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Journal of Chromatography A, 837 (1999) 107–116

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatographic separation and mass spectrometric identification of chlorophyll *b* allomers

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Received 22 September 1998; received in revised form 5 January 1999; accepted 11 January 1999

Abstract

Seven methanolic allomers of Chl *b* were separated and isolated by using sucrose column chromatography and normal-phase HPLC. Diastereomeric selectivity was achieved for 13^2(*R,S*)-methoxychlorophyll *b* and the magnesium complex of $3^1,3^2$-didehydro-15^1-hydroxy-15^1(*R,S*)-methoxy-7^1-oxo-rhodochlorin-15-acetic acid δ-lactone 15^2-methyl-17^3-phytyl ester. 13^2(*S*)-Hydroxy-10-methoxychlorophyll *b*, 13^2-hydroxychlorophyll *b* and the magnesium complex of $3^1,3^2$-didehydro-7^1-oxo-rhodochlorin-15-glyoxylic acid $13^1,15^2$-dimethyl-17^3-phytyl ester were also isolated in high purity. The allomers were mainly identified by using UV–Vis spectrophotometry and FAB–MS. The allomerization of Chl *b* clearly differed from that of Chl *a*. The most striking difference was the formation of 13^2(*S*)-hydroxy-10-methoxychlorophyll *b*, as the corresponding derivative has never been observed to form in the allomerization of Chl *a*.

Keywords: Allomers; Free radicals; Mass spectrometry; Fast atom bombardment; Chlorophylls

1. Introduction

The oxidation at C-13^2 by triplet oxygen in alcohol (Willstätter's allomerization [1,2]) occurs to all chlorophylls (Chls), which have an intact β-keto ester structure at the isocyclic ring (ring E, Fig. 1). The allomerization products (allomers) all have an oxygen atom bonded to a carbon, originally C-13^2. There is strong evidence for the free-radical allomerization mechanism but many details of the mechanism still demand research [3–6].

Chl allomers or their derivatives occur in Chl preparations, processed vegetables and green fruits [7], lake bottom sediments [6,8], and possibly in senescent plants [9]. Some derivatives are also potential photosensitizers for the photodynamic therapy of cancer. Hence, there is an obvious need for the characterization of the allomers.

In green algae and green plants the Chl *a*:Chl *b* ratio of the light-harvesting pigments is generally 3:1. The allomerization research concentrates on Chl *a* [3–6,10–13] and only few publications are available about the allomerization of Chl *b* [7,11,14,15]. Reasons for this can be the lack of starting material, the assumed analogy to the allomerization of Chl *a*, and the difficulties in the separation of the Chl *b* allomers.

In recent allomerization studies, high-performance liquid chromatography (HPLC) has been the most common method for the separation of the allomers

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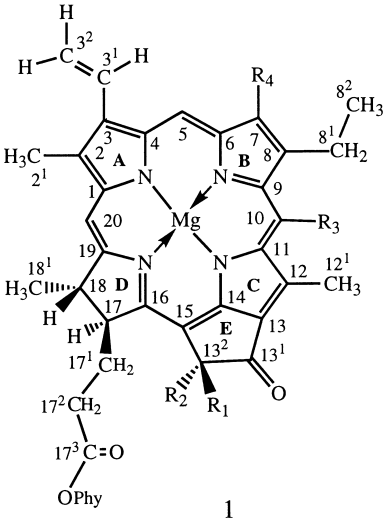
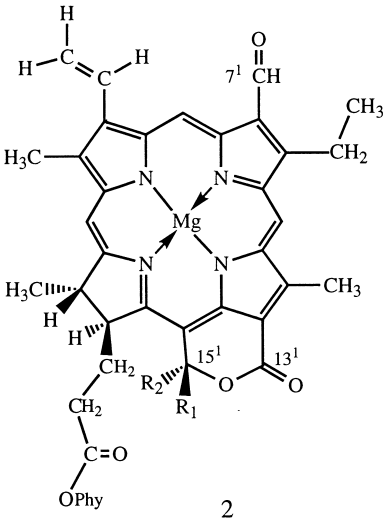
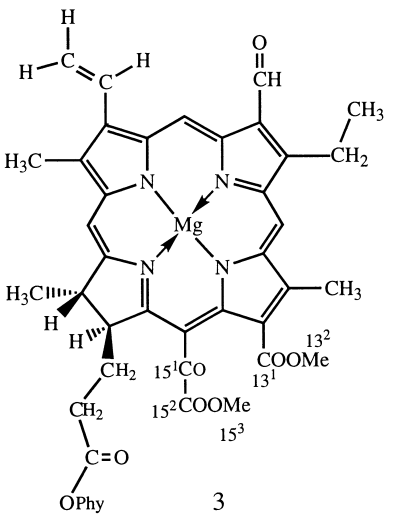
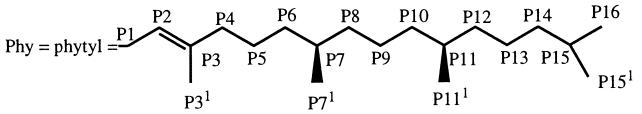
0021-9673/99/$ – see front matter
PII: S0021-9673(99)00059-X









No.	R_1	R_2	R_3	R_4	Semisystematic name [4,25]	Abbreviation
1a	H	$COOCH_3$	H	CH_3	13^2(*R*)-Chlorophyll *a*	Chl *a*
1b	H	$COOCH_3$	H	CHO	13^2(*R*)-Chlorophyll *b*	Chl *b*
1c	$COOCH_3$	OH	H	CHO	13^2(*R*)-Hydroxychlorophyll *b*	13^2(*R*)-HO-Chl *b*
1d	OH	$COOCH_3$	H	CHO	13^2(*S*)-Hydroxychlorophyll *b*	13^2(*S*)-HO-Chl *b*
1e	$COOCH_3$	OCH_3	H	CHO	13^2(*R*)-Methoxychlorophyll *b*	13^2(*R*)-MeO-Chl *b*
1f	OCH_3	$COOCH_3$	H	CHO	13^2(*S*)-Methoxychlorophyll *b*	13^2(*S*)-MeO-Chl *b*
1g	OH	$COOCH_3$	OCH_3	CHO	13^2(*S*)-Hydroxy-10-methoxychlorophyll *b*	13^2(*S*)-HO-10-MeO-Chl *b*
2a	$COOCH_3$	OCH_3	-	-	The magnesium complex of 3^1,3^2-didehydro-15^1-hydroxy-15^1(*R*)-methoxy-7^1-oxo-rhodochlorin-15-acetic acid δ-lactone 15^2-methyl-17^3-phytyl ester	15^1(*R*)-MeO-lact-Chl *b*
2b	OCH_3	$COOCH_3$	-	-	The magnesium complex of 3^1,3^2-didehydro-15^1-hydroxy-15^1(*S*)-methoxy-7^1-oxo-rhodochlorin-15-acetic acid δ-lactone 15^2-methyl-17^3-phytyl ester	15^1(*S*)-MeO-lact-Chl *b*
3	-	-	-	-	The magnesium complex of 3^1,3^2-didehydro-7^1-oxo-rhodochlorin-15-glyoxylic acid 13^1, 15^2-dimethyl-17^3-phytyl ester	15-Glyox-Chl *b*

Fig. 1. Structures, names and numbering system for Chl *a*, Chl *b* and the Chl *b* allomers.

[5–7,12,13,16]. The structural analysis of allomers has been based on electronic absorption spectrophotometry (UV–Vis), IR, NMR [17], mass spectrometry (MS) and surface-enchanced resonance Raman spectroscopy [6,18].

Fast-atom bombardment mass spectrometry (FAB–MS) has proven to be a very efficient technique in the characterization of tetrapyrroles. As FAB is based on the sputtering phenomenon [19], there is no need for volatility or thermal stability of the sample. The 'softness' makes FAB a very suitable method for the ionization of Chls and there are many publications dealing with the structural analyses of Chls and derivatives, including allomers, by using FAB [6,20–24].

In this work, seven methanolic allomerization products of Chl *b* were purified by sucrose column chromatography and normal-phase HPLC in combination, and characterized using UV–Vis, FAB–MS and NMR when required. There were clear differences in the allomerization of Chl *b* as compared to that of Chl *a*. The most notable difference was the formation of an entirely new Chl derivative, 13^2(*S*)-HO-10-MeO-Chl *b* (for structure see Fig. 1), whose analogue has never been detected in the allomerization of other Chls.

1.1. Experimental

1.1.1. Isolation and purity of chlorophyll b

Chl *b* was isolated from clover leaves by the method described previously [26], but since then modified for large-scale preparation. The purity of Chl *b* was confirmed by sucrose TLC [27], HPLC [LiChrosorb Si 60 column, 2-propanol–hexane eluent (6:94, v/v); for the specification of the column, see the experimental HPLC section] and ^{1}H NMR [28]. According to these analyses, the only impurities were water (water:Chl *b* ca. 1.5:1) and a trace amount of Chl *b′* (=13^2(*S*)-Chl *b*).

1.2. Allomerization reaction

Solid Chl *b* was dissolved in methanol (J.T. Baker, Deventer, The Netherlands, Catalog No. 8045, Absolute, ACS, 'Baker analyzed reagent', dried over 3 Å molecular sieves) to form a solution with a concentration of ca. 2 mg/ml in an Erlenmeyer flask provided with a loose glass stopper and wrapped with aluminium foil. The solution was continuously stirred magnetically at room temperature. The course of the reaction was followed by taking ca. 50 μl samples from the reaction mixture. These samples were immediately filtered, (diluted) and analyzed by HPLC [LiChrosorb Si 60 column, 2-propanol–hexane eluent (6:94, v/v)] and sucrose TLC. Chl *b* disappeared totally in 5 h and there were no changes observed in the product mixture after that period. The mixture was transferred with methanol into a small round-bottomed flask and evaporated to dryness with a rotary evaporator.

1.3. Solvents for liquid chromatography

All solvents used were analytical-reagent grade or HPLC grade. Hexane was from Prolabo (Paris, France) and it was distilled through a Vigreux column. 2-Propanol was from Merck (Darmstadt, Germany) or Lab-Scan (Dublin, Ireland). Tetrahydrofuran (peroxide-free, distilled before use) and methanol were from Rathburn (Walkerburn, Scotland).

1.4. Sucrose column chromatography

1.4.1. Column packing

Powdered sugar containing ca. 1.5% calcium phosphate (Sucros Oy, Kantvik, Finland) was used for the sucrose column chromatography. The mean particle size of the sucrose was 0.35 mm and it was passed through a 180 μm sieve before use. Two glass columns were packed by the slurry method [26] to give the packed columns 1 and 2. In the case of column 1, the height of the sucrose layer was 54 cm and the inside diameter was 6 cm. The corresponding measures for column 2 were 80 and 3 cm. After packing, columns 1 and 2 were wrapped with aluminium foil and used in separations 1 and 2, respectively.

1.4.2. Separation 1

The allomer solution was evaporated to dryness under reduced pressure. The residue (ca. 250 mg) was dissolved in 10 ml tetrahydrofuran and 40 ml eluent (2-propanol–methanol–hexane, 0.5:0.5:99, v/v/v), and the solution was introduced onto the top

of the sucrose layer in column 1. The collected effluent fractions were analyzed using sucrose TLC, UV–Vis spectrophotometry and HPLC. The effluent fractions containing a pure allomer were washed with distilled water, evaporated to dryness and dehydrated by the chloroform co-distillation method [29]. The other fractions were combined, washed with distilled water and evaporated to dryness with a rotary evaporator.

1.4.3. Separation 2

About 20 mg of allomers were dissolved in 0.2 ml tetrahydrofuran and 0.8 ml eluent (2-propanol–hexane, 0.8:99.2, v/v), and this solution was introduced onto the top of the sucrose layer in column 2. To speed up the elution, the principle of 'flash chromatography' was utilized. The effluent fractions were analyzed and handled as in separation 1. Separation 2 was repeated until the desired amounts of allomers were obtained.

1.5. High-performance liquid chromatography

1.5.1. Instrument

The HPLC instrument has been described earlier [5], except that the old gradient controller (Model 660 solvent programmer) was replaced with a Waters Automated Gradient Controller (Waters, Milford, MA, USA). Separations were monitored by recording the absorbance at 440, 450 or 460 nm with the photodiode array detector of the instrument.

1.5.2. Columns

Two silica columns were used: Zorbax Sil (250× 9.4 mm I.D., a semi-preparative column), particle size 5 μm (DuPont, Rockland Technologies, Newport, DE, USA) and LiChroCART HPLC cartridge 250-4 LiChrosorb Si 60 (250×4.0 mm I.D.), particle size 7 μm (Merck). The columns were protected with a precolumn (Guard-Pak Inserts Nova-Pak Silica; Waters).

1.5.3. Samples

The HPLC samples were dissolved in 2-propanol–hexane (1–6:99–94). All samples were filtered through MillexLCR 0.45 μm cartridges (Millipore).

1.6. Thin-layer chromatography

The TLC analyses for the allomer mixture, sucrose column fractions and purified compounds were performed on laboratory-prepared sucrose plates [27]. The developing solvent was 2-propanol–methanol–hexane (0.8:0.7:98.5, v/v/v) or tetrahydrofuran–2-propanol–hexane (0–0.5:1–1.5:98–99, v/v/v). The spots were detected visually and by UV fluorescence (366 nm).

1.7. UV–Visible spectra

The UV–Vis spectra were recorded on a Varian CARY 5E UV–Vis–NIR spectrophotometer at ambient temperature, directly upon the (diluted) effluent from the sucrose column. The absorption spectra of the purified and dried (by using the chloroform co-distillation method or an argon stream) compounds were measured in dry diethyl ether [analytical-reagent grade, Merck; dried and stabilized with 2,6-di-*tert*-butyl-4-methylphenol (BHT)]. In the HPLC analyses, the on-line UV–Vis spectra were recorded from 300 to 800 nm with the photodiode-array detector of the instrument.

1.8. Mass spectrometry

The FAB mass spectra of Chl *b* and its allomers were measured with a Finnigan MAT 8200 mass spectrometer equipped with a FAB gun. The temperature of the ion source was equal to room temperature, the xenon beam energy was ca. 8 kV, the accelerating voltage 3 kV, the resolution 1000 and the magnetic field scanning rate was 1 scan/8.5 s from m/z 100 to m/z 1000. The sample (dissolved in methanol) was introduced into the ion source with a static FAB probe; ca. 0.5 μl of 3-nitrobenzyl alcohol (3-NBA; 98%, Aldrich, Steinheim, Germany) and 0.5–1 μl of sample solution were injected on the stainless steel tip of the probe.

In a typical processing procedure of a FAB mass spectrum, about 20 scans were added together and the background was subtracted. There were some problems with the background subtraction, as the ion abundances of the background and the sample did not remain constant during the FAB–MS experiment. The difficulties in the background subtraction

were most evident with concentrated samples, since very little 3-NBA remained on the FAB probe tip after the allomer had decomposed. In these cases, 50–100 scans were added together and no background subtraction was made.

2. Results and discussion

2.1. Sucrose column chromatography

Separation 1 yielded only spectroscopically pure (s.p.) 15-Glyox-Chl *b* (Fig. 1) and therefore the other allomers had to be re-separated on the longer sucrose column 2 (separation 2). After the sucrose column separations, the effluent fractions were washed and dried as described in the experimental. The following compound fractions were obtained: (1) Mg-free compounds (traces); (2) 15-Glyox-Chl *b* (s.p.); (3) 13^2-MeO-Chl *b* epimer II (s.p.); (4) 15^1-MeO-lactone-Chl *b* epimers and 13^2-MeO-Chl *b* epimer I; (5) 13^2(*S*)-HO-10-MeO-Chl *b*, 13^2-HO-Chl *b* and a very small amount of 15^1-MeO-lactone-Chl *b* epimers. Fractions (4) and (5) were further purified on a semi-preparative Zorbax Sil column.

2.2. High-performance liquid chromatography

Normal-phase columns were chosen for the HPLC separations on the basis of a few preliminary experiments on reversed-phase HPLC columns and the previous experience obtained from the separations of the Chl *a* allomers [5].

In order to achieve a good separation of the Chl *b* allomers on silica columns, many eluent compositions with different amounts of 2-propanol, 1-propanol, methanol, tetrahydrofuran or mixtures of these in hexane were tested. Also some experiments using the gradient elution were performed. The best separations of the Chl *b* allomers were achieved using tetrahydofuran–hexane (12–13:88–87, v/v) as the eluent and the semi-preparative Zorbax Sil column (Fig. 2). Tetrahydrofuran in hexane afforded narrower peaks in the chromatogram than propanol in hexane. The most probable cause for the narrower peaks was the high disaggregating efficacy of tetrahydrofuran, which can coordinate both to the fifth and sixth coordination positions of the central magnesium atom of a Chl molecule. Chl *b* and its allomers have a higher tendency to aggregate than Chl *a* and its allomers, due to the formyl group at C-7.

Fractions (4) and (5) from the sucrose column 2 were purified further using the semi-preparative Zorbax Sil column. The eluent was tetrahydrofuran–hexane (12:88, v/v) and the injection concentration ca. 1 mg/100 μl for fraction (4). There were serious aggregation problems in the case of fraction (5), since the 13^2-hydroxy group of 13^2(*S*)-HO-10-MeO-Chl *b* and 13^2-HO-Chl *b* increased the aggregation tendency as compared to the other allomers of Chl *b*. For fraction (5), tetrahydrofuran could not be used as a disaggregating agent, because it caused pheophytinization (loss of magnesium) of 13^2(*S*)-HO-10-MeO-Chl *b*. Hence, the injection concentration of the allomer sample had to be kept below 0.2 mg/100 μl. Also a two-step HPLC procedure had to be applied in order to achieve a satisfactory purification for this fraction. Using 2-propanol–hexane (6:94) as the eluent, the 15^1-MeO-lact-Chl *b* epimers were first separated from 13^2(*S*)-HO-10-MeO-Chl *b* and 13^2-HO-Chl *b*. Then the latter derivatives were separated from each other employing 2-propanol–methanol–hexane (3.5:1.5:95) as the eluent.

2.3. Identification of allomers

A preliminary identification of the Chl *b* allomers was made on the basis of their elution orders on the sucrose column and on the normal-phase HPLC column and on the basis of their UV–Vis spectra (Fig. 3). The UV–Vis spectra were compared with the spectra of the Chl *a* allomers [5], the Chl *b* spectrum [30] and the earlier reported spectra of 15^1-MeO-lact-Chl *b* and 13^2-HO-Chl *b* [14,30].

15-Glyox-Chl *b* and the 15^1-MeO-lact-Chl *b* epimers were identified on the grounds of their UV–Vis spectra. The UV–Vis spectra of the 13^2-MeO-Chl *b* epimers and 13^2-HO-Chl *b* were almost indistinguishable from the spectrum of Chl *b* (Fig. 3), but it was possible to make a tentative identification by comparing the elution order (Fig. 2) with that of the analogous Chl *a* allomers [5]. The final identification of the purified allomers was made by

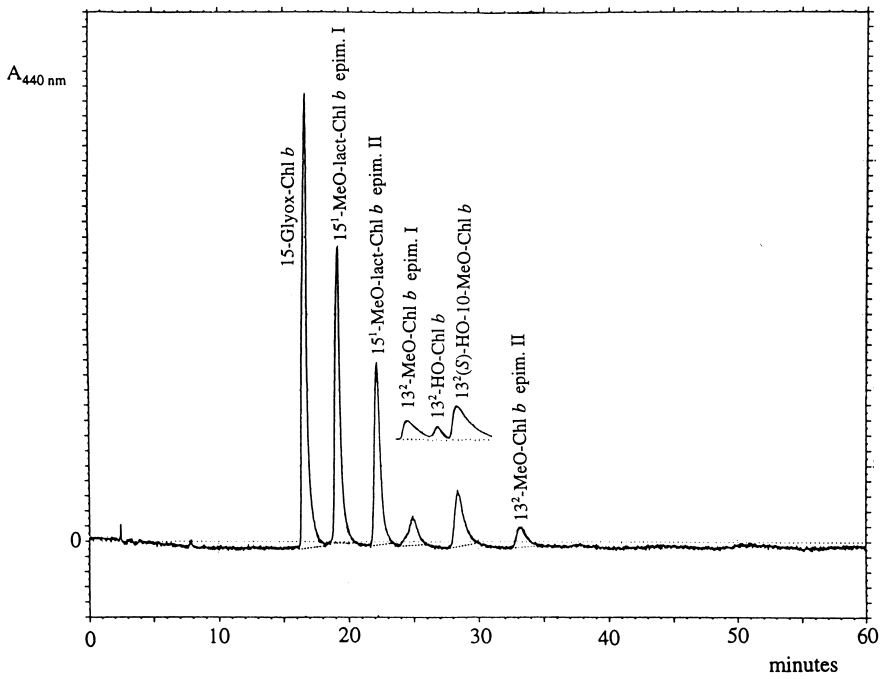


Fig. 2. A HPLC profile of the Chl *b* allomers on the semi-preparative Zorbax Sil column. Mobile phase: tetrahydrofuran–hexane (12:88, v/v); flow-rate: 4 ml/min. *Insert:* Part of another HPLC profile showing the presence of 13^2-HO-Chl *b*. I and II refer to the elution order of the epimers. These epimers have different absolute configurations at the oxidized carbon (originally C-13^2). The determination of the absolute configurations requires NMR [17] and is beyond this work.

using FAB–MS (vide infra) and in the case of 13^2(*S*)-HO-10-MeO-Chl *b* also NMR [31] .

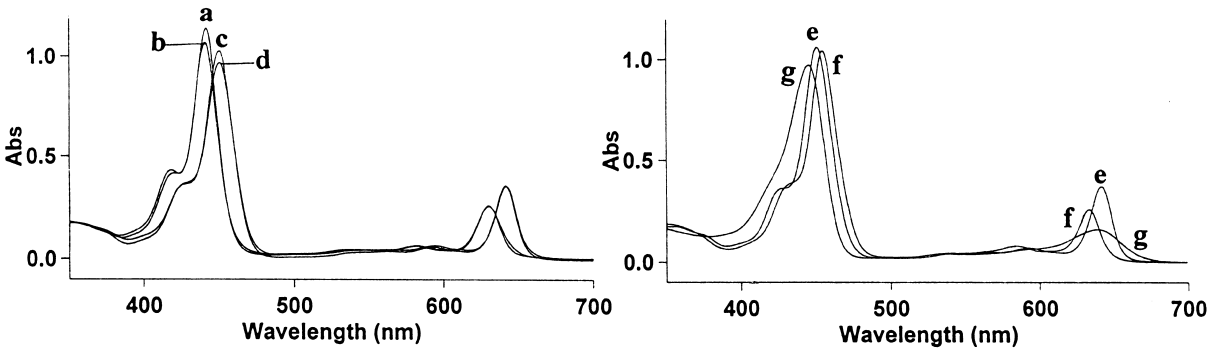
2.4. Mass spectrometry

The most important ions and their relative abundances in the FAB mass spectra of Chl *b* and its allomers are listed in Table 1. The structural assignments of the ions are presented in Table 2 and some ions are briefly discussed below.

In each of the FAB spectra of Chl *b* and its seven allomers, there occurred a clear π-radical cation ($M^{\cdot+}$) peak. Discussion about the formation of $M^{\cdot+}$ in FAB–MS when 3-NBA is used as a matrix can be found in the literature [22–24]. All the compounds of this work also formed a very intense $[M+1]^+$ peak, which corresponds to a mixture of the heavy isotope $M^{\cdot+}$ ion (Chl containing ^{25}Mg or ^{13}C) and the $[M+H]^+$ ion. In the spectra of the Chl *b* allomers, except the 13^2-MeO-Chl *b* epimer I, the abundance of the $[M+1]^+$ ion was clearly higher (ca. 85–137% of the abundance of $M^{\cdot+}$) than the calculated theoretical abundance (ca. 77–78.5% of the abundance of $M^{\cdot+}$). The high abundance of the $[M+1]^+$ peak is caused by the FAB process, which tends to produce even-electron ions such as the protonated molecules $[M+H]^+$ [19,24,32].

The FAB spectra of Chl *b* and its allomers showed the formation of the $[M-278]^+$ ion, formed by the loss of phytadiene via the McLafferty rearrangement, to give a carboxylic acid. The $[M-277]^+$ ion, which occurred at high abundance in the FAB spectra of the Chl *b* allomers, is formed by the loss of phytadiene from the $[M+1]^+$ ion or by the protonation of the $[M-278]^+$ ion.

There are two possible fragmentations for the formation of the $[M-337]^+$ ion, seen in the FAB spectra of Chl *b* and its allomers: $[M^{\cdot+}-C_{20}H_{38}-\cdot CH_2CO_2H]^+$ or $[M^{\cdot+}-C_{20}H_{38}-\cdot CO_2CH_3]^+$. As the $[M-337]^+$ ion has also appeared in the FAB spectrum of 13^2-demethoxycarbonyl-pheophytin *b* (pyropheophytin *b*) [24], the $[M-337]^+$ ion is probably



Spectrum	Compound	Peak positions (nm)						Ratio*
		Soret	I	II	III	IV	V	A_S/A_I
a	15^1-MeO-lact-Chl *b* epimer II	442	630	583		(538)	418	4.45
b	15^1-MeO-lact-Chl *b* epimer I	441	631	584		537	418	4.37
c	13^2-MeO-Chl *b* epimer I	451	641	594	(567)	(545)	428	2.89
d	13^2-MeO-Chl *b* epimer II	452	642	595	(567)	(545)	428	2.73
e	13^2-HO-Chl *b*	451	641	593	(567)	(545)	427	2.98
f	13^2(*S*)-HO-10-MeO-Chl *b*	455	633	585		537	(434)	4.06
g	15-Glyox-Chl *b*	447	639	(590)		535	(419)	6.24
	Chl *b* [30]	453	642	593	567		430	2.88

* Absorbance at Soret band divided by the absorbance at band I.

Fig. 3. UV–Vis spectra of the purified and dehydrated Chl *b* allomers recorded in dry diethyl ether.

(mainly) formed by the partial fragmentation of the 17-side-chain.

The $[M-321]^+$, ion now detected to form from Chl *b*, has not been reported in the earlier investigations of the mass spectrum of Chl *b* [20,21,33–35]. In this work, the $[M-321]^+$ ion was seen also in the FAB spectra of 15-Glyox-Chl *b,* the 15^1-MeO-lact-Chl *b* epimers and the 13^2-MeO-Chl *b* epimers. The residue to be cleaved is 43 a.m.u. after the loss of phytadiene. In the collisional activation mass spectrum of the $M^{\cdot+}$ from Chl *a*, the $[M-43]^-$ peak has been assigned to $[M-CO-\cdot CH_3]^-$ by Grese et al. [22], but they do not explain in further detail this fragmentation. It is noteworthy that the cleavage of CO is also mentioned in the structural assignment of the $[M-365]^+$ ion by Brown and Wilkins [34] and in our structural assignment of the $[M-364]^+$ ion (Table 2). It is also possible that the [M−321] fragmentation consists of a loss of phytadiene and a methyl radical and ethylene [36].

The FAB spectrum of 13^2(*S*)-HO-10-MeO-Chl *b* exhibited a clear signal corresponding to the $[M-353]^+$ ion. This fragmentation has been seen earlier also in the FAB spectra of Chl *a* and Chl *b* [35]. We have previously proposed that the loss of phytadiene and $\cdot CH_2COOH$ or $\cdot CO_2CH_3$ and the formation of a double bond between carbons 17 and 18, concomitant to the loss of ·H and $\cdot CH_3$, may explain the formation of the $[M-353]^+$ ion in the FAB spectra of 13^2(*R*)- and 13^2(*S*)-HO-Chl *a* [23]. This is a probable explanation also for the formation of this ion seen in the FAB spectrum of 13^2(*S*)-HO-10-MeO-Chl *b.*

2.5. Comparison between the allomerizations of chlorophylls a and b

The allomerization reaction of Chl *b*, described in this work, was performed under conditions analogous to those used in the allomerization of Chl *a* [5],

Table 1
The most important ions in the positive FAB mass spectra of Chl *b* and its allomers in 3-NBA matrix

Relationship to molecular ion	Chl *b*	15-Glyox-Chl *b*	15^1-MeO-lact-Chl *b*		13^2-MeO-Chl *b*		13^2-HO-Chl *b*	13^2(*S*)-HO-10-MeO-Chl *b*
			Epimer I	Epimer II	Epimer I	Epimer II		
M+23					959 (29)	959 (21)	945 (13)	975 (6)
M+1	907 (20)[a]	953 (100)	953 (39)	953 (26)	937 (34)	937 (56)	923 (37)	953 (37)
M+0	906 (25)	952 (92)	952 (36)	952 (23)	936 (45)	936 (66)	922 (38)	952 (27)
M−31					905 (3)	905 (9)		
M−59		893 (3)	893 (1)	893 (6)				
M−87		865 (4)						
M−255					681 (24)	681 (22)		
M−277	629 (53)	675 (92)	675 (25)	675 (11)	659 (24)	659 (39)	645 (62)	675 (58)
M−278	628 (100)	674 (88)	674 (22)	674 (7)	658 (24)	658 (34)	644 (100)	674 (76)
M−291	615 (21)							
M−293		659 (13)	659 (9)	659 (7)	643 (30)	643 (20)		
M−295							627 (75)	657 (90)
M−309		643 (27)	643 (21)	643 (18)	627 (76)	627 (62)		
M−321	585 (15)	631 (15)	631 (22)	631 (20)	615 (41)	615 (20)		
M−323					613 (23)	613 (22)		629 (29)
M−337	569 (28)	615 (88)	615 (100)	615 (100)	599 (100)	599 (100)	585 (92)	615 (100)
M−351	555 (11)	601 (30)	601 (15)	601 (18)	585 (45)	585 (25)		
M−353								599 (39)
M−364		588 (76)	588 (21)	588 (16)				
M−365	541 (16)			587 (34)			557 (44)	587 (34)
M−367					569 (38)	569 (23)		
M−369						567 (24)	553 (42)	583 (35)
M−381		571 (31)	571 (18)					
M−382				570 (19)				
M−383					553 (49)	553 (40)	539 (93)	569 (45)
M−395	511 (17)		557 (21)	557 (18)				
M−397		555 (36)			539 (63)	539 (39)	525 (63)	555 (42)
M−399								553 (50)
M−410					526 (56)	526 (40)		
M−411	495 (51)	541 (56)	541 (28)	541 (34)			511 (87)	541 (56)

[a] The relative abundances are given in parenthesis.

which enables one to compare the allomerizations of the two chlorophylls. We want to emphasize that we used methanol of the same purity for both allomerization reactions, because there are variations in base and/or metal-ion impurities in various commercial methanols [4].

According to the HPLC analysis, the principal allomerization products of Chl *b* in dry methanol were the 15^1-MeO-lact-Chl *b* epimers (46%, the epimer I/epimer II ratio 65:35) and 15-Glyox-Chl *b* (36%). Also the 13^2-MeO-Chl *b* epimers (7%, the epimer I/epimer II ratio 57:43) and 13^2(*S*)-HO-10-MeO-Chl *b* (8%) were formed (Fig. 2). Small amounts of other compounds, including 13^2-HO-Chl *b* ($\ll 0.5\%$), were detected when high concentrations of allomerized sample were injected into the HPLC column.

The composition of the allomer mixture produced by Chl *a* [15-Glyox-Chl *a* (7%), 15^1-MeO-lact-Chl *a* (52%), 13^2-HO-Chl *a* (18%) and 13^2-MeO-Chl *a* (21%) [5]] clearly differs from that produced by Chl *b*. The 15^1-MeO-lact-Chl derivatives were the main allomers in both cases, but a clearly higher amount of the 15-Glyox-Chl derivative was formed in the allomerization of Chl *b* than in that of Chl *a*. Another notable difference was that only a very small amount of 13^2-HO-Chl *b* was detected to form from Chl *b*, whereas a significant amount of 13^2-HO-

Table 2
Structural assignments for the ion species in the FAB mass spectra of Chl *b* and its allomers

Relationship to molecular ion	Structural assignment
M+23	$[M+Na]^+$
M+1	$[M+H]^+$
M+0	$M^{+\cdot}$
M−31	$[M-{}^{\cdot}OCH_3]^+$
M−59	$[M-{}^{\cdot}CO_2CH_3]^+$
M−87	$[M-{}^{\cdot}COCO_2CH_3]^+$
M−255	$[M-C_{20}H_{38}+Na]^+$
M−277	$[M-C_{20}H_{38}+H]^+$
M−278	$[M-C_{20}H_{38}]^+$
M−291	$[M-C_{20}H_{38}-13]^+$
M−293	$[M-C_{20}H_{38}-{}^{\cdot}CH_3]^+$
M−295	$[M-C_{20}H_{38}-{}^{\cdot}OH]^+$
M−309	$[M-C_{20}H_{38}-{}^{\cdot}OCH_3]^+$
M−321	$[M-C_{20}H_{38}-{}^{\cdot}CH_3-CO]^+$ or $[M-C_{20}H_{38}-{}^{\cdot}CH_3-CH_2=CH_2]^+$
M−323	$[M-C_{20}H_{38}-{}^{\cdot}COOH]^+$
M−337	$[M-C_{20}H_{38}-{}^{\cdot}CH_2CO_2H]^+$ or $[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3]^+$
M−351	$[M-C_{20}H_{38}-{}^{\cdot}CH_2CH_2CO_2H]^+$
M−353	$[M-C_{20}H_{38}-{}^{\cdot}CH_2CO_2H-{}^{\cdot}CH_3-{}^{\cdot}H]^+$ or $[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CH_3-{}^{\cdot}H]^+$
M−364	$[M-C_{20}H_{38}-{}^{\cdot}CH_2CO_2H-CO+H]^+$ or $[M-C_{20}H_{38}-{}^{\cdot}COCO_2CH_3+H]^+$
M−365	$[M-C_{20}H_{38}-{}^{\cdot}CH_2CO_2H-CO]^+$
M−367	$[M-C_{20}H_{38}-{}^{\cdot}OCH_3-{}^{\cdot}CO_2CH_3+H]^+$ or $[M-C_{20}H_{38}-{}^{\cdot}OCH_3-{}^{\cdot}CH_2CO_2H+H]^+$
M−369	$[M-C_{20}H_{38}-{}^{\cdot}CH_2CO_2H-{}^{\cdot}OCH_3-{}^{\cdot}H]^+$ or $[M-C_{20}H_{38}-{}^{\cdot}CH_2CO_2H-{}^{\cdot}OH-{}^{\cdot}CH_3]^+$
M−381	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CO_2H+H]^+$
M−382	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CO_2H]^+$
M−383	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CO_2H-{}^{\cdot}H]^+$
M−395	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CH_2CO_2H+H]^+$
M−397	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CH_2CO_2H-{}^{\cdot}H]^+$
M−399	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CO_2H-{}^{\cdot}OH]^+$
M−410	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CH_2CH_2CO_2H]^+$
M−411	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CH_2CO_2H-{}^{\cdot}CH_3]^+$
M−411−n·14	$[M-C_{20}H_{38}-{}^{\cdot}CO_2CH_3-{}^{\cdot}CH_2CO_2H-{}^{\cdot}CH_3-C_nH_{2n}]^+$

Chl *a* was formed from Chl *a*. A third clear difference between the two allomerizations was the formation of an entirely new Chl derivative, $13^2(S)$-HO-10-MeO-Chl *b*, whose analogue has never been observed in the *a*-series. The UV–Vis data (Fig. 3) support the conclusion that 13^2-HO-10-MeO-Chl *b* was formed also in the allomerization reaction carried out by Minguez-Mosquera et al. [7], although they have identified this compound erroneously as a lactone.

Acknowledgements

Financial support from the Academy of Finland and the Finnish Cultural Foundation is gratefully acknowledged.

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