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High-performance liquid chromatographic separation and isolation of the methanolic allomerization products of chlorophyll *a*

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ABSTRACT

Isocratic normal-phase HPLC on a silica column with diode-array detection provided a powerful means for the analytical separation and preparative isolation of seven methanolic allomerization products of chlorophyll *a*. Diastereomeric selectivity was achieved for $13^2(R,S)$-hydroxychlorophyll *a*, $13^2(R,S)$-methoxychlorophyll *a* and the Mg complex of $3^1,3^2$-didehydro-15^1-hydroxy-$15^1(R,S)$-methoxyrhodochlorin-15-acetic acid δ-lactone 15^2-methyl 17^3-phytyl ester. The Mg complex of $3^1,3^2$-didehydrorhodochlorin-15-glyoxylic acid $13^1,15^2$-dimethyl 17^3-phytyl ester was also isolated in high purity. Reversed-phase HPLC did not result in an acceptable separation in spite of using several different brands of reversed-phase C_{18} columns and mobile phase compositions. The identification of the allomerization products was based on UV–Vis and ^1H NMR spectra, retention times and co-elution with authentic samples. The observed formation of small amounts of numerous side-products is interpreted as further evidence for the free-radical mechanism of the allomerization.

INTRODUCTION

The chlorophylls (Chls) are a group of closely related compounds which play a central role in the photosynthetic conversion of light energy into chemical energy. It is well known that the chlorophylls are extremely susceptible to a number of chemical transformations which can occur during their extraction and separation. Typical reactions are loss of the central magnesium atom (pheophytinization), configurational change at C-13^2 (epimerization), photochemical degradation, transesterification or hydrolysis of the ester groups and oxidation of the isocyclic ring by molecular triplet oxygen (allomerization) [1–5].

The allomerization, first observed by Willstätter and Stoll [1], includes a complicated series of oxidation reactions at C-13^2 by molecular triplet oxygen ($^3O_2$) in alcohol solutions [2–14]. The allomerization reaction, which poses a serious problem in the isolation of chlorophylls from natural sources, may yield several oxidation products depending on the conditions and starting material. Several reaction mechanisms have been proposed for the allomerization of Chl *a* [2–4,6–17]. The free-radical mechanism, where Chl enolate anion is the key intermediate, is supported by ample experimental evidence [4,11], but some details of the reaction are still poorly understood.

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A variety of chromatographic procedures, including paper, thin-layer (TLC), conventional column (CC) and high-performance liquid chromatography (HPLC), have been developed and used for analytical and preparative separations of chlorophylls and their derivatives [18–23]. The first published HPLC separation for Chl compounds dates from 1975 [24]. Since then, the trend has been towards increased use of HPLC for the ultimate separation, isolation and analysis of these plant pigments [14,19–23,25–38]. The high resolving power, speed, increased sensitivity and several detection systems now available make modern HPLC superior to other LC techniques. In most studies, reversed-phase (RP) HPLC has been chosen in preference to the normal-phase (NP) separation. Schaber *et al.* [14] were the first to follow the allomerization reaction in methanol by RP-HPLC with tetrahydrofuran (THF)–MeOH–water (36:54:10, v/v/v) as the mobile phase. They obtained good separations for the $13^2(R)$- and $13^2(S)$-epimers of 13^2-hydroxy-Chl *a* and the $15^1(R)$- and $15^1(S)$-epimers of the 15^1-methoxylactone derivative, but they could not detect other allomerization products (see Fig. 1).

In this paper, we report the results of investigations on the allomerization of Chl *a* in methanol, performed under atmospheric oxygen, in darkness and at room temperature. We describe an improved method for the separation, isolation and identification of the main allomerization products and their epimers, using NP-HPLC under isocratic conditions and diode-array detection (DAD) for preliminary identification. The separated compounds were identified more conclusively by NMR spectroscopy.

EXPERIMENTAL

Isolation and purity of chlorophyll a

Chl *a* (Fig. 1) was isolated from clover leaves by the method described previously [41], but since then modified for large-scale preparation. The purity of the Chl *a* was confirmed by UV–Vis spectrophotometry, ^{1}H NMR, TLC and HPLC. The spectroscopic properties of the preparation were identical with those described earlier [42]. The ^{1}H NMR spectrum showed that the only impurity was water (present in a ratio of *ca.* 1:1). TLC on sucrose [43] yielded only one spot, whereas NP-HPLC [Zorbax Sil column (250 × 4.0 mm I.D.), particle size 5 μm (Rockland Technologies, Newport, DE, USA), eluent 1.5% (v/v) 2-PrOH (LiChrosolv; Merck, Darmstadt, Germany) in hexane (LiChrosolv, Merck), flow-rate 1 ml/min, detection at 420 nm] revealed a trace amount of Chl *a'* which may have formed during the analysis.

Allomerization experiments

Experiment 1. An amount of 31.4 mg of solid Chl *a* was dissolved in 15 ml of methanol (J.T. Baker, Denventer, Netherlands, Catalog No. 8045, Absolute, ACS, "Baker analyzed reagent", dried over 3 Å molecular sieves). This solution was continuously stirred magnetically at room temperature in an erlenmeyer flask provided with a loose glass stopper and wrapped with aluminium foil to avoid any effect of light. The course of the reaction was followed by TLC on sucrose [43] employing 1% (w/w) 2-PrOH (analytical-reagent grade, Merck) in light petroleum (analytical-reagent grade, Merck; b.p. 60–80°C, distilled through a Vigreux column) as the eluent. After 3 days, Chl *a* was completely converted into its allomers. Without stirring, the reaction reportedly takes 7 days [10]. The methanolic reaction mixture was poured into a separating funnel containing water–hexane (Extrapure, Merck; distilled through a Vigreux column) (1:1, v/v). The aqueous phase was saturated with sodium chloride and extracted several times with hexane until it was colourless. The combined hexane phases were washed with water and concentrated with a rotary evaporator. An aliquot was taken and evaporated to dryness. The residue was dissolved in diethyl ether and analysed by TLC on sucrose. According to this analysis, the composition of the products had not changed during the extraction process. The allomerization products were studied by RP-HPLC on C_{18} columns and by NP-HPLC on silica columns. The main extract in hexane was evaporated to dryness under an argon stream and the residue was dissolved in a minimum amount of

methanol for RP columns or of 0.5–1.5% (v/v) 2-PrOH in hexane for silica columns.

Experiment 2. The whole allomerization experiment was repeated with the difference that the amounts of Chl *a* and methanol were 304. 5 mg and 150 ml, respectively, and that light petroleum was used instead of hexane as extraction solvent. The mixture of allomerization products obtained was prefractionated on a sucrose column followed by HPLC purification of some fractions for NMR spectroscopy (see below).

Chromatography on a sucrose column

The conventional chromatographic procedure on a sucrose column [43] was employed in the preseparation of the allomerization products from experiment 2. Powdered sugar (Cultor, Kirkkonummi, Finland), passed through a 100-mesh sieve, was used in the separations. The sugar (1500 g) was suspended in 2500 ml of the eluent [light petroleum (analytical-reagent grade, Merck, b.p. 60–80°C) or hexane (Extrapure, Merck), both distilled through a Vigreux column, containing 1% (w/w) of 2-PrOH] to form a slurry, which was then poured into a glass column (6 cm O.D.) to form a sucrose layer, 54 cm high. The mixture of allomerization products was evaporated to dryness and dehydrated by the chloroform co-distillation method [42]. The solvent residues were removed on a vacuum line at room temperature. The dry product was monomerized by dissolving it in 20 ml of diethyl ether [analytical-reagent grade, Merck; dried and stabilized with 2,6-di-*tert.*-butyl-4-methylphenol (BHT)] and evaporating nearly to dryness. The product was dissolved in a minimum amount of the eluent [0.5–1% (w/w) 2-PrOH in distilled hexane or light petroleum] and the solution was introduced on to the top of the sucrose layer. The separation was performed in the dark by wrapping the column with aluminium foil. The flow-rate of the mobile phase was 4–5 ml/min. The collected fractions were analysed using sucrose TLC, UV–Vis spectrophotometry and HPLC. After the analyses, the fractions containing a pure component, were combined, washed several times with distilled water and concentrated to a small volume. Final purification was achieved by NP-HPLC using a semi-preparative Zorbax Sil column.

High-performance liquid chromatography

The HPLC experiments, both analytical and semi-preparative, were performed with a Waters (Milford, MA, USA) liquid chromatograph consisting of two Model 501 pumps controlled by a Model 660 solvent programmer, a Rheodyne (Cotati, CA, USA) Model 7125 injector (10-, 100- and 1000-μl loops) a Waters Model 990 photodiode-array detector, an NEC APC III computer with chromatographic software and a Waters Model 990 recorder. Samples were monitored by absorption at 420 and 430 nm. All HPLC columns were operated under isocratic conditions at ambient temperature. The flow-rate was 3–4 ml/min for the semi-preparative columns and 0.8–1.5 ml/min for the analytical columns.

The reversed-phase separations were performed on five different C_{18}-modified silica columns: Resolve C_{18} (150 × 3.9 mm I.D.), particle size 5 μm (Waters); μBondapak C_{18} (300 × 3.9 mm I.D.), 10 μm (Waters); Novapak C_{18} (150 × 3.9 mm I.D.), 4 μm (Waters); LiChrosorb RP-18 (250 × 4.0 mm I.D.), 5 μm (Merck); and Zorbax ODS (250 × 9.4 mm I.D.), 5–6 μm (semi-preparative column) (DuPont, Wilmington, DE, USA). The mobile phase solvents for RP-HPLC were methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF) (all of LiChrosolv grade, Merck), used in different binary, ternary and quaternary combinations with water (distilled, deionized).

The normal-phase separations were carried out with silica columns: Zorbax Sil (250 × 9.4 mm I.D.), 5–6 μm (semi-preparative column) (DuPont); LiChrospher Si 100 (250 × 4.0 mm I.D.), 10 μm (Merck); LiChroCART HPLC cartridge 250-4 Superspher Si 60 (250 × 4.0 mm I.D.), 4 μm (Merck); and Zorbax Sil (250 × 4.6 mm I.D.), 5 μm (Rockland Technologies). The eluent was usually 2-PrOH (LiChrosolv, Merck) in hexane (LiChrosolv, Merck) with the concentration of the former varying from 0.8 to 2.0% (v/v). In some experiments the mobile phase composition was MeOH–2-PrOH–hexane (0.4:0.8:98.8, v/v/v). The semi-preparative NP

Zorbax Sil column was used both for analytical and preparative purposes.

The HPLC solvents were filtered through a 0.45-μm membrane filter (Millipore, Milford, MA, USA) and thoroughly degassed by application of ultrasound before use. Millex LCR 0.45-μm cartridges (Millipore) were used for the filtration of the HPLC samples and the cartridges were always flushed carefully with the sample solvent. The HPLC columns were thoroughly flushed at the end of each day with a polar solvent; methanol or acetonitrile was used for RP columns and methanol for silica columns.

Component identification was based on UV–Vis and ^{1}H NMR spectra, retention times and co-elution with authentic samples.

Isolation of allomerization products by semi-preparative HPLC

After chromatography on a sucrose column, the fraction (band 4) containing both epimers of the 15^1-MeO-lactone derivative and the other epimer of 13^2-MeO-Chl *a* in addition to numerous minor components (see Fig. 5b) was further fractionated on a semi-preparative Zorbax Sil column. A 1-ml volume of the 2-PrOH–hexane (0.8:99.2, v/v) solution, containing *ca.* 4 mg of the aforementioned mixture, was injected and the column was eluted isocratically with the same solvent. Each component fraction was collected in repeated separations until the amount of the component was sufficient for NMR analyses. The combined fractions, containing a pure component, were washed with water to remove 2-PrOH, hexane was evaporated and the residue was dried by the chloroform co-distillation method. The solids were stored at −18°C.

Thin-layer chromatography

The TLC analyses for separate compounds, mixtures of allomerization products and fractions from the sucrose column were performed on laboratory-prepared sucrose plates, using 1% (w/w) 2-PrOH in light petroleum (analytical-reagent grade, Merck; b.p. 60–80°C, distilled through a Vigreux column) as eluent [43].

UV–visible spectra

Perkin-Elmer Model 550 and 554 spectrophotometers were used for the spectrophotometric characterization of the compounds dissolved in diethyl ether (analytical-reagent grade, Merck; dried) at ambient temperature. In the HPLC separations, the on-line UV–Vis spectra were recorded from 200 to 800 nm with the photodiode-array detector.

Nuclear magnetic resonance spectra

The ^{1}H NMR spectra were recorded on a Varian Gemini-200 instrument and a Varian Unity 500 MHz spectrometer, using 5-mm sample tubes. The NMR samples were dissolved in acetone-d_6 (Aldrich, 99.5, 99.98% ^{2}H, or Merck, 99.8% ^{2}H) to give the concentrations presented in Table I. The ^{1}H chemical shifts (δ) are given in ppm downfield from the internal standard, tetramethylsilane (TMS). The spectra were assigned by comparison with the spectrum of Chl *a* [42] and by using the two-dimensional NMR techniques heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond multiple quantum coherence (HMBC) [44–46].

Preparation of authentic 13^2(R,S)-hydroxy-Chl a

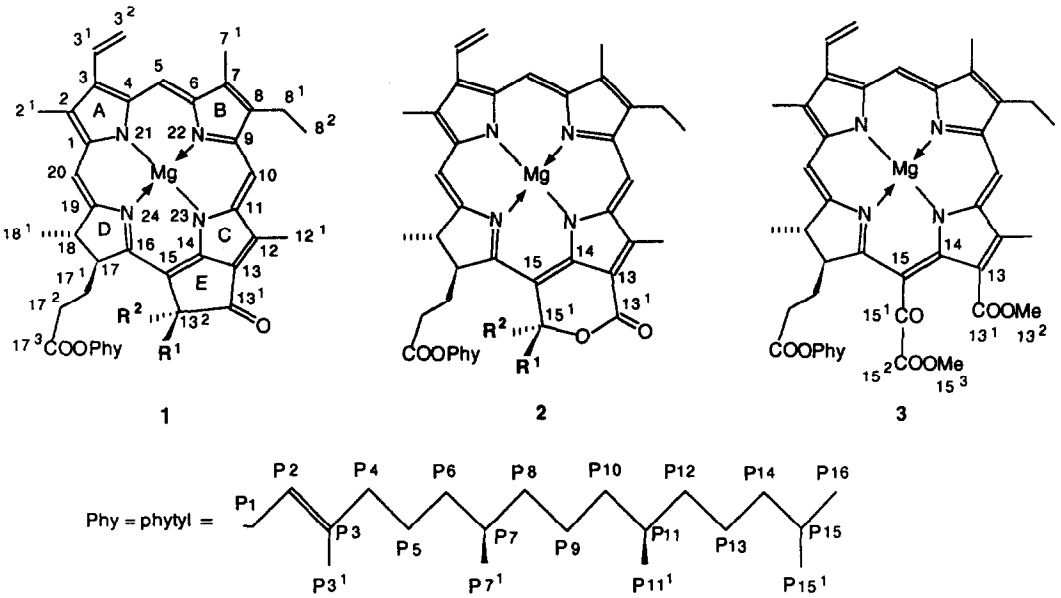
Authentic 13^2(*R*,*S*)-OH-Chl *a* was obtained by further purification of the preparations synthesized earlier in two ways: by horseradish peroxidase-catalysed H_2O_2 oxidation of Chl *a* solubilized with Triton-X 100 [47] and by selenium dioxide oxidation of Chl *a* in pyridine solution under argon [48]. The preparations were re-purified using chromatography on a sucrose column (3 cm O.D., height of the sucrose layer 45 cm). For the chromatography, the preparation was dissolved in 1 ml of diethyl ether plus 4 ml of the eluent. 13^2-Hydroxy-Chl *a* was eluted with 1% (v/v) 2-PrOH–hexane or MeOH–2-PrOH–hexane (0.4:0.8:98.8, v/v/v). The column, wrapped with aluminium foil, was operated in the dark at a flow-rate of 1 ml/min. The collected fractions were analysed by HPLC and the purest fractions were combined, washed with water and dried by the chloroform co-distillation method.

The solvent was evaporated to dryness and the solid was stored at −18°C.

RESULTS AND DISCUSSION

Allomerization reaction of Chl a

Fig. 1 shows the structures and numbering system for Chl *a* (**1a**) and the allomerization products relevant to this study. Except for a trace amount of Chl *a'* (**1b**), no other components could be detected in the original Chl *a* preparation by NP-HPLC. Methanolic solutions of Chl *a* [2.3 m*M*; 31.4 mg in 15 ml (experiment 1) and 304.5 mg in 150 ml (experiment 2)] were found to allomerize completely in 3 days when magnetically stirred under atmospheric oxygen at room temperature in darkness. The seven allomerization products identified were as follows:



No.	R^1	R^2	Semisystematic name [4,39]	Trivial name [8,40]	Abbreviation
1a	H	$COOCH_3(13^4)$	$13^2(R)$-Chlorophyll *a*	Chlorophyll *a*	Chl *a*
1b	$COOCH_3(13^4)$	H	$13^2(S)$-Chlorophyll *a*	Chlorophyll *a'*	Chl *a'*
1c	$COOCH_3(13^4)$	OH	$13^2(R)$-Hydroxychlorophyll *a*	10(*R*)-Hydroxychlorophyll *a*	$13^2(R)$-HO-Chl *a*
1d	OH	$COOCH_3(13^4)$	$13^2(S)$-Hydroxychlorophyll *a*	10(*S*)-Hydroxychlorophyll *a*	$13^2(S)$-HO-Chl *a*
1e	$COOCH_3(13^4)$	$OCH_3(13^{3'})$	$13^2(R)$-Methoxychlorophyll *a*	10(*R*)-Methoxychlorophyll *a*	$13^2(R)$-MeO-Chl *a*
1f	$OCH_3(13^{3'})$	$COOCH_3(13^4)$	$13^2(S)$-Methoxychlorophyll *a*	10(*S*)-Methoxychlorophyll *a*	$13^2(S)$-MeO-Chl *a*
2a	$COOCH_3(15^3)$	$OCH_3(15^{2'})$	The magnesium complex of $3^1,3^2$-didehydro-15^1-hydroxy-$15^1(R)$-methoxy-rhodochlorin-15-acetic acid δ-lactone 15^2-methyl-17^3-phytyl ester	Mg-10(*R*)-Purpurin 7-lactone methyl ether methylphytyl ester	$15^1(R)$-MeO-lactone-Chl *a*
2b	$OCH_3(15^{2'})$	$COOCH_3(15^3)$	The magnesium complex of $3^1,3^2$-didehydro-15^1-hydroxy-$15^1(S)$-methoxy-rhodochlorin-15-acetic acid δ-lactone 15^2-methyl-17^3-phytyl ester	Mg-10(*S*)-Purpurin 7-lactone methyl ether methylphytyl ester	$15^1(S)$-MeO-lactone-Chl *a*
3			The magnesium complex of $3^1,3^2$-didehydro-rhodochlorin-15-glyoxylic acid $13^1,15^2$-dimethyl-17^3-phytyl ester	Mg-Purpurin 7-dimethyl-phytyl ester	15-Glyox-Chl *a*

Fig. 1. Structures, names and numbering system for Chl *a* and Chl *a'* and their allomerization products.

15^1-MeO-lactone derivative (two epimers, **2a** and **2b**), 13^2-HO-Chl *a* (two epimers, **1c** and **1d**), 13^2-MeO-Chl *a* (two epimers, **1e** and **1f**) and 15-glyox-Chl *a* (**3**). The concentration of Chl *a* has been postulated to affect the number and nature of the products formed in the allomerization reaction [7,12].

Separation and isolation of the allomerization products of Chl a

Column chromatography. The conventional column chromatographic separation of the allomerization products from experiment 2 gave, after eluting with solvents of several polarities, five coloured bands arranged one above another in order of decreasing adsorptivity on sucrose. The order of the bands from the bottom of the sucrose column according to NP-HPLC analysis was (1) pheophytins *a'* and *a*, (2) 15-glyox-Chl *a*, (3) 13^2-MeO-Chl *a* (one epimer), (4) 15^1-MeO-lactone derivative (two epimers), overlapped by the other epimer of 13^2-MeO-Chl *a* (Fig. 2a) and (5) 13^2-HO-CHl *a* (two epimers), overlapped by an unknown impurity and small amounts of 13^2-MeO-lactone epimers and of the other 13^2-MeO-Chl *a* epimer (Fig. 2b). The preparative sucrose column was capable of separating as stereochemically pure compounds 15-glyox-Chl *a* and one epimer of 13^2-MeO-Chl *a*.

Reversed-phase HPLC. Because RP chromatography is used in at least half of all HPLC separations [49], it is always a good first choice for the separation of soluble organic compounds. In addition, Schaber *et al.* [14] used RP-HPLC for following the allomerization reaction. Therefore, also we selected this HPLC method for our first trial. Five C_{18} columns from different manufacturers were tested along with a Zorbax ODS semi-preparative column similar to that used by Schaber *et al.* [14]. Starting with different binary MeOH–water and ACN–water mobile phases, we soon found that the amount of water could be at most 5% (v/v), otherwise the Chl derivatives seemed to form various water adducts that were adsorbed strongly on the RP column and could be washed out only with 100% MeOH. We then tried the Zorbax ODS column using THF–

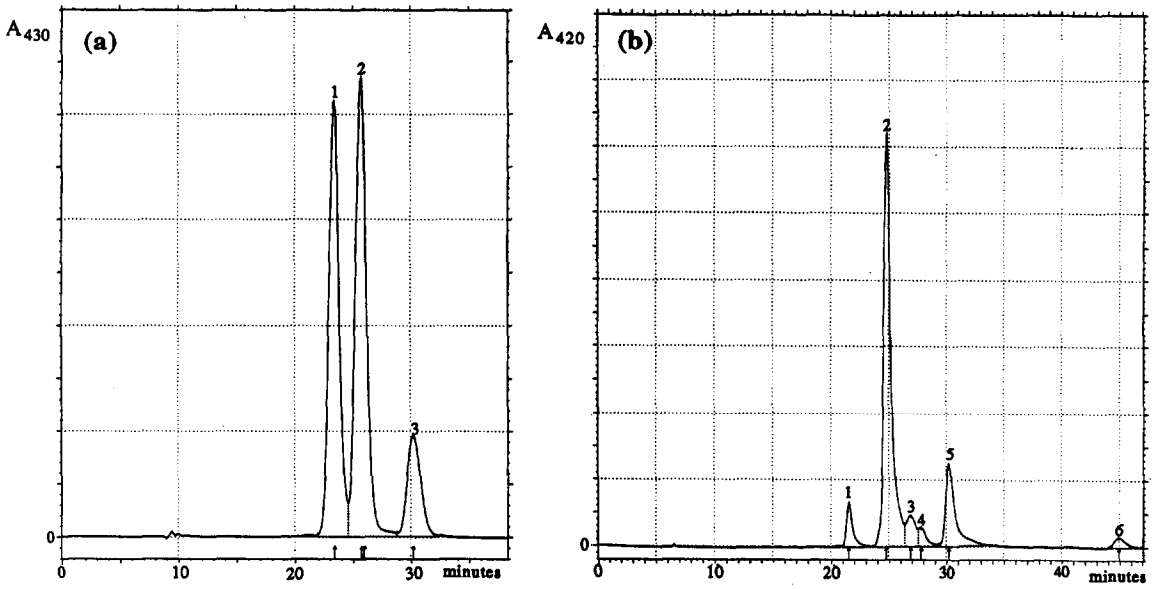


Fig. 2. (a) NP-HPLC of band 4 from the preparative sucrose column (allomerization products from experiment 2). Column, LiChrospher Si 100 (250 × 4.0 mm I.D.), particle size 10 μm; mobile phase, 2-PrOH–MeOH–hexane (0.8:0.4:98.8, v/v/v); flow-rate, 0.8 ml/min. Peaks: 1 and 2 = 15^1-MeO-lactone-Chl *a* epimers; 3 = 13^2-MeO-Chl *a* epimer 2. (b) NP-HPLC of band 5 from the preparative sucrose column (allomerization products from experiment 2). Column, Zorbax Sil (250 × 9.4 mm I.D.), 5–6 μm; mobile phase, 1.25% (v/v) 2-PrOH in hexane; flow-rate, 3 ml/min. Peaks: 1 = unidentified; 2 and 5 = 13^2-HO-Chl *a* epimers; 3 and 4 = 15^1-MeO-lactone-Chl *a* epimers; 6 = 13^2-MeO-Chl *a* epimer 2.

MeOH–water (36:54:10, v/v/v) as the mobile phase, because Schaber *et al.* [14] were able to separate the epimers of 13^2-HO-Chl *a* and those of the 15^1-MeO-lactone derivative with this system. THF coordinates to the fifth and/or sixth coordination position of the central magnesium atom of the allomerization products, thus inhibiting the formation of water adducts. In spite of several attempts under these chromatographic conditions, we were able to resolve only the 15^1-MeO-lactone epimers, the other components, except 15-glyox-Chl *a*, being eluted unresolved (Fig. 3a). 15-Glyox-Chl *a* showed an increased affinity for the RP column, eluting only when the column was flushed with methanol. Attempts were made to improve the separation by varying the proportions of THF, MeOH and water in the ternary mobile phase and some quaternary solvent systems were also tested. The best, but still incomplete, RP-HPLC separation, was achieved by using the Zorbax ODS column and THF–MeOH–water (10:85:5, v/v/v) as the mobile phase (Fig. 3b).

Normal-phase HPLC. The allomerization mixture contains several components, some of which are eluted very close together and hence can be separated only with difficulty. Because we could not achieve the desired separation by the RP-HPLC method, in spite of using several different RP columns and mobile phase combinations, we attempted NP-HPLC on a silica column. This method has often shown a unique capability to resolve isomers, and is also a good choice for preparative-scale HPLC. Watanabe *et al.* [33], for example, achieved a good separation for Chl *a*, *a'*, *b* and *b'* and the corresponding pheophytins using isocratic HPLC on a silica column.

Four silica columns from two manufacturers (Merck and DuPont) were tried in the NP separations. All silica columns afforded better separations than the RP columns. The Zorbax Sil semi-preparative column was the best as it separated all allomers including their epimers (Fig. 4). It is noteworthy that not even the analytical Zorbax Sil column was capable of separating the allomers as effectively as the corresponding semi-preparative column. This demonstrates again that similarity of the chromatographic material does not ensure reproducibility of the retention properties for different batches of the same product, which has been and still is a problem in HPLC. Fig. 4 shows that the elution order of the allomerization products on a silica column did not meet expectations, as the more polar 13^2-HO-Chl *a* epimers were eluted before 13^2-MeO-Chl *a* epimers. For some reason, the latter epimers are retarded more strongly than the former in the polar stationary

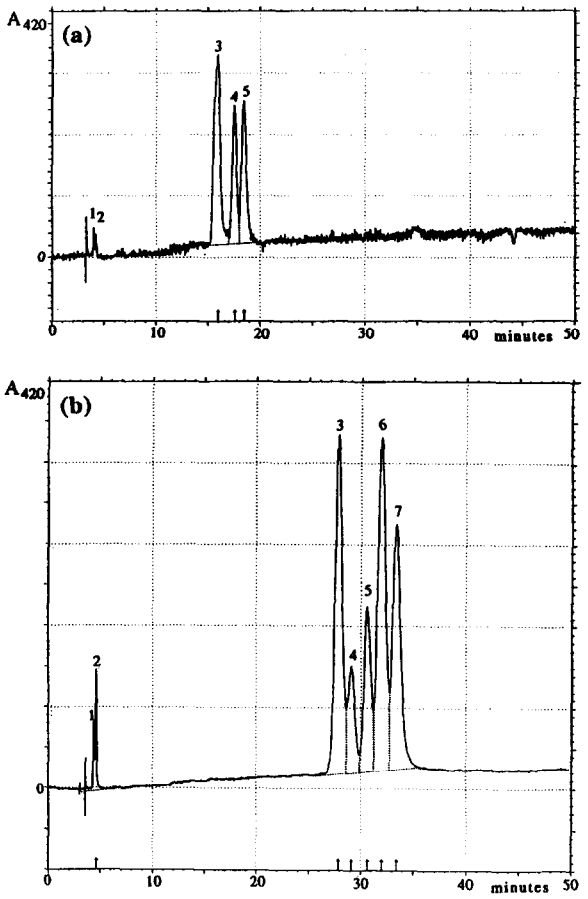


Fig. 3. RP-HPLC separation of the allomerization products on the Zorbax ODS column (250 × 9.4 mm I.D.), 5–6 μm. (a) Mobile phase, THF–MeOH–water (36:54:10, v/v/v); flow-rate, 3 ml/min. Peaks: 1 and 2 = unidentified, possibly pheophytins; 3 = 13^2-MeO-Chl *a* and 13^2-HO-Chl *a* epimers; 4 and 5 = 15^1-MeO-lactone-Chl *a* epimers. (b) Mobile phase, THF–MeOH–water (10:85:5, v/v/v); flow-rate, 3 ml/min. Peaks: 1 and 2 = unidentified, possibly pheophytins; 3, 4 and 5 all have UV–Vis spectra very similar to that of Chl *a* but the components were not identified more precisely; 6 and 7 = 15^1-MeO-lactone-Chl *a* epimers.

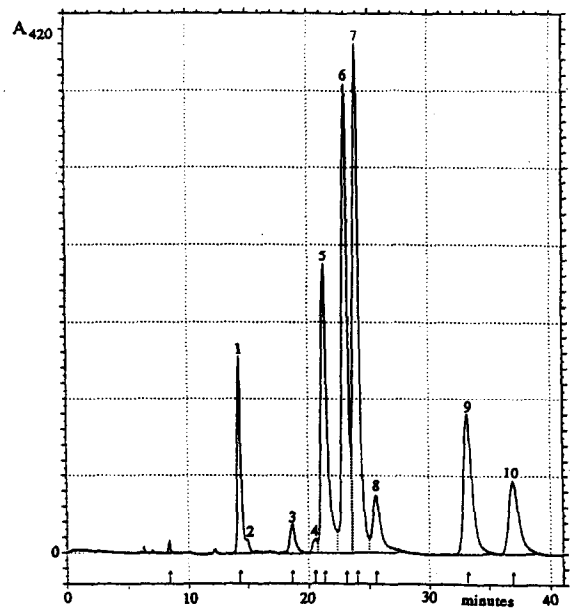


Fig. 4. NP-HPLC separation of the allomerization products on the Zorbax Sil column (250 × 9.4 mm I.D.), 5–6 μm. Mobile phase, 1.5% (v/v) 2-PrOH in hexane; flow-rate, 4 ml/min. Peaks 1 = 15-glyox-Chl *a*; 2, 3 and 4 = unidentified; 5 and 8 = 13^2-HO-Chl *a* epimers; 6 and 7 = 15^1-MeO-lactone-Chl *a* epimers; 9 and 10 = 13^2-MeO-Chl *a* epimers.

phase of silica. The principal allomerization products were the $15^1(R)$- and $15^1(S)$-epimers of the 15^1-MeO-lactone derivative (*ca.* 52% altogether). The amounts of $13^2(R,S)$-HO-Chl *a* and $13^2(R,S)$-MeO-Chl *a* were 21% and 18%, respectively, and the amount of 15-glyox-Chl *a* was 7%. The ratios of epimers 1 and 2, where 1 and 2 denote the elution order of the epimers in NP-HPLC, were 46:54 for the 15^1-MeO-lactone derivative, 79:21 for 13^2-HO-Chl *a* and 62:38 for 13^2-MeO-Chl *a*.

The mobile phase consisted of 0.8–2.0% (v/v) 2-PrOH in hexane or MeOH–2-PrOH–hexane (0.4:0.8:98.8, v/v/v). A very small change (0.1%) in the mobile phase composition affected the NP-HPLC separation results. The addition of MeOH to the mobile phase facilitated the chromatography of the 13^2-HO-Chl *a* epimers. The advantageous effect of MeOH may be attributed to its capability of preventing aggregation of the derivatives. The 13^2-HO-Chl epimers showed a particularly strong and concentration-dependent tendency for aggregation, owing to the presence of the central Mg atom and 13^2-hydroxyl and the 13^1-carbonyl groups in these molecules.

Bands 2 and 3 from the sucrose column (see above) contained pure 15-glyox-Chl *a* and one epimer of 13^2-MeO-Chl *a*, respectively. The chromatogram in Fig. 2a, in contrast, shows that band 4 from the sucrose column contained both epimers of 13^2-MeO-lactone-Chl *a* and the other epimer of 13^2-MeO-Chl *a*. The semi-preparative Zorbax Sil column was used for further purification of band 4. Figs. 5a and b show the analytical and preparative chromatograms, respectively, of band 4 run on the Zorbax Sil semi-preparative column. Fig. 5b shows that the 4-mg sample exhibits a behaviour similar to that of the analytical sample except for the slightly enhanced overlap between peaks 1 and 2 and the change in the retention time of component 4. After repeated separations the amount and purity of collected 13^2-MeO-lactone epimer 1 were 6 mg and 99.9%, respectively, and those of epimer 2 were 10.2 mg and 93–94%. The only impurity in the epimer 2 preparation was epimer 1.

Detection and identification of the allomerization products

Diode-array detection (DAD) has become established as a powerful LC detection method over the last 8 years [50–55]. DAD produces large amounts of multi-wavelength chromatographic and spectroscopic data in a single run. A variety of graphical and numerical strategies have been developed for the presentation of the data [50,53,54]. Most commonly, DAD is used to produce the on-the-fly UV–Vis spectra of the chromatographic peaks. This kind of spectrum was recorded here under isocratic HPLC conditions at the retention time of each allomer peak (Fig. 6) to facilitate its preliminary identification. Chl *a* and the derivatives substituted at C-13^2 have very similar UV–Vis spectra. Consequently, the 13^2-HO-Chl *a* and 13^2-MeO-Chl *a* epimers could not be identified solely on the basis of their UV–Vis spectra. Retention time comparisons and co-elution with authentic compounds and NMR spectroscopy were also needed. Nevertheless, 15-glyox-Chl *a* and the 15^1-MeO-lactone derivatives were distinguish-

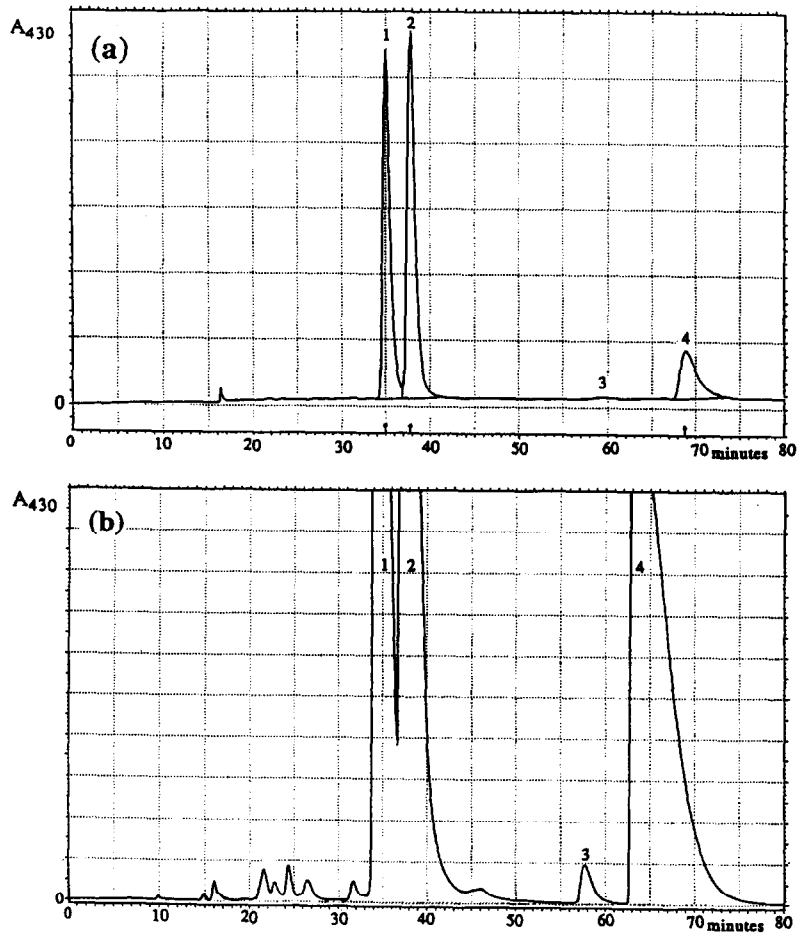


Fig. 5. (a) Analytical and (b) preparative (4 mg) NP-HPLC of the 13^2-MeO-lactone fraction (band 4) from the preparative sucrose column. Column, Zorbax Sil (250 × 9.4 mm I.D.), 5–6 μm; mobile phase, 0.8% (v/v) 2-PrOH in hexane; flow-rate, 4 ml/min. Peaks: 1 and 2 = 15^1-MeO-lactone-Chl *a* epimers; 3 and 4 = 13^2-MeO-Chl *a* epimers.

able by UV–Vis spectra from the other allomerization products.

Although it has been known for a long time that UV–Vis spectra are insufficient for the reliable identification of Chl allomers, NMR spectroscopy has rarely been used for this purpose. The ^{1}H and ^{13}C NMR resonances for the macrocycle of 13^2-HO-Chl *a* have been totally assigned [47,48] and the ^{1}H NMR spectrum of the 13^2-MeO-lactone derivative of Chl *a* has been partly interpreted [12,14]. In this study, the ^{1}H NMR spectra (Table I) were used to identify the allomerization products more conclusively. For about half of the Chl derivatives in Table I, the assignments have already been confirmed using the HMQC and HMBC techniques. For the other derivatives, the mutual order for several assignments is only tentative and requires confirmation by the aforementioned two-dimensional NMR techniques. Such experiments are in progress.

Mechanism of allomerization

Our allomerization results are different from those reported by Schaber *et al.* [14] in that we observed 15-glyox-Chl *a* and $13^2(R)$- and $13^2(S)$-MeO-Chls in addition to the diastereomers of 13^2-HO-Chl *a* and the 15^1-MeO-lactone derivative (major products). Several minor components could also be detected among the allomers (Fig.

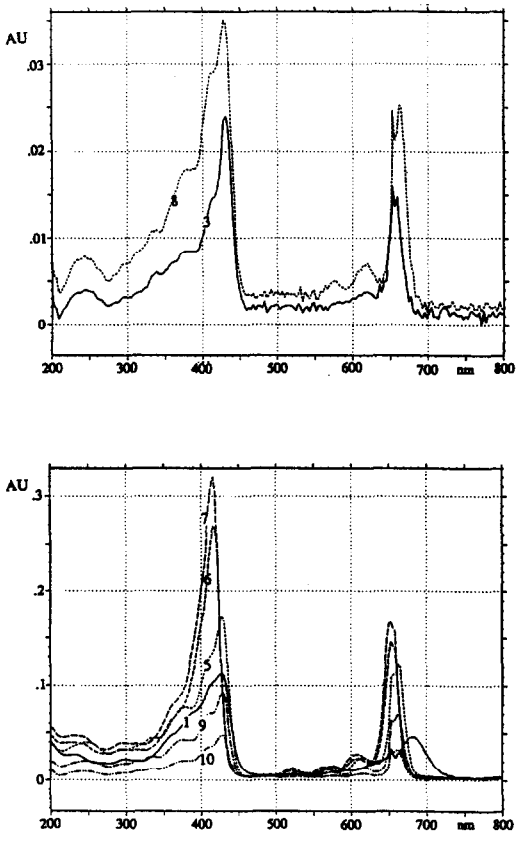


Fig. 6. UV–Vis spectra of the allomerization products of Chl *a* recorded from 200 to 800 nm with the diode-array detector under the chromatographic conditions of Fig. 4. The numbers on the spectra correspond to the numbers of the chromatographic peaks in Fig. 4. The shoulder in the spectra near 650 nm depends on the detector.

5b), but we did not investigate these. According to Schaber *et al.* [14], the 13^2-HO-Chl *a* epimers are major products and are formed in both presence and absence of extraneous nucleophiles (*e.g.*, MeOH), while the epimers of the 15^1-MeO-lactone derivative are formed only when Chl *a* is allowed to allomerize in a polar hydroxylic solvent [14]. They could not detect any 15-glyox-Chl *a* or 13^2-MeO-Chl *a* by RP-HPLC. These differences in results probably arise from differences in the reaction and fractionation conditions used by the two groups. We performed the reactions with continuous stirring and in darkness. Further, the methanol we used was perhaps different from that used by Schaber *et al.* [14] (unfortunately, they did not mention the purity or source of the methanol), and also we used NP-HPLC instead of RP-HPLC.

The free-radical chain mechanism involving triplet oxygen, the Chl C-13^2 radical and the Chl 13^2-hydroperoxide derivative [4,11] can account for the allomerization products formed in this study. Radical reactions are typical of the triplet oxygen. Singlet oxygen cannot be formed in the dark. The large number of the allomerization products (Fig. 5b) supports the radical mechanism. The unexpected ratio of *ca.* 4:1 for the 13^2-HO-Chl *a* epimers can be explained by their formation via two possible routes: (1) the termination reaction of the Chl C-13^2 radical with hydroxyl radical and (2) the homolytic cleavage of the Chl 13^2-hydroperoxide followed by hydrogen atom addition. Moreover, steric reasons may result in unexpected epimeric ratios.

CONCLUSION

The spontaneous allomerization reaction for Chl *a* in methanol (2.3 m*M*) in complete darkness produced as principal products 15^1-MeO-lactone derivatives (52%), consisting of 15^1-diastereomers in a ratio of 46:54. The other products were 13^2-HO-Chl *a* (21%, 13^2-diastereomers in a ratio of 79:21), 13^2-MeO-Chl *a* (18%, 13^2-diastereomers in a ratio of 62:38) and 15-glyox-Cl *a* (7%). In addition, some minor components were detected, but not identified.

The separation, isolation and structural identification of the individual components in the complex mixture of the Chl *a* allomers have posed an analytical problem of practical interest. In this study, the RP-HPLC experiments were carried out with various RP columns and mobile phase compositions, but no RP method resulted in the desired separation. Silica columns proved to have the unique capability of separating all allomerization products and their epimers. Of the silica columns tested in this study, the Zorbax Sil semi-preparative column provided the best separation and a powerful means for the preparative isolation of the allomerization products in milligram amounts. The amounts isolated were sufficient for the characterization of the compounds by NMR techniques. The pre-

TABLE I

^{1}H NMR CHEMICAL SHIFTS (δ^{TMS} IN PPM) FOR CHL *a* AND ITS ALLOMERS IN ACETONE-d_6

Proton[a]	Chl *a* (11.2 m*M*)	15-Glyox-Chl *a* (10.9 m*M*)	15^1-MeO-lactone-Chl *a*		13^2-MeO-Chl *a*		13^2-HO-Chl *a*	
			Epimer 1[b] (8.5 m*M*)	Epimer 2[b] (14.5 m*M*)	Epimer 1[b] (27.0 m*M*)	Epimer 2[b] (2.4 m*M*)	Epimer 1[b] (15.0 m*M*)	Epimer 2[b] (10.0 m*M*)
10-H	9.72	9.52	9.79	9.80	9.78	9.79	9.78	9.79
5-H	9.39	9.28	9.51	9.52	9.42	9.45	9.46	9.46
20-H	8.56	8.39	8.66	8.68	8.61	8.61	8.62	8.61
3^1-H_X	8.12	8.01	8.13	8.13	8.12	8.13	8.14	8.14
3^2-H_B (*trans*)	6.22	6.16	6.23	6.23	6.23	6.24	6.22	6.22
3^2-H_A (*cis*)	6.01	5.96	6.01	6.01	6.02	6.03	6.02	6.02
13^2-OH	–	–	–	–	–	–	6.05	6.02
13^2-H	6.18	–	–	–	–	–	–	–
P2-H	4.95	5.16	5.19	5.14	5.16	5.21	5.14	5.22
17-H	4.16	4.66	4.72	4.85	4.45	4.27	4.68[c]	4.15[c]
18-H	4.55	4.31	4.46	4.45	4.54	4.57	4.55[c]	4.55[c]
P1-CH_2	4.29	4.42	4.45	4.40	4.43	4.48	4.40[c]	4.46[c]
8^1-CH_2	3.80	3.72	3.82	3.83	3.82	3.83	3.83	3.83
13^2-CH_3	–	3.97[c]	–	–	–	–	–	–
13^4-CH_3	3.81	–	–	–	3.62	3.59[c]	3.60[c]	3.58[c]
15$^{2'}$-CH_3	–	–	3.84	3.49	–	–	–	–
15^3-CH_3	–	3.84[c]	3.61	3.65	–	–	–	–
12^1-CH_3	3.59	3.43[c]	3.77	3.77	3.65	3.66[c]	3.64[c]	3.64[c]
2^1-CH_3	3.34	3.23[c]	3.35	3.35	3.35	3.36[c]	3.36[c]	3.36[c]
7^1-CH_3	3.29	3.20[c]	3.31	3.31	3.28	3.31[c]	3.31[c]	3.31[c]
13$^{3'}$-CH_3	–	–	–	–	3.15	3.40[c]	–	–
17^1-CH_2, 17^2-CH_2	2.58–2.12	2.28–1.82	2.47–2.10	2.46–2.24	2.38–1.96	2.70–2.45	2.34–2.22	2.83–2.43
P4-CH_2	1.84	1.90	1.91	1.88	1.89	1.93	1.85	1.79
8^2-CH_3	1.70	1.65	1.70	1.70	1.73	1.72	1.71	1.71
18^1-CH_3	1.76	1.74	1.55	1.69	1.62	1.56	1.65	1.56
P3^1-CH_3	1.51	1.58	1.59	1.55	1.57	1.61	1.56	1.62

[a] See Fig. 1 for numbering of carbon atoms.
[b] Epimer 1 = faster moving epimer and epimer 2 = slower moving epimer in NP-HPLC.
[c] HMQC and HMBC experiments are necessary for the final assignments.

parative-scale isolation of Chl derivatives has formerly relied largely on conventional column chromatography (CC). In this study, however, conventional CC on sucrose had to be complemented by HPLC in order to achieve the separation of all allomerization products including the C-13^2/15^1 epimers. Although conventional CC requires large amounts of column material and solvents, it seems likely to maintain its position as a good preparative preseparation method. The determination of the absolute configuration at C-13^2 or C-15^1 for the isolated allomerization products by NMR is in progress. We should subsequently be able to specify which peaks in the HPLC traces belong to the 13^2/15^1(*R*)-epimers and which to the 13^2/15^1(*S*)-epimers. Additional studies to clarify the allomerization mechanism in further detail are in progress.

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