isolation of the silylated enol of Me-pheophorbide *a* was applied to the isolation of Chl *a* (except for the eluent), only a small amount of a yellow-green pigment was observed on the sugar column; the principal components in this case were Chl *a* and its oxidation products.

Purity of solvents and reagents. Chlorotrimethylsilane (CTMS) was of technical grade (Aldrich) and was further purified by distillation. Triethylamine (99 %, Aldrich) was also distilled before use. Ethyl bromoacetate (The Matheson Co.) was used without further purification. Other solvents and reagents were of reagent grade purity and were also used without further purification.

Spectroscopic measurements. VIS spectra were recorded with a Cary 14 Spectrophotometer. ^{1}H NMR spectra were measured with a Varian 220 MHz High-Resolution NMR spectrometer provided with a Fourier transform pulsing system. Hexamethyldisiloxane (HMS) was used as an internal standard in these measurements.

IR spectra were recorded with a Beckman Model IR 7 spectrophotometer.

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