Tetrahedron Vol 40, No. 4, pp. 659 to 664, 1984
Printed in Great Britain

0040-4020/84 $3.00 + .00
Pergamon Press Ltd.

CHLOROPLAST BIOGENESIS 45:

MOLECULAR STRUCTURES OF PROTOCHLOROPHYLLIDE (E443 F625) AND OF CHLOROPHYLLIDE A (E458 F674)

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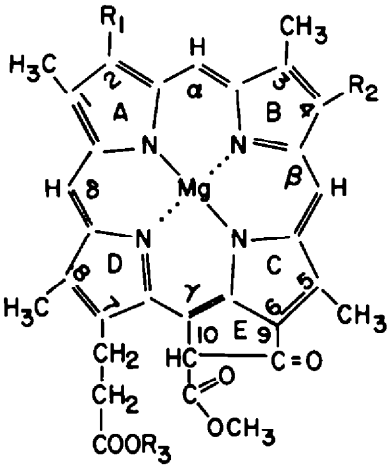
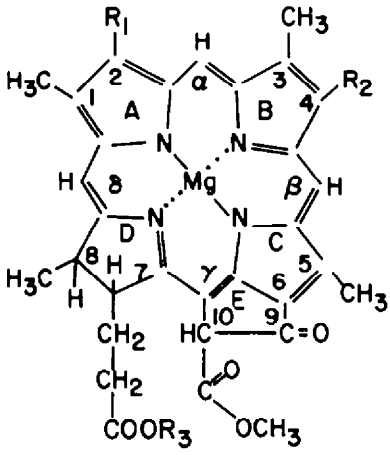
(*Received in U.S.A.* 12 *September* 1983)

Abstract - The divinyl structure of protochlorophyllide (E443 F625) and of its chlorophyllide a (E458 F674) photoreduction product was ascertained by nuclear magnetic resonance spectroscopy and by fast atom bombardment mass spectroscopy. These two phorbins are two newly discovered intermediates of the chlorophyll a biosynthetic pathway in higher plants. Both tetrapyrroles exhibited recognizable ABX spin-spin splitting patterns which are characteristic of divinyl tetrapyrroles. Fast atom bombardment mass spectroscopic data further confirmed the presence of two vinyl groups per molecule of protochlorophyllide (E443 F625) and of chlorophyllide a (E458 F674).

It has recently been demonstrated that, contrary to previous beliefs, most of the 2-MV chlorophyll a (Chl a) of green plants is formed via a divinyl (DV) biosynthetic route, i.e. via DV protochlorophyllide (Pchlide) (E443 F625) (1b, Fig. 1) and via DV chlorophyllide a (Chlide a) (E458 F674) (2c, Fig. 1) (1, 2). In this context, E and F refer to the Soret excitation and fluorescence emission maxima of the two tetrapyrroles, in ether at 77°K. DV Chlide a (E458 F674) is then reduced to 2-MV Chlide a (E447 F674) (2b, Fig. 1) (2) which in turn is converted to 2-MV Chl a (2a, Fig. 1) by esterification of the propionic acid residue at position 7 of the macrocycle. The preliminary identification of Pchlide (E443 F625) and of Chlide a (E458 F674) as DV tetrapyrroles, i.e. with vinyl groups at position 2 and 4 of the phorbin macrocycle (Fig. 1), was recently reported (3). Etiolated cucumber cotyledons were first induced to accumulate massive amounts of Pchlide (E443 F625) which was then photoconverted to Chlide a (E458 F674) by a 2.5 ms actinic light pulse. The presence of two vinyl groups in the newly formed Chlide a (E458 F674) was then demonstrated by catalytic hydrogenation and by primary chemical derivatization coupled to spectrofluorometric analysis of the reaction products (3). Finally DV Chlide a (E458 F674) was converted back to DV Pchlide (E443 F625) by chemical oxidation (3). Because of the very important biosynthetic implications of the ubiquitous occurence of DV Pchlide and of DV Chlide a in green(ing) plant tissues (1), it is very desirable to further substantiate the DV nature of these two important phorbin intermediates by some independent techniques, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS). In what follows, we describe the vinyl proton resonances of Pchlide (E443 F625) and of Chlide a (E458 F674), and deacribe the fast atom bombardment mass spectroscopic profiles of these two phorbins. Altogether, the chemical derivatization results reported in (3) and the NMR and MS data reported in this paper ascertain unambiguously the DV chemical nature of



1 Protochlorophyllide Derivatives



2 Chlorophyllide Derivatives

	R_1	R_2	R_3	Compound
1a	$CH=CH_2$	CH_2-CH_3	H	MV Pchlide
1b	$CH=CH_2$	$CH=CH_2$	H	DV Pchlide
2a	$CH=CH_2$	CH_2-CH_3	$C_{20}H_{39}$	MV Chl a
2b	$CH=CH_2$	CH_2-CH_3	H	MV Chlide a
2c	$CH=CH_2$	$CH=CH_2$	H	DV Chlide a

Fig. 1. Chemical structures of some relevant protochlorophyllides (Pchlides) and chlorophyllides (Chlides).

Pchlide (E443 F625) and of Chlide a (E458 F674).

RESULTS AND DISCUSSION

NMR Analysis of Pchlide (E443 F625) and of Chlide a (E458 F674).

Vinyl groups at position 2 of various tetrapyrroles usually exhibit an easily recognizable ABX spin-spin splitting pattern (4). The possible presence of a second vinyl group at position 4 of the macrocycle is likely to complicate this ABX pattern however. For example, in highly assymetrical phorbins such as DV Pchlide and DV Chlide a two adjacent sets of ABX splitting profiles may be expected, one for the 2nd position and one for the 4th position of the macrocycle, as was observed for chlorophyll c_2 (5) and for 2,4-DV Chl a (6).

One of the characteristic features of tetrapyrroles consists in the downfield appearance of meso-proton singlets (4). Furthermore it is well established that the meso-proton resonances in porphyrins experience (a) a stronger ring current than in chlorins, in which the 7-8 double bond has been reduced (Fig. 1, 1 vs 2), (b) are therefore more deshielded and (c) are observed at lower fields than in chlorins. The same phenomenon was also observed in this work, with the meso-proton singlets of Pchlide (E443 F625) being observed at lower fields than in Chlide a (E458 F674) (Table I). The order of the β, α, δ resonance assignment was by analogy to that of Chl a (7). As expected the γ-meso proton signal is missing from these profiles, since in phorbins the γ-position is part of ring E (Fig. 1). In the 8.20 - 8.30 ppm region, the NMR profiles of Pchlide (E443 F625) were characterized by two sets of doublet of doublets, instead of the single set of doublet of doublets usually observed in MV tetrapyrroles (4). From an analysis of the ABX splitting pattern (Fig. 2 A) and an evaluation of the spin-spin coupling constants (J) of Pchlide (E443 F625) (Table I) this particular profile is best rationalized by the

Table 1. Chemical shifts and coupling constants of the meso and vinyl protons of methyl Pchlide (E443 F625) and methyl Chlide a (E458 F674). The chemical shifts (δ, ppm) were reported downfield from TMS. The pigments were dissolved in $CDCl_3$ containing 2% CD_3OD by weight. The data for Chl a is from ref. 7.

Proton	Pchlide (E443 F625) Methyl ester (7.9×10^{-4} M)	Chlide a (E458 F674) Methyl ester (3.6×10^{-4} M)	2-MV Chl a (5×10^{-2} M in acetone - d_6)
		δ (ppm)	
β-H	10.19 (s)	9.80 (s)	9.57 (s)
α-H	10.01 (s)	9.43 (s)	9.27 (s)
δ-H	9.84 (s)	8.39 (s)	8.40 (s)
$4\text{-}H_X$	8.25(dd; J_{AX}=11.2; J_{BX}=18.0)		--
$2\text{-}H_X$	8.23(dd; J_{AX}=11.8; J_{BX}=18.1)	8.01 (m)	8.04(dd;J_{AX}=12.0; J_{BX}=18.0)
$4\text{-}H_B$	6.35(d; J_{BX}=18.0)*	6.22(dd;J_{AB}=1.47; J_{BX}=17.8)	--
$2\text{-}H_B$	6.33(d; J_{BX}=18.1)*	6.21(dd;J_{AB}=1.47; J_{BX}=17.6)	6.14(dd;J_{AB}=1.0; J_{BX}=18.0)
$4\text{-}H_A$	6.14(d;J_{AX}=11.2)*	6.04(dd;J_{AB}=1.47; J_{AX}=11.5)	--
$2\text{-}H_A$	6.12(d;J_{AX}=11.8)*	6.01(dd;J_{AB}=1.47; J_{AX}=11.7)	5.92(dd;J_{AB}=1.0; J_{AX}=12.0)

*At 11% of CD_3OD, geminal AB spin-spin splitting was observed and the following coupling constants were calculated: 4 - J_{AB} = 1.83 and 2 - J_{AB} = 1.52

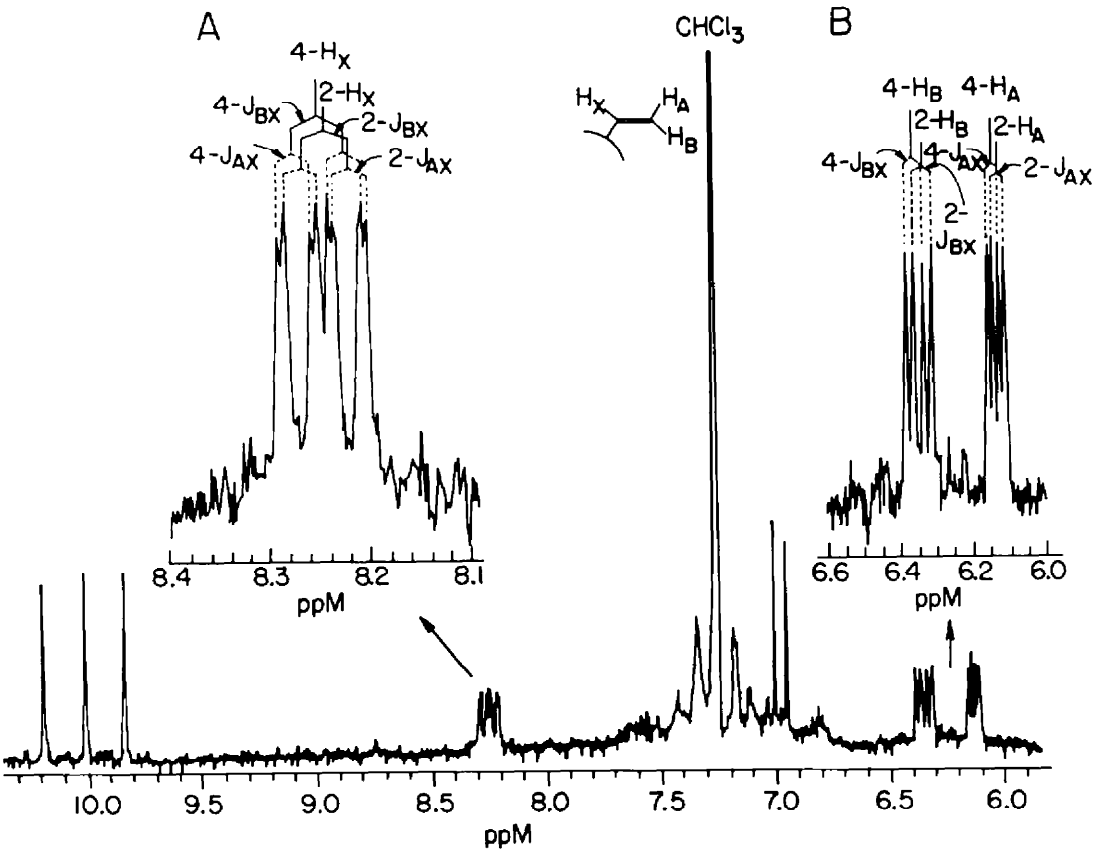


Fig. 2. NMR profile of the vinyl protons of Pchlide (E443 F625). The symmetrical signals centered on $CHCl_3$ (7.26 ppm) are spin sidebands. The two inserts represent the expanded regions of the Hx protons and the geminal H_A, H_B protons.

presence of two groups of X-vinyl protons i.e. 4-H_x and 2-H_x, per Pchlide molecule, instead of only one group of X-vinyl protons, either at position 2 or at position 4 of the macrocycles, of an isometric mixture of two compounds (Fig. 2 A, Table 1). This assignment is further substantiated by a 1:1:1:2 ratio of integration of the three meso protons to the H_x vinylic protons as well as by the absence of an ethyl resonance in the higher field region. It is furthermore justified by the observation that the Pchlide (E443 F625) and the Chlide a (E458 F674) used in this work were free of 2-vinyl, 4-ethyl and of 4-vinyl, 2-ethyl contaminants (3). Since the putative 4-vinyl protons of Pchlide (E443 F625) are closer to the electron-withdrawing groups of ring E (1b, Fig. 1), they are more deshielded than the vinyl protons at position 2 of the macrocycle, and are therefore more likely to resonate at lower fields, as depicted in Fig. 2A and in Table 1. A similar multiplet profile centered at 8.01 ppm was also observed for the X-vinyl protons of Chlide a (E458 F674) (Table 1). The center of this multiplet occurred at a slightly higher field than in Pchlide (E443 F625), probably due to the weaker ring current experienced by the Chlide a protons as a result of the C_7-C_8 bond reduction in this chlorin (2c, Fig. 1). The extent of overlap of the 4-H_x and 2-H_x signals in Chlide a (E458 F674) made it difficult to analyze in details the various proton resonances as was done for the 2- and 4-H_x signals of Pchlide (E443 F625).

In the 6.10 - 6.40 ppm region each one of the geminal vinyl protons (AB part of the vinyl resonances, Fig. 2B) of Pchlide (E443 F625) exhibited two well separated sets of doublet of doublets, instead of the one set of doublet of doublets, which is usually observed in MV tetrapyrroles (4) (Fig. 2B, Table 1). The lower field doublet of doublets are assigned to the two H_B protons which are trans-coupled with the H_x protons, while the higher field doublet of doublets are assigned to the two H_A protons which are cis-coupled to the $H_x$protons (Table 1). The geminal coupling constants (J_{AB}) were not resolved at the CD_3OD concentrations (2% by weight) used in these experiments. However when the concentration of CD_3OD was raised to 11% by weight, geminal coupling constants of 1.83 Hz (4-J_{AB}) and 1.52 Hz (2-J_{AB}) were observed (Table 1). Here again the ratio of integration of the meso protons to each of the geminal AB protons amounted to 1:2. This in turn further confirmed the presence of two vinyl groups, one at position 2 and the other at position 4 of the Pchlide (E443 F625) macrocycle. In this case too, since the 4-vinyl protons are closer to the electron-withdrawing groups of ring E (1b, Fig. 1) they are more deshielded than the 2-vinyl protons and their resonances were therefore observed at lower fields, as depicted in Fig. 2B and in Table 1.

In Chlide a (E458 F674), the two sets of geminal vinyl protons were each well resolved into two adjacent sets of doublet of doublets, a typical ABX spin system (Table 1). Finally the proton chemical shifts of Chlide a (E458 F674) were invariably observed at slightly lower fields than for 2-MV Chl a (4, 7, Table 1), most likely as a result of the stronger ring current generated by Chlide a (E458 F674). This is also consistent with the presence of two vinyl groups instead of one vinyl group in Chlide a (E458 F674).

Altogether, the foregoing NMR results strongly indicated that Pchlide (E443 F625) and Chlide a (E458 F674) were 4-vinyl-4-desethyl phorbins.

Mass Spectroscopic Analysis of Demetalated Pchlide (E443 F625) and of Demetalated Chlide a (E458 F674).

Further confirmation of the molecular structure of Pchlide (E443 F625) and of Chlide a (E458 F674) was derived from FAB mass spectroscopic analysis, after their respective conversion to methyl pheoporphyrin a_5 and methyl pheophorbide a following esterification and demetalation.

The mass spectrum of demetalated Chlide a (E458 F674) methyl ester (2c - Mg, R_3 = CH_3) exhibited molecular ions at m/z 605 ($MH^{+\cdot}$) and 604 ($M^{+\cdot}$) (Fig. 3A). The latter was fully consistent with the calculated 604 molecular weight (MW) of DV pheophorbide a methyl ester ($C_{36}H_{36}N_4O_5$). The lability of the peripheral substituents at C_{10} (ring E) and at C_7 (ring D) was rather obvious in this chlorin. The fragmentation pattern of the protonated free base was assigned as follows: at m/z 545 from the loss of $HCOOCH_3$, 519 from the loss of

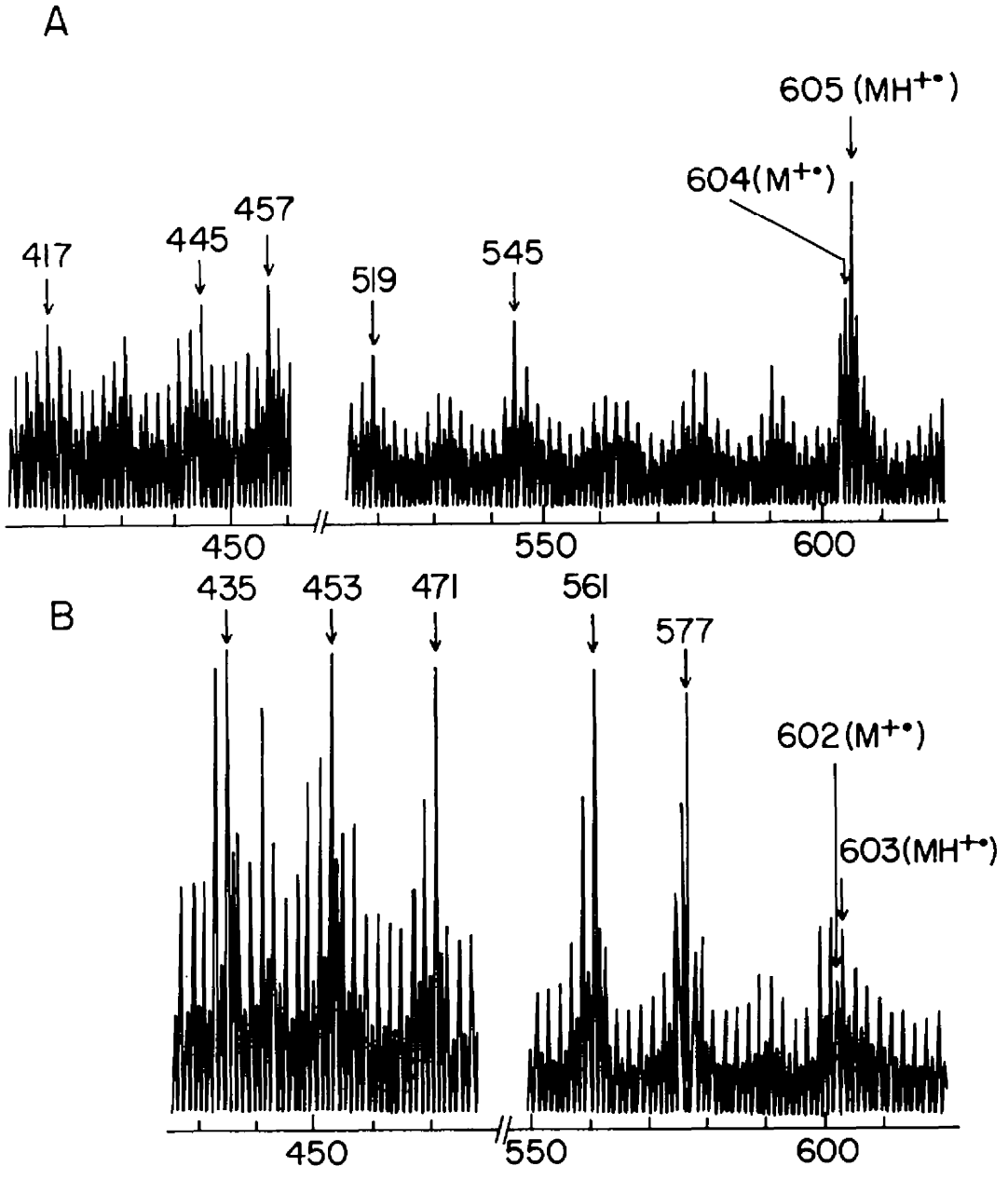


Fig. 3. Positive ion regions of the FAB mass spectra of (A) demetalated Chlide a (E458 F674) methyl ester and (B) demetalated Pchlide (E443 F625) methyl ester.

$CH_2CH_2COOCH_3$ and the acquisition of a proton, 457 from the loss of $HCOOCH_3$ plus $CH_2CH_2COOCH_3$ plus H, 445 from the loss of $C_4H_4O_3$ (ring E) plus $HCOOCH_3$ and 417 from the loss of $C_4H_4O_3$ plus $CH_2CH_2COOCH_3$ plus H.

Demetalated Pchilde (E443 F625) methyl ester (1b - Mg, R_3 = CH_3) exhibited molecular ions at m/z 603 ($MH^{+\bullet}$) and at 602 ($M^{+\bullet}$) (Fig. 3B) the latter was consistent with the calculated 602 MW of DV pheoporphyrin a5 methyl ester ($C_{36}H_{34}N_4O_5$). This also indicated that this compound differed from DV pheophorbide a only by the presence of an additional double bond, probably at the C_7-C_8 positions. The FAB mass spectrum of this tetrapyrrole also exhibited an interesting

fragmentation pattern (Fig. 3B). Two fragments were observed at m/z 577 and 561, which were tentatively attributed to the loss of an acetylene group and a ketene group respectively from the protonated free base. The largest fragment peaks occurred at m/z 471, 453 and 435 and could have been produced by the loss of ring E ($C_4H_4O_3$) plus CH_3OH (471) followed by the elimination of one (453) and two (435) water molecules respectively. There was no evidence for the loss of either the carbomethoxy group at C_{10} or the methyl propionate group at C_7.

Altogether the NMR and MS data concurred in ascertaining the divinyl nature of Pchilde (E443 F625) and of its photoproduct, i.e. Chlide a (E458 F674).

EXPERIMENTAL

Cucumber seeds, Cucumis sativus L. cv. Beit Alpha were germinated in moistened vermiculite, in the dark for 4 days (8). Pchlide (E443 F625) accumulation was induced by subjecting 5 g batches of excised, hookless, cotyledons to three successive light (2.5 ms) - dark (60 min) cycles at room temperature (9). The accumulated Pchlide (E443 F625) was then converted to Chlide a (E458 F674) by a fourth 2.5 ms light pulse (9); in that case the tissue was immediately frozen in liquid N_2 in order to prevent further conversion of Chlide a (E458 F674) to 2-MV Chlide a (E447 F674) (2). About 200 grams of cotyledons either enriched in Pchlide (E443 F625) or in Chlide a (E458 F674) were extracted, thirty g at a time in 200 ml of acetone: 0.1 N NH_4OH (9:1 v/v). The fully esterified tetrapyrroles were extracted into hexane, while the mono- and dicarboxylic tetrapyrrole pools remained in the hexane-extracted acetone fraction and were extraced in ether (3). The ether extracts containing either Pchlide (E443 F625) or Chlide a (E458 F674) were methylated, for 20 min in an ice bath, with an excess of freshly prepared diazomethane (3). The ether extracts were then concentrated under N_2 gas and the methylated pigments were purified on thin layers of silica gel H, developed in toluene: ethyl acetate: ethanol (8:2:2 v/v/v) at 4°C (3). The segregated pigments were eluted in ether (3), dried under N_2 gas and were stored in liquid N_2 until enough material was prepared for NMR analysis. Prior to NMR analysis the methylated pigments were repurified on thin layers of silica gel H as just described. The ^1H NMR spectra were recorded on a 360 MHz Nicolet NT-360 FT NMR spectrometer. The pigments were dissolved in $CDCl_3$ containing about 2% CD_3OD by weight to a concentration of about 10^{-3} to 10^{-4} M. The recording was usually terminated after 200 to 2000 pulses and a 0.2 Hz line broadening function was applied to each free induction decay prior to transformation. All chemical shifts were with reference to an internal standard of tetramethylsilane (TMS). The FAB mass spectra were recorded on a VG ZAB-1F mass spectrometer. Mg-free pigments were prepared by acidification of diethyl ether solutions of the methylated pigments with 2 N HCl. The reaction mixtures were then neutralized with sodium bicarbonate and washed with water until neutral. The resulting ethereal solutions were concentrated under nitrogen gas and purified by chromatography on thin layers of silica gel H, developed in toluene: ethyl acetate: ethanol (8:2:2 v/v/v) at 4°C. The purified samples were dissolved in thioglycerol and gave satisfactory positive ion spectra.

Acknowledgements:

This work was supported by NSF grants PCM 7811559, PCM 83-07660 and by funds from the Illinois Agricultural Experiment Station to C. A. Rebeiz. The NMR spectra were recorded at the NSF Regional Instrumentation Facility at the University of Illinois at Urbana, and was supported in part by NSF grant CHE 79-16100 to the University of Illinois. The FAB spectra were recorded in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois and was supported in part by grant GM 27029 from the National Institute of General Medical Sciences.

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