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STRUCTURE AND CONFORMATION OF PHOTOSYNTHETIC PIGMENTS AND RELATED COMPOUNDS. 13. IDENTIFICATION OF LOCALIZED VIBRATIONAL MODES IN CHLOROPHYLL A DERIVATIVES

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Abstract – A Resonance Raman investigation of chlorophyll a and meso-chlorinated and ring V hydroxylated derivatives and pheophytins reveals localized vibrational modes in the chlorophyll series. Both chlorination and hydroxylation give rise to changes in both band intensity and frequency throughout the spectrum. Chlorination affects mainly the core sensitive bands with C_aC_m and C_aN character. Hydroxylation leads to changes in the carbonyl and ring V associated bands. Small, but real spectral differences were found between the 10-epimers.

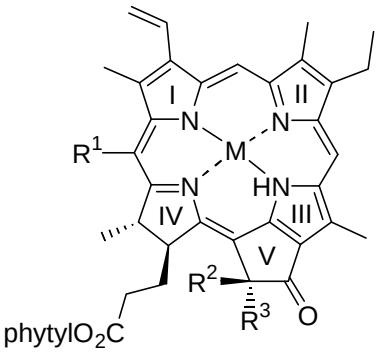
INTRODUCTION

Chlorophylls are the main accessory and reaction center pigments in the photosynthetic apparatus of Higher Plants and algae. Due to their fundamental relevance these porphyrin-based heterocyclic pigments have been studied in detail.[1] A fundamental question relates to the conformational flexibility of these pigments, i.e. whether and to what extend the tetrapyrrole macrocycle is flexible and can occur in different conformations.[2] Next to static X-ray crystallography,[3] vibrational spectroscopic techniques such as resonance Raman (RR) are crucial techniques for an investigation of their solution conformation and for the elucidation of their *in vivo* function.[4] More specific studies have addressed the metal and axial ligand

† Dedicated to the memory of the late Professors Terese M. Cotton and Robert A. Uphaus.

coordination in chlorophyll a (Chl a),[5] normal mode characteristics in synthetic chlorins,[6] liquid crystals,[7] metallochlorins,[8,9,10] amongst others.

While most of these studies have used either unaltered Chl's or synthetic analogues, not much attention has been given to chemically, slightly altered Chl deriviatives. The only exception is the study of the chlorophyll allomerization reaction, which provides useful information on Chl alteration and structural parameters.[11] Here we provide data on meso-chlorinated Chl's, some allomerization derivatives and the related free base pheophytins in order to elucidate any localized vibrational modes suitable for conformational analysis.



M = Mg(II)

Chl a	$R^1 = R^2 = H$, $R^3 = CO_2Me$
10-HO-Chl a	$R^1 = H$, $R^2 = OH$, $R^3 = CO_2Me$
δ-Cl-Chl a	$R^1 = Cl$, $R^2 = H$, $R^3 = CO_2Me$
δ-Cl-10-HO-Chl a	$R^1 = Cl$, $R^2 = OH$, $R^3 = CO_2Me$
Chl a'	$R^1 = R^3 = H$, $R^2 = CO_2Me$
Pheo a	M = 2H

Figure 1. Compounds studied.

In particular, the 10-hydroxylated derivatives are very suitable for RR studies because the absence of the acidic proton at position 10 prevents epimerization which otherwise occurs rapidly in polar organic solvents. These compounds were studied with Soret excitation in dry diethylether (Et_2O) and in tetrahydrofuran (THF) to provide two different solvent systems with distinct coordination at the central magnesium atom, the coordination number being five in ether and six in THF.[12] The chlorophyll epimers (Chl a')[13] and the corresponding pheophytins (Pheo) were prepared and studied for comparison. These derivatives are structurally different variants of the chlorophylls, which can be employed in the elucidation of the vibrational spectra. They are known to play an important role in photosynthesis; two bacteriopheophytins are an integral part of the reaction center of photosynthetic bacteria and they have also been shown to occur in the photosystems of Higher Plants.[14] Both chlorination and hydroxylation give rise to changes in both band intensity and frequency throughout the spectrum. Chlorination effects mainly the core sensitive bands with C_aC_m and C_aN character. Hydroxylation leads to changes in the carbonyl and ring V associated bands. Small, but real spectral differences were found between the 10-epimers. This investigation continues our earlier studies on the conformation and structure of photosynthetic pigments[15] and complements related studies on the mass spectrometric analysis of ring V chlorophyll derivatives.[16]

RESULTS

The structures of Chl a and its derivatives are displayed in Figure 1. The meso substituted chloro-derivatives (δ-Cl-Chl a) is modified (relative to Chl a) at the δ-position and the ring V modifications

are hydroxylation (10-OH-Ch1 a) and epimerization (Ch1 a') at position 10. The absorption spectra (see experimental section) are very similar to that of Ch1 a; however there is a 6 nm bathochromic shift of the Q_y-band of δ-Cl-Ch1 a and a slight shift in the Soret region. The similarity of the absorption spectra indicates that the π-system is not greatly perturbed by the Cl-substituent and that the red shifts may be caused by the inductive effect of the halogen substitution. As expected, the 10-OH-Ch1 a substitution has very little effect on the electronic structure of the macro cyclic ring because this position is out of the π-configuration pathway. Similarly, only small effects are seen for the epimers.

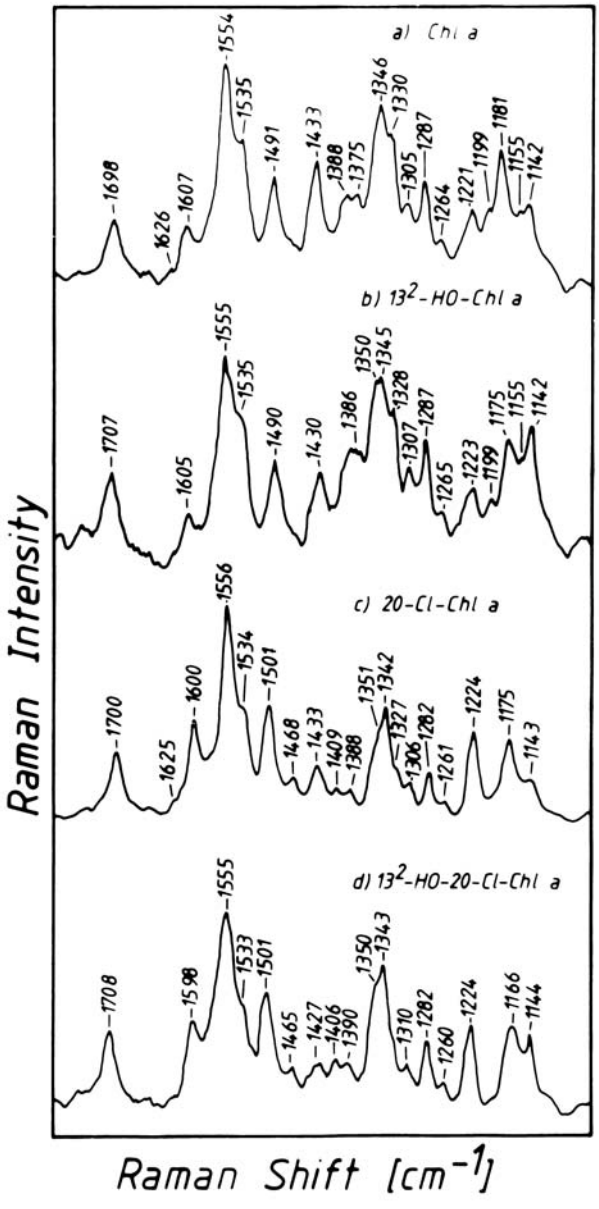


Figure 2. RR spectra of Chl a derivatives in Et_2O with 413.2 nm excitation. Chl concentration was approximately 1mM for all samples. Laser power, 15 mW.

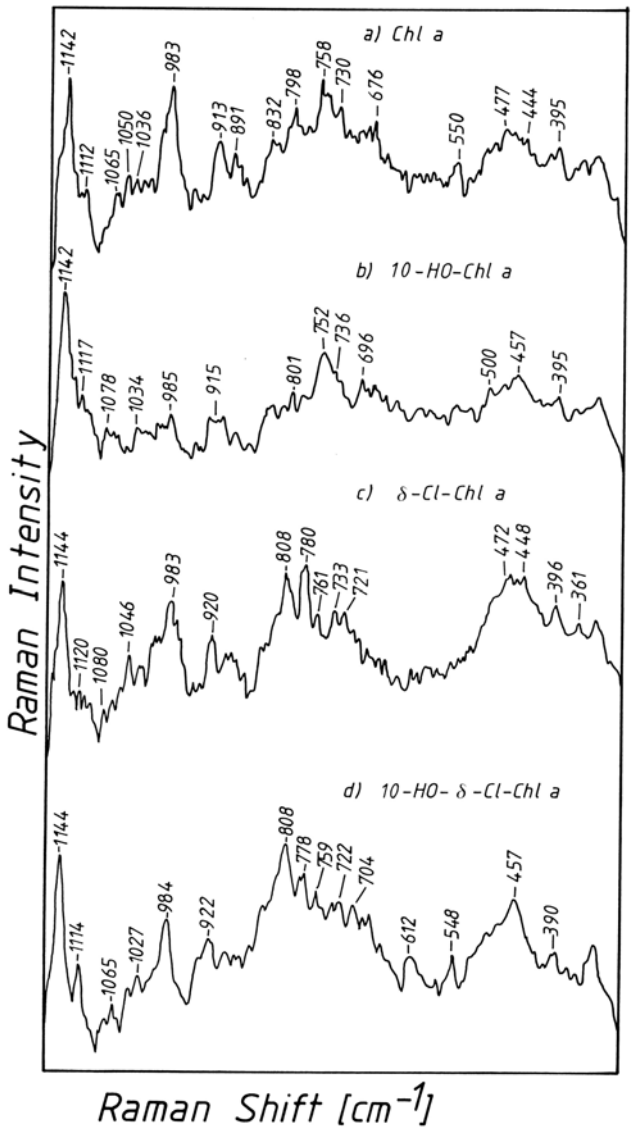


Figure 3. RR spectra (low frequency region) of Chl a derivatives in Et_2O with 413.2 nm excitation. Chl concentration was approximately 1mM for all samples. Laser power, 15 mW.

Resonance Raman Spectra of Hydroxy- and Chloro-Substituted Ch1 *a*

The high frequency Soret excitation spectra of Ch1 a and the 10-OH and δ-Cl-derivatives are displayed in Figure 2 and tabulated in Table 1. Excitation at 413.2 nm affords good quality spectra throughout the high frequency region (1000-1800 cm^{-1}) and reasonable quality spectra in low frequency region (200-1000 cm^{-1}). The high frequency region is characterized by in-plane stretching and bending vibrations and the low frequency portion of the spectrum is interpreted as arising predominantly from in- and out of plane bending vibrations. The high frequency RR spectra of 10-OH and δ-Cl-derivatives look fairly similar to unmodified Ch1 a, which suggests that there are not major perturbation of the chlorine ring structure and that the Ch1 a normal coordinate calculations may also pertain to the chemically modified chlorophyll ring structure.

The bands that shift upon 10-OH substitution (Figure 2a compared to b) are: 1698 → 1707 cm^{-1}, 1433 → 1430 cm^{-1}, 1221 → 1223 cm^{-1} and 1881 → 1175 cm^{-1} (Table 3). The core-size sensitive vibrations at 1607, 1554 and 1491 cm^{-1} remain constant. The effects of δ-Cl-substitution (Figure 2a vs. c) are: 1607 → 1600 cm^{-1}, 1491 → 1501 cm^{-1}, changes in the 1388 and 1375 cm^{-1} bands to two bands at 1409 and 1388 cm^{-1}, 1346 →1342 cm^{-1}, 1287 →1282 cm^{-1},1221 → 1224 cm^{-1}, loss of intensity of the 1199 cm^{-1} band and shift of the 1181 cm^{-1} band to 1175 cm^{-1}. The 1181 cm^{-1} band is similarly affected by 10-OH-substitution. The C_9=O ketone stretching frequency remains fairly constant at 1698-1700 cm^{-1} as does the most intense band in the spectrum at 1555 cm^{-1}.

The high frequency RR spectrum of di-substituted 10-OH-δ-Cl-Ch1 a derivative in Figure 2d is essentially the summation of the individual substitution effects seen in the Figure 3b and c. The 10-OH band shift relative to Ch1 a are observed at 1698 → 1708cm^{-1}, 1433 → 1427 cm^{-1}, 1221 → 1224 cm^{-1} and downshift of the 1881 cm^{-1} band. The δ-Cl individual substituent band shifts are observed at 1607 → 1598 cm^{-1}, 1491 → 1501 cm^{-1}, changes in the 1388-1375 cm^{-1} region, 1346 →1343 cm^{-1}, 1287 → 1282 cm^{-1}, loss of the 1199 cm^{-1} band and the downshift of the 1182 cm^{-1} band to 1166 cm^{-1}. These frequency downshifts also seem to be additive for the 10-OH-δ-Cl- derivative which displays a 1182 to 1166 (delta = 16 cm^{-1}) shift (relative to Ch1 a) compared to a 7 cm^{-1} shift for the individually substituted compounds. The same band shifts are observed with 406.7 nm laser excitation (data not shown).

Figure 4 presents the high frequency RR spectra of Chl a and its derivatives in THF (see also Table 1). In THF the central Mg^{2+} is six coordinate whereas in ether it displays typical five coordinate marker vibration.[12] The differences in the RR spectrum of Chl a between five-coordinate Mg^{2+} in the ether and six coordination are a 5-10 cm^{-1} downshift of the vibrations >1400 cm^{-1}. The ring vibration frequency shifts to 1597, 1547, 1530, 1485 and 1431 cm^{-1} are caused by expansion of hydrophorbin in ring as the central metal moves into the plane of the ring. The C_9=O stretching frequency is sensitive to coordination number as seen by the 7 cm^{-1} downshift. Krawczyk has discussed the sensitivity of this band to both coordination number

and dielectric constant of the environment.[17] The vibrations <1400 cm^{-1} are essentially constant with changes in coordination number with the exception of the shift of the 1286 cm^{-1} band to 1289 cm^{-1}.

Table 1. Comparison of Soret excitation RR spectra of Chl a derivatives with coordination numbers [CN] of five (in ether) and six (in THF) at the central magnesium atom [cm^{-1}].

Chl a		**10-HO-Chl a**		**δ-Cl-Chl a**		**10-HO-δ-Cl-Chl a**	
CN 5	**CN 6**	**CN 5**	**CN 6**	**CN 5**	**CN 6**	**CN 5**	**CN 6**
1698	1696	1707	1701	1700	1701	1708	1704
1626				1625			1626
1607	1597	1605	1595	1600	1590	1598	1590
			1576		1575		1571
1554	1547	1555	1547	1556	1544	1555	1545
1533	1533	1535	1532	1534	1527	1533	1528
1491	1485	1490	1486	1501	1495	1501	1494
				1468	1462	1465	1462
1433	1431	1430	1428	1433	1430	1427	1426
1388	1398		1386	1409	1400	1406	1399
1375	1374	1386	1373	1388	1390	1390	1392
		1350		1351		1350	1350
1345	1342	1345	1343	1342	1342	1343	1343
1332	1327	1328	1327	1327	1328	1327	1328
1305	1309	1307	1307	1306	1311	1310	1310
1287	1289	1287	1289	1282	1284	1282	1286
1264	1265	1265	1264	1261	1261	1260	1260
	1232		1230				
1221	1220	1223	1222	1223	1226	1224	1224
1199	1203	1199	1199				
1182	1180	1175	1175	1175	1174	1166	1168
1155		1155	1163				
1142	1145	1142	1143	1145	1143	1144	1144
1112		1117		1120		1114	
1065		1078		1080		1065	
1050				1046			
1036		1034				1027	
983		985		983		984	
913		915		920		922	
891							
832							
798		801		808		808	
				780		778	
758		752		761		759	
730		736		733			
				721		722	
		696				704	
676							
						612	
550						548	
		500					
477				472			
444		457		448		457	
395		395		396		396	

Hydroxylation of Chl a at the C-10 position results in the following band shifts in THF (Figure 4b): 1696 → 1701 cm^{-1}, 1597 → 1595 cm^{-1}, the appearance of the distinct shoulder at 1576 cm^{-1}, 1431 → 1428 cm^{-1}, 1220 →1222 cm^{-1} and 1180 → 1175 cm^{-1}. The band shifts and magnitudes are similar to those observed

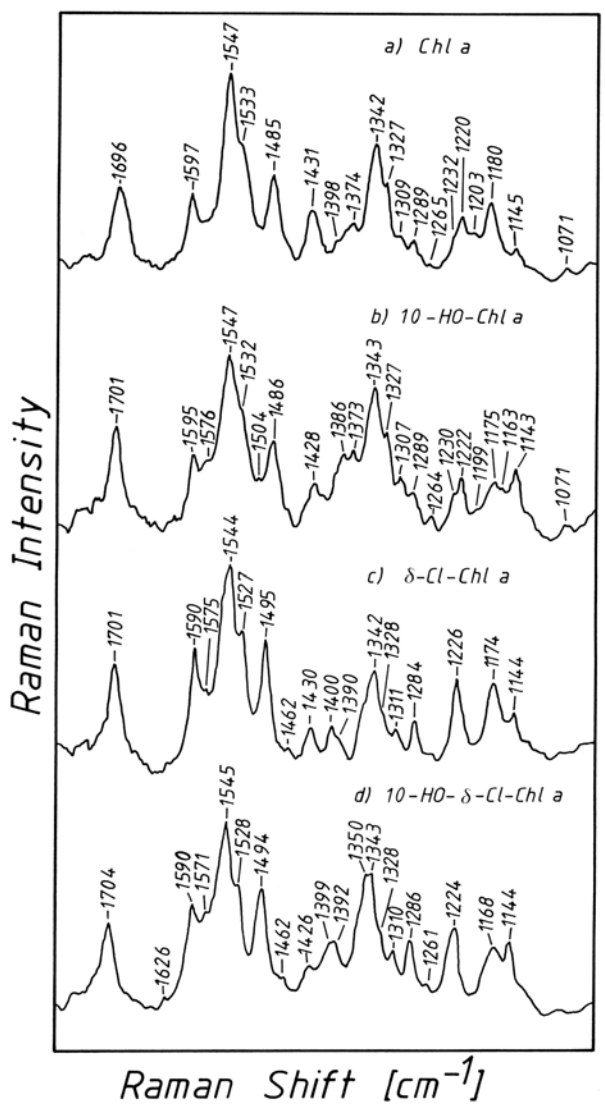


Figure 4. RR spectra of Chl a derivatives in THF with 413.2 nm excitation. Chl concentration was approximately 1mM for all samples. Laser power, 10 mW.

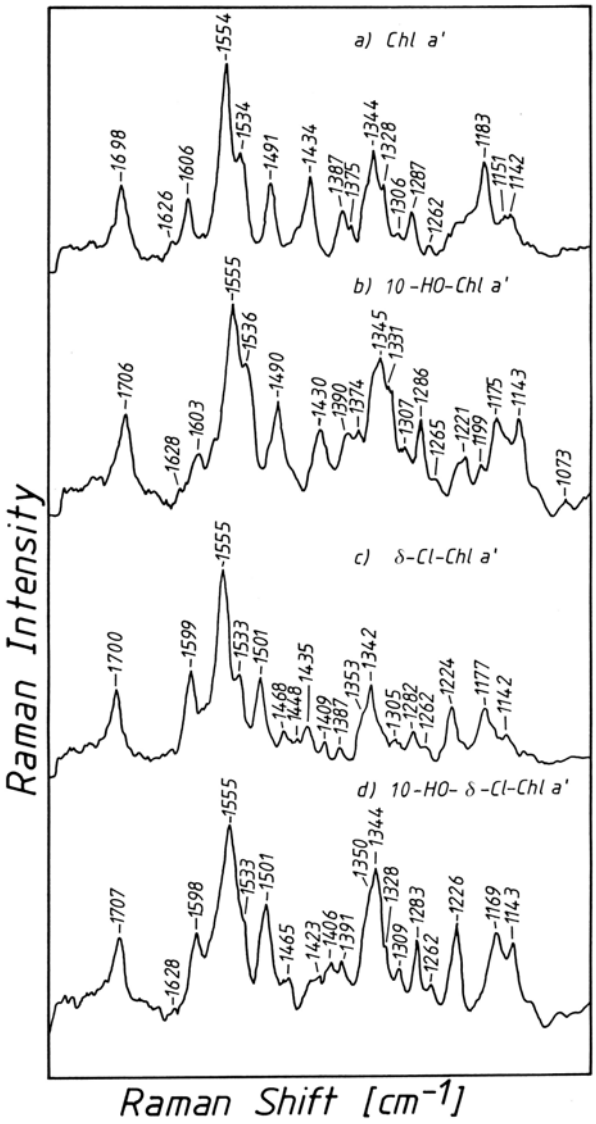


Figure 5. RR spectra of 10S-configurated epimers of Chl a derivatives in Et_2O with 413.2 nm excitation. Chl concentration was approximately 1mM for all samples. Laser power, 15 mW.

in the ether with the minor exceptions of a) a larger shift of the γ (C_9=O) in THF, b) the small (2 cm^{-1}) shift of the 1597 cm^{-1} band in THF and c) the appearance of the band at 1576 cm^{-1}. The additional shifts of two high frequency bands could be caused by an enhanced sensitivity of the macrocyclic ring owing the "strain" induced by six coordination.

Chlorination at the δ-methine position produces the following shifts relative to Chl a in THF (Figure 4c): 1696 → 1701 cm^{-1}, 1597 → 1590 cm^{-1}, the appearance of shoulder at 1575 cm^{-1}, 1547 → 1544 cm^{-1}, a shift of the 1533 cm^{-1}, shoulder to 1527 cm^{-1}, 1485 → 1495 cm^{-1}, changes in the 1400-1375 cm^{-1} region, 1289 →1284, 1220 → 1226 cm^{-1}, disappearance of the 1203 cm^{-1} band and 1180 → 1174 cm^{-1} shift. Again, these shifts, directions and magnitudes are very similar to those observed in the ether with the exceptions of the additional shifts present in the highest frequency bands, the presence of a new shoulder at 1626 cm^{-1} and the

absence of the band shift at 1342 cm^{-1}. The shoulder at 1626 cm^{-1} could be γ (C=C) and it is observed in these samples because of the downshift of the intense 1597 cm^{-1} band. The disubstituted derivative (Figure 4d) displays the additive effects of both 10-HO and δ-Cl substitutions. Table 2 compiles the frequency shifts observed upon hydroxylation and chlorination.

Chemical changes in chlorine periphery have been known to alter the equilibrium constant of ligand binding but the high frequency vibrational shifts observed here for the series of derivatives in one solvent are not consistent with changes in ligation. In particular, the opposing shifts of -8 cm^{-1} and +10 cm^{-1} of the 1597 and 1485 cm^{-1} sore-size sensitive bands, respectively, that typically display parallel shifts, argue against simple changes in ligation state. The additivity of the vibrational shifts implies that the chemical changes in one part oh the chlorophyll molecule do not effect all of the molecular vibrations and two different sets of vibrations are being altered with the ring V hydroxy- and δ-methine chloro-substitutions.

Table 2. Shifts of RR frequencies compared to Chl a upon hydroxylation and chlorination [cm^{-1}]. Excitation wavelength was 413.1 nm and spectra were recorded in diethyl ether.

Band in Chl a	**Assignment from Ref. 6a**	**Shift upon hydroxylation of Chl a**	**Shift upon chlorination of Chl a**	**Shift upon chlorination and hydroxylation of Chl a**	**Shift upon hydroxylation of δ-Cl-Chl a**
1698	CO(V) C_aC_m(γ)	+9	+2	+10	+8
1607	C_aC_m(α,β)	–2	–7	–9	–2
1491	C_aC_m(δ)	–1	+10	+10	0
1433	δCH(V) C_aC_b(I,II,III)	–3	0	–6	(–6)
1388	C_aC_m(δ) C_aC_b(II)	–2		#	#
1375	C_aN(IV) C_aC_b(I,III)	(0)		#	#
1346	C_aN(I,III,IV)	–1	–4	–3	+1
1330	δC_mH(δ) C_aN(I)	–2	–3	–3	–1
1305	C_aN(II,IV)	+2	+1	+5	+4
1287	δC_mH(α,β) C_aN(II,IV)	0	–5	–5	0
1221	δC_mH(δ) δC_bH(IV)	0 +2	–5 +3	–5 +3	0 0
1181	C_mC_{10}(V) δC_aNC_a(I)	–6	–6	–15	–9

Not possible to make assignment with confidence.

Epimers

Soret excitation RR spectra of the epimers of Chl a and its hydroxy- and chloro-substituted derivatives are displayed in Figure 5. Chl a' and δ-Cl-Chl a' were prepared according the method of Hynninen and Lötjonen.[18] Epimerization proceeds via the C-10 acidic proton which deprotonates under basic conditions allowing scrambling of the stereochemistry at the 10-carbon position. Because of this, the epimers of the

10- hydroxy derivatives of Chl a were made by the hydroxylation of previously prepared samples of Chl a'. Therefore, hydroxy derivatives are especially stable for racemization.

The spectra of the series of compounds in Figure 5 display the same overall pattern of shifts seen in Figures 2 and 3. Therefore, epimerization is a minor perturbation on the Ch1 a vibrational structure. However, there are small, but consistent, changes between the corresponding compounds in Figures 3 and 5. The band shifts are tabulated in Table 3. The bands that shifts (most consistently) throughout the series are: a slight downshift of γ C_9=O in the chlorinated derivatives, b) a 1-2 cm^{-1} downshift of the 1600-1610 cm^{-1} band, c) an upshift in the band at 1430 cm^{-1}, d) a shift of the 1220 cm^{-1} band, and e) a 2-3 cm^{-1} upshift of the 1165-1180 cm^{-1} band. These vibrations are the same bands that are affected by hydroxylation, which indicates that the Chl vibrations that are affected by epimerization are those located at ring V.

In this matrix of band shifts there are several of the above vibrations that remain constant (Table 2). In some cases these bands are at the extreme of the observed range, for example, there is no noticeable downshift of the 1598 cm^{-1} band of 10-hydroxy-δ-chloro-Chl a. Also, the spectrum of the 10-OH derivative displays two bands in this group which do not shift at 1430 and 1175 cm^{-1}. The acidic proton of this derivative has already been replaced by a bulky OH-group so a change in stereochemistry at the C-10 position may not induce an additional increase in the ring strain. This may explain why this derivative is least sensitive to epimerization.

Table 3. Shifts of RR frequencies of Chl a upon epimerization at C-10 [cm^{-1}]. Excitation wavelength was 413.1 nm and spectra were recorded in diethyl ether.

Band in Chl a	Assignment from Ref. 6a	Chl a'	10-HO-Chl a'	δ-Cl-Chl a'	10-HO-δ-Cl-Chl a
1698	CO(V) C_aC_m(γ)	0	–1	0	–1
1607	C_aC_m(α,β)	–1	–2	–1	0
1433	δCH(V) C_aC_b(I,II,III)	+1	0	+2	–4
1221	δC_mH(δ) δC_bH(IV)	0	–2	0	–2
1181	C_mC_{10}(V) δC_aNC_a(I)	+2	0	+2	+3

Pheophytins

The high and low frequency RR spectra of hydroxy and chloro-derivatives of Pheo a are presented in Figure 6 and 7. The RR spectrum of Pheo a differs from Chl a by 5-30 cm^{-1} upshift of most of the vibrations in the core-size sensitive region (>1400 cm^{-1}). This is the result of the smaller core size of Pheo a relative to Chl a.[3a] The major bands that shift between Pheo a and 10-OH-Pheo a are; γ C_9=O shifts from 1710-1716 cm^{-1}, the 1307 cm^{-1} band appears to split into two bands at 1310 and 1296 cm^{-1} and the 1130 band shifts down to 1126 cm^{-1}. The difference between Pheo a and δ-Cl- Pheo a are: 1710 → 1715 cm^{-1}, 1498 → 1512 cm^{-1},

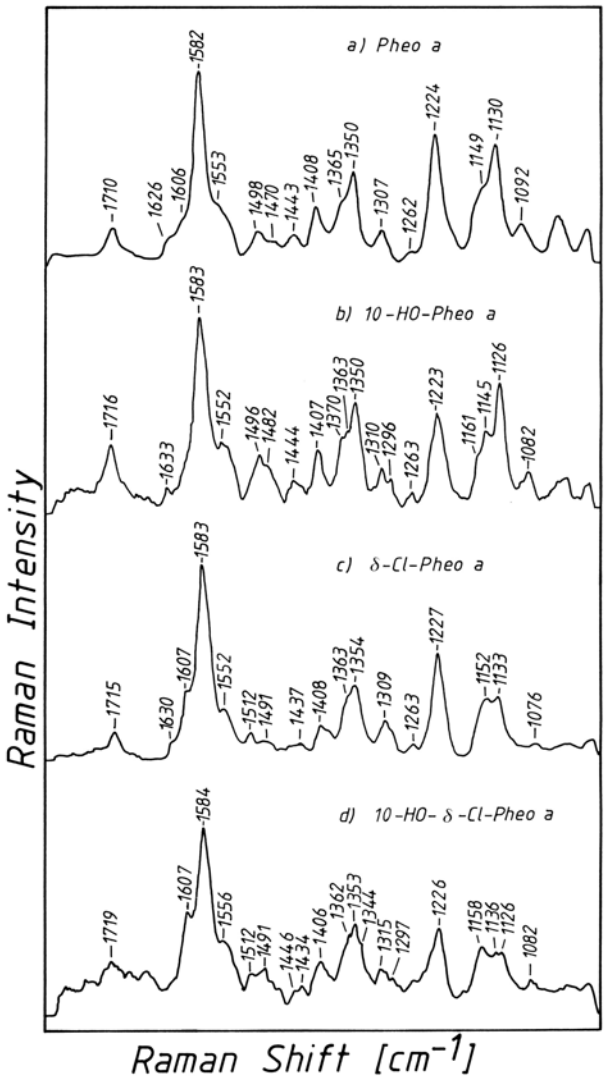


Figure 6. RR spectra of Pheo a derivatives in Et_2O with 413.2 nm excitation. Pheo concentration was approximately 1mM for all samples. Laser power, 15 mW.

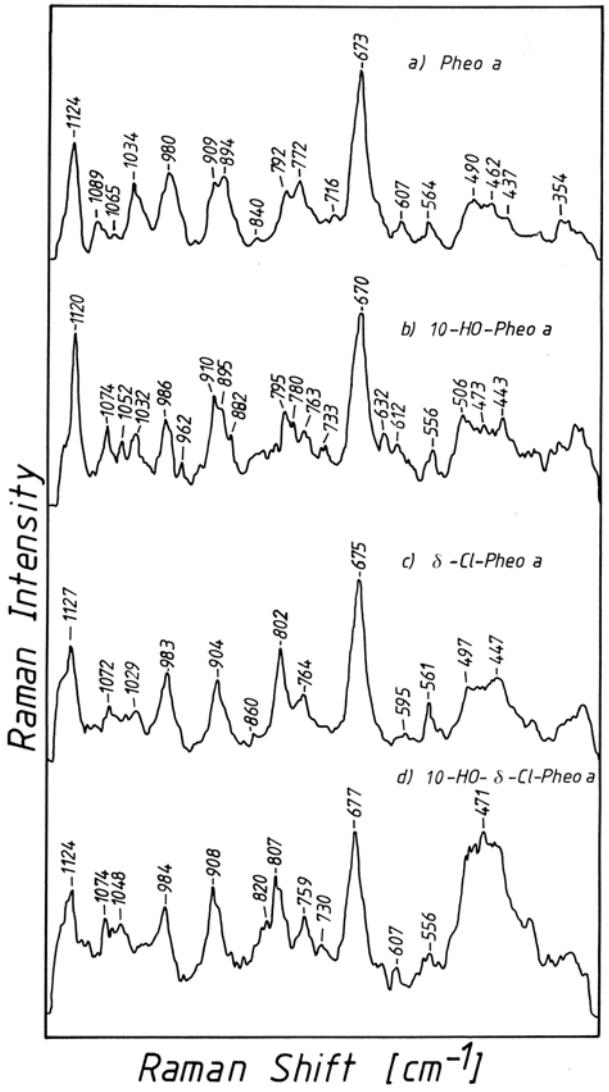


Figure 7. RR spectra (low frequency region) of Pheo a derivatives in Et_2O with 413.2 nm excitation. Pheo concentration was approximately 1mM for all samples. Laser power, 15 mW.

1350 → 1354cm^{-1}, 1307 → 1309 cm^{-1}, 1224 → 1227 cm^{-1} and 1130 → 1133 cm^{-1}. The disubstituted 10-OH-δ-Cl- Pheo a derivatives displays the following band shifts; the frequency upshift of γ C_9=O from 1710 → 1719 cm^{-1}, a decrease in intensity and possible frequency downshift of the 1630 cm^{-1} band, 1498 → 1512 cm^{-1}, 1350 → 1353 cm^{-1}, 1307 → 1315 cm^{-1}, 1224 → 1226 cm^{-1}, and a frequency shift of 1130 → 1126 cm^{-1}. Although the band shifts are not as distinct as those observed for Chl a, a similar pattern emerges. These is a frequency upshift of γ C_9=O and the core-size marker band at 1498 cm^{-1}. The larger downshift of the highest frequency core-size marker band is less obvious than for the Chl a derivatives. In the low frequency RR spectrum of the pheophytin derivatives (Figure 7) all bands are shifted.

Table 4. RR frequencies of Pheo a derivatives in diethyl ether when excited with 413.1 nm laser light [cm^{-1}].

Pheo a	10-HO-Pheo a	δ-Cl-Pheo a	10-HO-δ-Cl-Pheo a	Assignments from ref. 6a
1710	1716	1715	1719	C_9=O
1626	1633	1630		
1606		1607	1607	$C_aC_m(\alpha,\beta)$
1582	1583	1583	1584	C_bC_b
1553	1552	1552	1556	C_bC_b
1498	1496	1512	1512	$C_aC_m(\delta)$
1470	1482	1491	1491	
1443	1444		1446	
		1437	1434	
1408	1407	1408	1406	
1370				
1365	1363	1363	1362	
1350	1350	1354	1353	
			1344	
1307	1310	1309	1315	
1262	1263	1263		
1224	1223	1227	1226	δC_bH(IV), $\delta C_mH(\delta)$
	1161			
1149	1145	1152	1158	
1130	1126	1133	1136	C_aN(II), δC_bMg, δC_aNCa(I)
1089	1074	1072	1074	
1065	1052		1048	
	1032	1029		
980	986	983	984	
	962			
909	910	904	908	
894	895			
	882			
840		860	820	
792	795	802	807	
772	780			
	763	764	759	
716	733		730	
673	670	675	677	
	632			
607	612	595	607	
564	556	561	556	
490	506	497		
462	473		471	
437	443	447		

DISCUSSION

Chlorophyll a – Band Shifts and Assignments

Chemical substitution(s) of complex molecules can aid in the interpretation of their vibrational properties; However, the perturbations must be small in order to correlate the vibrations from one molecule to another. Here we consider the following possibilities to interpret the hydroxy- and chloro-substituted Chl a derivatives: 1) ring puckering or out-of-plane distortions, 2) changes in vibrational mode composition and 3) a localization of vibrational modes in the specific quadrants of Chl a macrocycle. Ring strain induced by

chemical substitutions can be alleviated by flexibility in the chlorophyll macrocyclic ring.[2,19,20] This S_4-ruffing or ring puckering effects is manifest as a frequency downshift of the core-size sensitive vibrations, e.g., D_{2d} form of NiOEP.[21] For the hydroxy-substituted derivative two core-size sensitive bands at 1600 and 1430 cm^{-1} shift down slightly in the frequency relative to Chl a.[22] This may be an indication of ring ruffling. However, for δ-Chloro-substituted Chl a both frequency downshifts (1609 cm^{-1}) and upshifts (1490 cm^{-1}) are observed for the methane bridge stretching vibrations. This is inconsistent with the shifts expected for the chlorin core ruffling.

The possibilities of changes in vibrational mode composition can be recognized as inconsistent band shifts. For example, deuteration produces frequency downshifts in C-H bending and stretching modes as the result of a pure isotope effect but the observation of frequency upshifts in these bands would be indicative of a remixing of the vibration modes.[6a,23] To establish a consistent pattern of band shifts of the hydroxy- and chloro-substituted derivatives, we have obtained RR spectra of these compounds in two different coordination states: five-coordinate in ether and six-coordinate in THF. In both ligation states the corresponding bands shift, both up and down in frequency, with approximately the same magnitude. This is an indication that the character of the vibrational modes remains constant.

The third possibility for the Chl a derivatives vibrational band shifts is that of localized mode description of the vibrations. This has been previously suggested by Boldt et al.[6a] In this case we would expect to observe a possible inconsistent pattern of band shifts because vibrations in one part of molecule would be affected differently than those in another quadrant.

The observed high frequency band shifts for δ-chloro-Chl a are at 7 cm^{-1} downshift of the 1600 cm^{-1} band and a 10 cm^{-1} upshift of 1490 cm^{-1} band. Both of these vibrations are core-size sensitive and have been assigned as having predominantly C_aC_m character. Because of the opposite frequency shifts in these vibrations with similar mode character, the band shifts observed for δ-chloro- and also the 10-OH-Chl a derivatives are interpreted as arising, in part, from localized vibrational mode.

10-Hydroxy Derivative

The type of localized vibrations that one might expect to shift upon hydroxylation at the C-10 position can be determined by examination of Ch1 a structure. These projected vibrational shifts are: addition of γ (C10-OH) in the range 1000-1150 cm^{-1}, and shifts in γC_mC_{10}, γC_9=O, γ C_aC_m(δ), γ C_{2aa}N (III, IV) and (possibly) δC_bH (IV). There are no new bands observed in the crowded 1000-1200 cm^{-1} region, so we are unable to detect the appearance of γC_{10}-OH band. As predicted the C_9 ketone stretching band is affected by C_{10}-hydroxylayion. This band shifts up by 2 cm^{-1} and 5 cm^{-1} in ether and THF, respectively, owing to the increase in the ring strain.

Two core-size sensitive vibrations at 1607 and 1433 cm^{-1} in ether and 1597 and 1431 in THF are slightly sensitive to hydroxylation and have been assigned as , γ C_aC_m(α, β) at 1600 cm^{-1} and [ð CH_2 (V) and γ

C_aC_m(ð,δ)] at 1430 cm^{-1} (Table 2). These shifts may be indicative of core ruffling. The mid-frequency range vibrations that are shifted by hydroxylation at 1220 and 1180 cm^{-1} are assigned to vibrations # 28 and 30, respectively, of Boldt et al.[6a] The band at 1220 cm^{-1} contains predominantly δC_bH (IV), γ C_aN (IV) and δC_mH (δ) character. In addition to ring V, it is not surprising that the pyrroline ring IV is also affected by hydrixy-substitution at C_{10}. Vibration #30[6a] is assigned as γC_mC_{10} (V) and δC_bH (IV); its 5-7 cm^{-1} downshift upon hydroxylation is consistent with the electron-withdrawing nature of the C_{10}-OH group and the possible ring distortions.

Previous work has investigated the effects of strain in ring V by a comparison of decarboxylated chlorophyll derivatives.[8,20c] In this case as the ring strain is relieved by pyrolysis, the bands of Cl-Fe^{3+}-pheophorbide a at 1623 and 1494 cm^{-1} shift up in frequency by 2-6 cm^{-1} , and the 1227 cm^{-1} band shifts down by 8 cm^{-1} . The opposing shifts of the high frequency bands for the hydroxy-substitution (increasing ring strain) and pyrolysis (decreasing strain in ring V) suggest a common mechanism for these spectral effects, possibly changes in ring planarity and more importantly, because these vibrations seem to be ring V specific they are consistent with localized mode picture of Chl a.

δ-Chloro Derivative

In the localized mode model of chloro-substituted Chl a we would expect to observed a new band in the range of 650-750 cm^{-1}, loss of δC_mH (δ), and a new band that correspond to δC_mCl (δ) in the low frequency region. Chloro-substitution at this position should also affect γC_aC_m(δ) and possibly other methine bridge stretching mode, γ C_aN on ring I and IV and γC_aC_b(I and IV). No low frequency γ C_mCl bending or stretching modes are observed (due to the quality of the spectra). The predicted Cl-sensitive vibrations in the high frequency region do compare fairly well with the assignments of the observed Ch1 a derivative band shifts. These assignments are given in Table 2. The observed core-size sensitive vibration band shift include a 7 cm^{-1} downshift of the 1607 cm^{-1} band [γC_aC_m(α, β)] and a 10 cm^{-1} upshiftof the 1491 cm^{-1} band [γC_aC_m(δ), γC_aC_b(I)]. These large opposing frequency shifts underscore the difference in the mode character for these vibrations. Boldt et al. assign the corresponding band of the Chl a 1491 cm^{-1} in Ni^{2+}pyropheophorbide at 1552 cm^{-1} as γC_aC_b(I), γC_aC_b(III).[6a] Because of the strong core-size sensitivity of the 1491 cm^{-1} band [9-11] we suggest that this band should be more properly assigned as vibration # 13 (11) γC_aC_m(δ), γC_aC_b(I)]. This is also consistent with the large upshift of this band resulting from electron withdrawal from π-system by the δ-chloro substituent. Again, similar to hydroxy-substituted Chl a, the lack of an observed shift in all the high frequency core-size sensitive modes leads us to conclude that a localized vibrational mode picture is most consistent with the data.

The other vibrations affected by chloro-substitution are 1696 cm^{-1} γ (C_9=O) in THF only 1398 cm^{-1} (γ C_aN (IV), 1286 cm^{-1} [δC_mH (α, β), γ C_aN (II, IV)], 1220 cm^{-1} [δC_bH (IV), δ C_aN (IV), δC_mH (δ)] and 1182 cm^{-1} (γC_mC_{10} (V)). The atoms associated with these modes are all located in the immediate vicinity of the

δ-Cl-position. Some of these vibrations , i.e., (e.g.), the ketone stretching vibration at 1700 cm^{-1} and the highest frequency core-size marker at 1597 cm^{-1}, γ (C_aC_m), and the 1220 cm^{-1} band and cyclopentanone ring mode γC_mC_{10} at 1180 cm^{-1} also shift upon C-10 hydroxylation. There is obviously a change in electron density induced by Chloro-substituent that weakens the γ C_aN bonds near it and γC_mC_{10}. The upshift of the γ C_9=O vibration could be caused by steric strain induced in ring V by redistribution of electron density of the halogen substituent or by a decrease in overlap of the ketone group with the aromatic π-system. There are two possible interpretations of the observed shifts; there are a) core-ruffling induced by δ-chloro-substituent and b) a redistribution of charge due to the inductive effect of the halogen atom. The disubstituted 10-OH and δ-Cl-Chl a derivatives are simply a summation of the effects and band shifts observed for the singly-substituted compounds. This provides more evidence against changes in vibrational mode composition. The opposing shifts of the high frequency core-size marker vibrations support a localized mode description of Chl a.

Epimers

The most consistent band shifts observed for the epimers of Chl a and its derivatives (Figure 5) are a 1-2 cm^{-1} downshifts of the 1600 cm^{-1} band and a 2-3 cm^{-1} uoshift of the 1800 cm^{-1} band. The less consistent, but real band shifts of this series of compounds are slight downshifts of 1707 cm^{-1} (γC_9=O) and 1430 cm^{-1} bands for the hydroxy- and δ-Cl-substituted derivatives, respectively, and a 2 cm^{-1} upshift of the 1220 cm^{-1} band. This set of vibrations has already been assigned in Table 2 with reference to 10-hydroxy substitution of Chl a. It is reasonable to assume that the addition of steric strain in ring V by epimerization should affect the same vibrations that were modified by the introduction of a OH-group at position 10. These two substitutions clearly identify the ring V sensitive vibrations of Chl a. The magnitude of the shifts is smallest for 10-OH- Chl a. The largest perturbation of ring V is most likely the OH-substitution, while a reversion in stereochemistry in this carbon atom only induces minor additional band shifts. These small band shifts also make identification of epimeric forms of Chl a by resonance Raman spectroscopy very difficult. The main identifications are 1-3 cm^{-1} shifts of the 1600 and 1180 cm^{-1} bands.

Pheophytin a

Only limited spectral information is available on pheophytins. Because some parallel vibrational shifts of hydroxy- and chloro- substituted Chl's a and pheo's a are observed, it was possible to assign some of the high frequency bands of Pheo a (Table 4). The band shifts noted upon hydroxylation that can be assigned by analogy with Chl a shifts are a 6 cm^{-1} upshift of the 1710 cm^{-1} γC_9=O band, and a 4 cm^{-1} downshift of ring V-specific γC_mC_{10}(V), vibrations at 1130 cm^{-1}. For the δ-Cl-derivative, the core-size marker bands that shift are at 1626 and 1498 cm^{-1}. These bands shift by 19 and 14 cm^{-1}, respectively. The exact position of the 1626 cm^{-1} band is less certain because of its low intensity and the presence of several shoulders in this region; however, these bands do correlate with shifts in the core sensitive vibrations of chlorinated Chl a at

1600 and 1490 cm^{-1}. These bands are assigned as $\gamma C_aC_m(\alpha, \beta)$ and $\gamma C_aC_m(\delta)$, respectively. The band that remains constant at 1583 cm^{-1} may be analogous to the 1555 cm^{-1}, band of Chl a. The 10-30 cm^{-1} upshift of these core-size marker bands in pheo a relative to Chl a is expected for the smaller core-size of the metal-free derivatives. The other pheophytin vibrations that correlate with the Chl a derivatives are observed at 1220 and 1130 cm^{-1}. These bands are assigned to [δC_bH (IV), γC_aN (IV), δC_mH (δ)] and γC_mC_{10} (V), respectively.

CONCLUSIONS

The hydroxy- and chloro substitutions of Chl a are small enough perturbations on the chlorophyll molecule so that the observed band shifts can be interpreted within the framework of the Chl a vibrations. The vibrational shifts are indicative of a localized mode description of Chl a , as previously suggested by Boldt et al.[6a] The shifts caused by 10-hydroxy substitution are localized mainly within or near ring V while δ-chloro substitution results in shifts of methine bridge stretching vibrations and also bands in ring IV and V region of the molecule. The shift induced by epimerization of the Chl a derivatives at the C-10 position are the same as those observed for 10-hydroxy substitution, but smaller in magnitude. These shifted vibrations are thus at ring V sensitive vibrations. Because the band shifts are small, it is difficult to identify epimers from their RR spectra alone. This localized mode description of Chl a may be an aid in the interpretation of specific protein effects in intact protein systems.

EXPERIMENTAL

Chl a was isolated from *Scenedesmus obliquus* mutant C-6E according to published procedure.[24] Hydroxylated chl a derivatives were prepared using the method of Pennington et al.[25] or by subjecting Chl a to a separation on silica gel thin layer plates, which results in the oxidation of the chlorophylls.[26] 20-Cl-Chl a was prepared from Chl a by chlorination with chloroperoxidase.[27]

The 10-epimers of Ch1 a and δ-Cl-Ch1 a were prepared by epimerization of the parent chlorophylls in triethylamine[18] and subsequent separation of the equilibrium mixture was carried out with HPLC. The hydroxylated 10*S*-epimers were always formed as side products in the formation of 10*R*-configurated epimers and were separated from the reaction mixture by TLC.[26] The corresponding pheophytins were derived from the parent chlorophylls by acidification with trifluoroacetic acid (H_2O/light petroleum mixture.[28] All pigments were extensively purified by reverse phase HPLC, using HPLC grade solvents.

Samples for resonance Raman spectroscopy were placed in 5 mm Pyrex tube, vacuum degassed by using the freeze-pump-thaw technique, and sealed under vacuum. The setup for Raman measurements has been described elsewhere.[29] For laser excitation sources a Coherent Innova 100 Kr^+ and Coherent Innova 90-5 Ar^+ laser were used. Typical laser powers employed were 10-15 mW and the Raman scattered light was

collected in the backscattering geometry. An 1800 groove.mm^{-1} grating was used in the spectrograph (Spex Trilemate) stage and indene was used for frequency calibration. The integration time for each spectrum was 200 seconds. All spectra were collected for the diode array response and for background fluorescence. The reproducibility of the data in one set-up was ±1 cm^{-1}. Between different set-up's, i.e. involving a new calibration the reproducibility was ±5 cm^{-1} Only samples of one set-up were compared with each other.

Absorption Spectra: Chl a (Et_2O): 661, 615, 578, 428 nm, (THF): 664, 627, 436 nm; 10-HO-Chl a (Et_2O): 661, 614, 427 nm, (THF): 664, 627, 436 nm; δ-Cl-Chl a (Et_2O): 666, 626, 590, 432 nm, (THF): 668, 635, 438 nm; 10-HO-δ-Cl-Chl a (Et_2O): 666, 623, 585, 431 nm, (THF): 668, 631, 437 nm; Pheo a (Et_2O): 667, 613, 530, 503, 410 nm; 10-HO-Pheo a (Et_2O): 667, 613, 530, 503, 409 nm; δ-Cl-Pheo a (Et_2O): 675, 617, 542, 512, 413 nm; 10-HO-δ-Cl-Pheo a (Et_2O): 675, 616, 542, 512, 413 nm. The 10-epimers of the different Chls show the same absorption specta at the 10*R*-configurated parent Chls.

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