A comparative study of the allomerization reaction of chlorophyll *a* and bacteriochlorophyll *a*

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A combined spectroscopic and chromatographic approach has been employed to study the products of allomerization of chlorophyll *a* (chl *a*), bacteriochlorophyll *a* (bchl *a*) and bacterioviridin (bvir) under a variety of conditions. Using high-performance liquid chromatography, mass spectrometry (MS), tandem-MS, NMR spectroscopy, UV–VIS absorption spectroscopy and protected surface-enhanced resonance Raman spectroscopy we have identified the allomers formed under all the conditions. Analysis of the different product distributions enables us to reach conclusions about the reaction mechanism. Water is identified as the source of hydroxyl in the allomerization reaction and it is firmly established that a C(13^2)-CO_2Me group is required for allomerization to occur. Under identical allomerization conditions, bchl *a* yields a distribution of products that is different from those given by chl *a* and bvir. This observation has allowed us to demonstrate that it is the bonding at the C(7)–C(8) position of chlorophylls and not the presence of a conjugated carbonyl functionality that influences the reactivity in ring E.

Introduction

Methanolic solutions of chlorophyll *a* (**I**; Fig. 1) (chl *a*) that are exposed to oxygen undergo replacement of the H-atom in the C(13^2) position by an oxygen-containing moiety in a reaction called allomerization.[1] Allomerization occurs both by chemical and enzymatic pathways and leads to complex mixtures of products known as chl allomers.[2] Although the process has been recognized for over 50 years there remains uncertainty as to the structures of some of the allomers and the mechanism of their formation.[3] Allomerization has been implicated as an early stage reaction in the breakdown of chlorophylls in the natural environment.[4] For example, allomers have been postulated as early intermediates in the breakdown of chlorophylls both in oxygenated sediments[5] and during autumn leaf senescence.[6] Recently, there has been renewed interest in the structures and occurrences of the allomers, in particular using modern spectroscopic techniques for more complete structural identification. The allomerization reaction is also important in food science where there is a need to control the stability of chlorophylls in foods subjected to modern processing methods. Processes such as cooking, heat processing and freeze preparation are known to cause discolouration of fruits and vegetables due to the conversion of the constituent chlorophylls to their pheophytins.[7] The alkaline wash procedures used for processing certain fruits are thought to promote allomerization of the constituent chlorophylls and pheophytins and considerable effort has been directed at identifying the reaction products.[8–10]

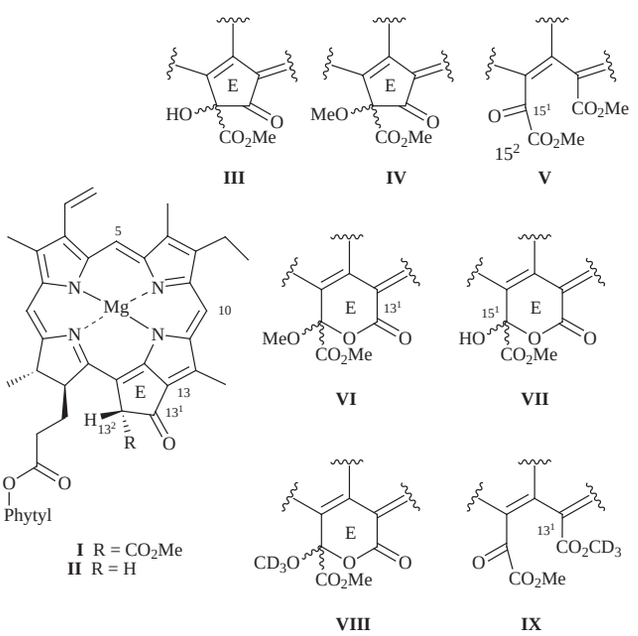


Fig. 1 Structural formulae of (**I**) chl *a*, (**II**) pyrochl *a*, (**III**–**IX**) the allomers of chl *a*; for brevity only ring E is shown for the allomers

The polar nature of chlorophylls and their oxidation products permits their separation by high-performance liquid chromatography (HPLC). Schaber *et al.*[11] were the first to utilize the technique to examine allomers, using reversed-phase HPLC (RPHPLC) to study the allomers of chl *a*. More recently, Kuronen *et al.*[12] demonstrated conditions under which normal phase HPLC (NPHPLC) also affords good separation of the allomers. The detection method most commonly employed for HPLC is UV–VIS absorption, including methods that permit examination of spectra on-line (*e.g.* multichannel diode array detection). Unfortunately, allomers often have similar absorption spectra to their parent compounds as well as to one another.[13] As a result, the assignment of UV–VIS absorption spectra is somewhat speculative, and routine identification of transformation products is difficult. The use of HPLC coupled with mass spectrometry (HPLC–MS) in conjunction with UV–VIS absorption spectra has been demonstrated to give improved results in this area.[14–16]

Despite the advances in HPLC–MS, the identification of chlorophyll allomers still depends largely on their separation and purification followed by spectroscopic analysis. A wide variety of spectroscopic techniques have been used to elucidate the structures of the allomers of chl *a*. Mass spectrometry (MS) has been used extensively since Hunt *et al.* first used ^{252}Cf plasma desorption MS to study the structures of two chl *a* allomers.[17] More recently, the use of fast-atom bombardment (FAB) MS has been demonstrated.[9,18,19] The application of NMR spectroscopy[11,12,20,21] has culminated in the complete assignment of the ^{1}H and ^{13}C spectra of seven allomers including three pairs of C(13^2) or C(15^1) diastereomers (Fig. 1, **III** to **VI**).[22] Despite its power in structural determination, NMR suffers from a relative insensitivity. Recently, we have demonstrated the power of protected surface-enhanced resonance Raman spectroscopy (PSERRS) in the study of the allomers of chl *a*.[23]

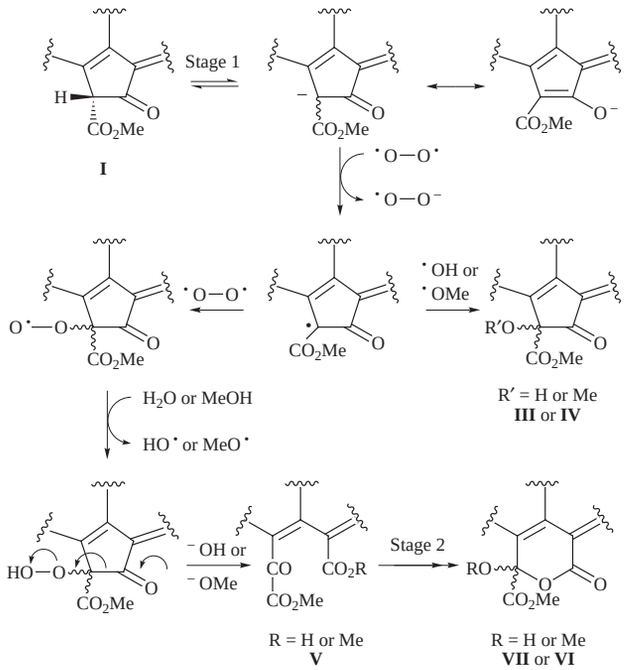


Fig. 2 Mechanism of chl *a* allomerization proposed by Hynninen;[26] for brevity, only ring E is shown. Figures refer to the structure numbers in Fig. 1.

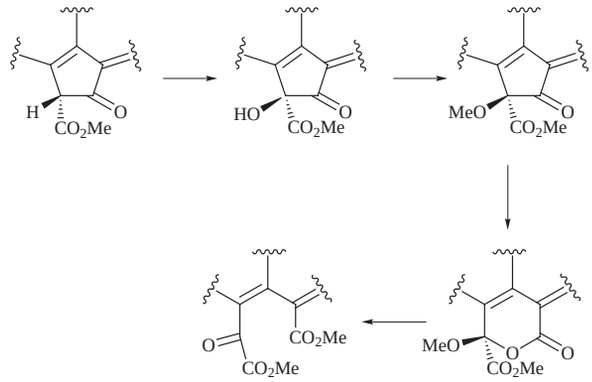


Fig. 3 Allomerization pathway proposed by Brereton *et al.*;[16] for brevity, only ring E is shown

Using a combination of chromatographic, mass spectrometric and spectroscopic techniques we have identified four allomers of chl *a*, namely C(13^2)-OH chl *a* (**III**), Mg–purpurin 7 dimethyl phytyl ester (**V**), C(15^1)-OMe lactone chl *a* (**VI**) and C(15^1)-OH lactone chl *a* (**VII**; Fig. 1) formed in the allomerization reaction. For brevity, these allomers are termed the OH allomer, the purpurin allomer, the OMe-lactone allomer and the OH-lactone allomer, respectively, throughout. All but the purpurin allomer can exist as C(13^2) or C(15^1) diastereomers. Here we draw from results taken from all of the spectroscopic techniques discussed above to elucidate the structures and distributions of the allomers and to obtain information on the reaction mechanism.

The mechanism suggested for the allomerization of chl *a* has undergone a number of revisions. Hynninen and Assandri proposed a mechanism involving reaction of the chl *a* enolate anion with $^1O_2$.[24] Subsequently, however, it was confirmed that allomerization occurs readily in the dark, thereby precluding the involvement of light, a requirement for the generation of $^1O_2$.[25] Further, it was shown that the radical scavenger β-carotene inhibits allomerization and, accordingly, Hynninen suggested a radical mechanism.[25] This mechanism has since been modified[26] and is shown in Fig. 2. Later work led Brereton *et al.*[16] to suggest an alternative allomerization pathway on the basis of product distributions measured by HPLC and HPLC–MS (Fig. 3).

Our interest in the mechanism of allomerization stems from the suggestion that allomers may be intermediates in the formation of sedimentary aetioporphyrins.[5] Accordingly, we have set out to compare the degradation pathways of chlorophylls under oxic and anoxic conditions. As part of this study we intend to compare and contrast the reactions of bacteriochlorophyll *a* (bchl *a*) and chl *a* as these compounds occur in phototrophic organisms that exist in anoxic and oxic aquatic environments, respectively. A prerequisite, however, is a knowledge of the comparative oxidative transformation pathways of chlorophylls and bacteriochlorophylls under laboratory conditions.

In this paper we discuss the mechanisms proposed for chl *a* allomerization. We have used a combined chromatographic and spectroscopic approach to identify the products of chl *a* allomerization under a variety of conditions. These results, and those obtained from the use of isotopic labelling, reveal a number of features of the reaction mechanism. Product distributions are discussed in terms of the two main mechanistic schemes proposed. A similar approach has been used to study the allomerization of bchl *a*. Previous studies of the allomerization of bchl *a* have been far fewer than those of chl *a.* We have identified the major products of bchl *a* allomerization and, by making comparisons with those observed for chl *a*, we have reached conclusions regarding the structural differences that induce different oxidative transformation pathways in tetrahydro- and dihydroporphyrins.

Experimental

Compound preparation

Chl *a* was isolated from spinach leaves and purified by chromatography on sucrose according to methods described previously.[27] The number of chromatographic separations required was reduced by performing an additional purification step involving precipitation of chlorophylls from the crude acetone extract using dioxane–water.[28] Pyrochlorophyll *a* (pyrochl *a*) was prepared from chl *a* following literature procedures.[29] Bchl *a* was extracted from *Chromatium D* cell paste and was similarly purified by chromatography without intermediate steps of purification.[30] Bacterioviridin (bvir) was prepared by oxidation of bchl *a* following literature procedures.[31]

Solvents

HPLC grade solvents (Fisons) were used throughout. In some cases, methanol was dried thoroughly by refluxing over sodium sulfate for at least 24 h before allomerization reactions were performed; CD_3OD (99.99% + D) was purchased from Sigma and used as received.

Allomerization conditions

Allomerization reactions were performed by stirring a methanolic solution of chl *a* or bchl *a* (>95% pure by HPLC and FABMS) in the dark in contact with the air. Volumes were typically 5 cm^3 and the chl concentration was typically 10^{-3} mol dm^{-3}. Reaction progress was monitored using RPHPLC and/or UV–VIS absorption spectroscopy.

Analytical methods

Analytical RPHPLC was performed using a system comprising a Waters 717 Autosampler, Waters 600-MS system controller, and a Waters 996 PDA detector. Instrument control was performed using Waters Millennium 4010 software running on a Viglen 486 PC. A pre-column was attached to the main column which consisted of two 15 cm ODS-2 Phase-Sep RP columns of internal diameter 4.6 mm and particle size 3 μm. The column was operated at ambient temperature at a flow rate of 0.7 cm^3

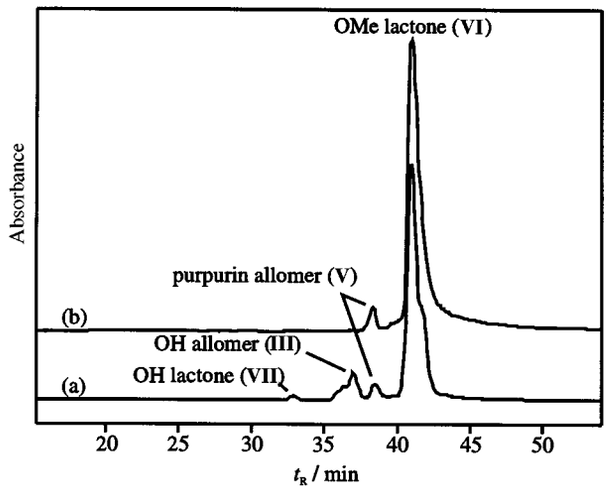


Fig. 4 Partial chromatogram, monitored at 425 nm, from RPHPLC analysis of chl *a* allomerization products from reaction in (a) undried methanol and (b) rigorously dried methanol. Unlabelled peaks correspond to diastereomers (see text).

min^{-1} and was eluted with a mobile phase consisting of mixtures of acetonitrile, ethyl acetate, methanol and water using a gradient programme.[32] Semi-preparative RPHPLC was performed on a system comprising a Waters 510 pump, Hewlett-Packard 1040A PDA detector and Hewlett-Packard 85B data collection system. A column identical to those described above was operated at ambient temperature at a flow rate of 1 cm^3 min^{-1}. The mobile phase consisted of 86% methanol, 11% acetone and 3% water (v/v).

HPLC–MS was performed using the system and conditions described previously.[33] Briefly, a Waters MS 600 Silk Quaternary HPLC system, fitted with a Rheodyne 7125 injection valve, was linked to a Finnigan MAT TSQ 700 quadrupole mass spectrometer *via* a Finnigan MAT atmospheric pressure chemical ionization interface. HPLC conditions were identical to those detailed above for analytical HPLC.

UV–VIS absorption spectra were recorded on a Perkin-Elmer Lambda 15 double beam spectrometer controlled by Perkin-Elmer PECSS software running on a Viglen 386 PC. PSERR spectra were recorded using a laser system and spectrometer that has been described in detail elsewhere.[34] The silver substrate used was a silver hydrosol prepared by standard borohydride reduction methods.[35] PSERR-active solutions were prepared by methods described elsewhere.[23,32,35]

FABMS was performed on a VG Autospec instrument in positive ion mode. The sample was dissolved in CH_2Cl_2 and introduced to a matrix of 9:1 nitrobenzyl alcohol–2,2′-thiodiethanol [bis(2-hydroxyethyl) sulfide] (v/v). This matrix has been designed to yield a significant amount of the $M^{\bullet+}$ species in chl *a*.[36] To promote ionization the sample was bombarded with a fast-moving beam of Cs^+ ions at an angle of 30°. The Cs^+ ion beam was provided by a VG FAB gun operating at 20 keV and a current of 2 mA.

Results and discussion

Products of the allomerization of chl *a* in methanol

Allomerization of chl *a* in methanol that had not been subjected to rigorous drying yielded a complex mixture of products with a distribution similar to those reported previously.[11,12] Despite the complexity of the reaction mixture its individual components could be separated using RPHPLC [Fig. 4(a)].The first of the allomers to elute, with retention time (t_R) 32.9 min, was the least abundant of the products. The UV–VIS absorption spectrum of this component in acetonitrile solution (Soret max. 416 nm, Q_y max. 652 nm, $r = 2.05$ where r is the ratio of peak heights at λ_{max} of the Soret and Q_y bands) exhibits a lack of structure on the blue side of the Soret band by comparison with that of chl *a*, indicating disruption of ring E.[2] FABMS exhibited a parent molecular ion at *m/z* 924, an $[M + H]^+$ ion at *m/z* 925, and a fragmentation pattern similar to that of a component assigned as C(15^1)-OH lactone chl *a* by Grese *et al.*[18] Accordingly this first allomer is assigned as the OH-lactone; confirmation of this assignment comes from the PSERR spectrum discussed later. As might be expected for this structure, the PSERR spectrum obtained is very similar to that of the OMe-lactone allomer[23] since altering the C(15^1) substituent is unlikely to lead to significant alteration in the electronic structure of the macrocycle.[37]

The second of the oxidation products to elute, t_R 37.0 min, exhibited a UV–VIS absorption spectrum in acetonitrile solution (Soret max. 428 nm, Q_y max. 661 nm, $r = 1.3$) which was indistinguishable from that of chl *a*. Accordingly, structural assignment of this species was performed by FABMS and PSERRS. The FABMS displayed a parent molecular ion at *m/z* 908, an $[M + H]^+$ ion at *m/z* 909, and a fragmentation pattern characteristic of C(13^2)-OH chl *a*.[19] The 457.9 nm excited PSERR spectrum displayed an upshift of the ν C(13^1)=O band from its position of 1685 cm^{-1} in the spectrum of chl *a* to 1696 cm^{-1}. This upshift has been observed previously in the spectrum of the OH allomer,[23] thus confirming its assignment.

Unlike the other oxidation products, the next of the allomers to elute (t_R 38.6 min) displayed an extremely characteristic UV–VIS absorption spectrum (Soret max. 418 nm, Q_y max. 657 nm, $r = 2.3$). Characteristics observed were a loss of intensity and a broadening of the Q_y band compared with the spectra of chl *a* and the other allomers. We assign this product as Mg–purpurin 7 dimethyl phytyl ester (**V**; Fig. 1) as both the FABMS, where the most abundant ion is the $[M + H]^+$ species at *m/z* 939, and PSERR spectra closely resemble those published previously.[16,23] Cleavage of ring E during the formation of this species leads to the characteristic changes in UV–VIS absorption spectrum.

The most abundant product was the last to elute, t_R 41.0 min, and displayed a UV–VIS absorption spectrum similar to that of the OH-lactone. The FABMS displays a parent molecular ion at *m/z* 938 and an $[M + H]^+$ ion at *m/z* 939; in this instance the most dominant ion in the spectrum is seen at *m/z* 601. We assign this species as C(15^1)-OMe lactone chl *a* (**VI**; Fig. 1) as the ^1H NMR spectrum in [$^2H_6$]acetone closely resembles that published previously.[22] The relatively high yield of this oxidation product in the allomerization reaction is consistent with this assignment as the OMe-lactone allomer has been shown to be the most abundant product in several previous studies.[11,12,16] The dominance of the ion at *m/z* 601 in the FABMS spectrum is consistent with a concerted process resulting in the loss of the C(15^2)-CO_2Me substituent and the phytyl side chain as phytadiene, with proton transfer back to the charge-retaining fragment.[36]

Chl *a* allomerization products in CD_3OD

Replacement of methanol by undried CD_3OD yielded a mixture of products which gave a chromatogram identical to that shown in Fig. 4(a). The UV–VIS absorption spectra of the oxidation products were also identical to those of the products formed in methanol. Furthermore, the FABMS of the oxidation products which eluted at t_R 32.9 and 37.0 min were also unchanged; the OH-lactone allomer (**VII**) and OH allomer (**III**) are products of allomerization both in deuteriated and in non-deuteriated methanol. However, in the FABMS of the products which elute at t_R 38.6 and 41.0 min all of the ions show an increase in mass of 3 Da by comparison with those of the products formed in normal methanol. This is consistent with incorporation of OCD_3 rather than OMe.

The deuteriated allomer which elutes first, t_R 38.6 min, displayed a parent molecular ion at *m/z* 941; from consideration of the fragmentation pattern we assign this component product as Mg–purpurin 7 methyl [$^2H_3$]methyl phytyl ester (**IX**; Fig. 1).

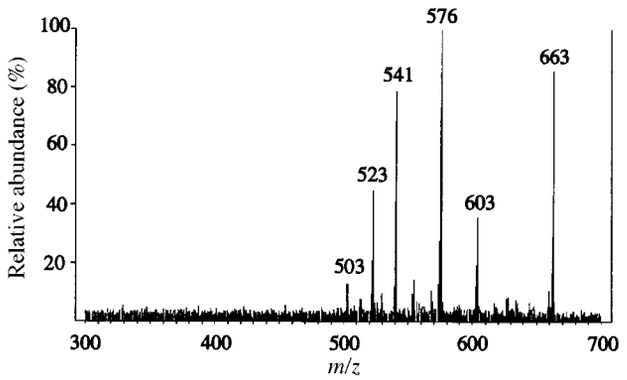


Fig. 5 FABMS–MS spectrum of **IX**, daughters of the major fragment ion at *m/z* 663

The parent molecular ion gave rise to significant amounts of daughter ions at *m/z* 663 and 576. FABMS–MS showed that the *m/z* 663 ion, which corresponds to loss of the phytyl side chain as phytadiene with back donation of a proton to the charge retaining fragment,[36] produced daughter ions of *m/z* 603, 576, 541, 523 and 503 (Fig. 5). The ion at *m/z* 603 represents the loss of the $C(15^2)$-CO_2Me group with proton abstraction from the charge-retaining fragment, and *m/z* 576 represents the loss of the entire C(15) substituent. Subsequent loss of the $C(13^1)$-OCD_3 substituent led to the ion at *m/z* 541 and the ion at *m/z* 523 represents the complete loss of the C(13) substituent. If the substituents at $C(13^1)$ and $C(15^2)$ in **IX** were reversed, *i.e.* the CO_2CD_3 group was in the $C(15^1)$ position and the CO_2CH_3 group was in the $C(13^1)$ position, then the fragmentation pattern from the *m/z* 663 ion would be different. Loss of $C(15^1)$-CO_2CD_3 with proton abstraction from the charge-retaining fragment would yield an ion at *m/z* 600; complete loss of the C(15) side chain would give rise to an ion at *m/z* 573 and subsequent loss of the C(13) substituents as described above would yield ions at *m/z* 542 and 524, respectively.

The second deuteriated oxidation product, t_R 41.0 min, gave a FABMS fragmentation pattern which is identical to that of the OMe-lactone allomer (**VI**; Fig. 1) except that all the ions have an increase in mass of 3 Da. Consequently, we assign this structure as $C(15^1)$-OCD_3 lactone chl *a* (**VIII**; Fig. 1). The position of incorporation of OCD_3 was confirmed as $C(15^2)$ by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of the OMe-lactone allomer has recently been fully assigned;[22] the 15^2 proton resonances appear at 3.84 (*S*-epimer) and 3.49 ppm (*R*-epimer). The corresponding region in the spectrum of the OCD_3-lactone allomer does not display these resonances and the positions of other resonances are not significantly altered, although the spectrum of the deuteriated species is significantly broader. Loss of the 15^2 proton resonances confirms that OCD_3 is incorporated at this position.

The role of water in the reaction mechanism

Previous studies of the allomerization of chl *a* have discussed the involvement of hydroxyl radicals and hydroxide ions although none has attempted to demonstrate the source of these. In the mechanism proposed by Hynninen,[26] an hydroxyl radical reacts with the chl *a* radical in a termination step to produce the OH allomer, and hydroxide or methoxide ion can react with chl *a* hydroperoxide to form the OH-lactone or OMe-lactone allomer as the final product (Fig. 2). The product distributions of the allomerization reactions under the various conditions employed here provide evidence that water is the source of the hydroxyl species. On allomerization of chl *a* in undried methanol the product distribution was similar to that observed previously.[11] The OMe-lactone allomer was the major product, but the OH allomer was also formed in significant yield. The OH-lactone allomer and the purpurin allomer were formed as minor products. Allomerization of chl *a* in CD_3OD yielded similar proportions of the deuteriated counterparts, *i.e.* $C(15^1)$-OCD_3 lactone chl *a* (**VIII**) and Mg–purpurin 7 methyl [$^2H_3$]methyl phytyl ester (**IX**; Fig. 1), as well as the protiated $C(13^2)$-OH and OH-lactone allomers. These results demonstrate clearly that the source of the $C(13^2)$ hydroxyl is not methanol, from which incorporation of OD would result. Reaction of chl *a* in rigorously dried methanol produced only the OMe-lactone and the purpurin allomers, with the OMe-lactone allomer being the most abundant [*cf.* Fig. 4(b)]. The absence of the hydroxylated allomers confirms water as the source of the hydroxyl in the reaction. This was further confirmed by addition of water (up to 30%) to the allomerization reaction mixture; the yield of the hydroxylated allomers increased by sixteen times as a result.

The mechanism of the allomerization reaction of chl *a*

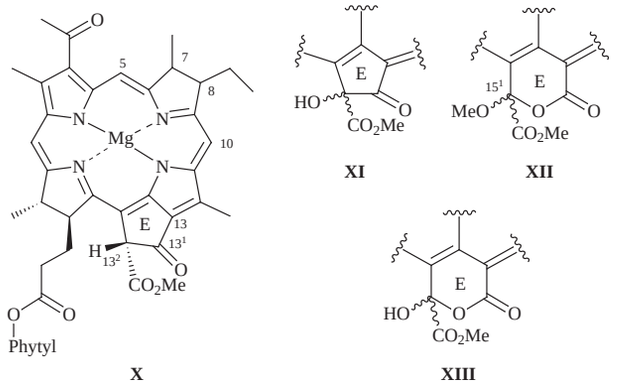
Brereton *et al.*[16] suggested a pathway of allomerization in which the OMe allomer (**IV**; Fig. 1) is formed from the OH allomer (Fig. 3). The pathway suggests that the OMe allomer subsequently reacts to give the OMe-lactone and purpurin allomers (Fig. 3).[16] In contrast, other workers have suggested that the OH allomer does not undergo further allomerization.[11] In re-examining the reactions of the OH allomer we found that it shows no reactivity to allomerization in methanol, even when the reaction conditions are made more forcing by increasing the temperature to 50 °C. After a period of one month the only change was found to be a small degree of pheophytinization, whereas allomerization of chl *a* is complete within 3 days. This finding contradicts the reaction mechanism suggested by Brereton *et al.*[16]

An explanation for the lack of reactivity of the OH allomer can be derived from stage 1 of the reaction mechanism proposed by Hynninen (Fig. 2).[26] Here it is suggested that allomerization of chl *a* requires proton abstraction, either from the $C(13^2)$ position or from the $C(13^1)$-OH group of the enol. In forming the OH allomer the $C(13^2)$ hydrogen is replaced by an -OH group. Consequently, neither $C(13^2)$ proton abstraction nor enolization can occur, and allomerization is prevented. This stage of the reaction mechanism has been studied further using pyrochl *a* (**II**; Fig. 1) which differs from chl *a* by the absence of the $C(13^2)$-CO_2Me substituent (Fig. 1). Unlike the OH allomer, pyrochl *a* possesses a $C(13^2)$ proton and might be able to undergo allomerization. In agreement with previous results,[38] pyrochl *a* was found to be stable to allomerization under the conditions described above. The absence of the $C(13^2)$-CO_2Me substituent reduces the acidity of the $C(13^2)$ proton in pyrochl *a* compared with that in chl *a,* such that enolization or proton abstraction is inhibited and hence allomerization is suppressed. Thus, our results lend support to stage 1 of the reaction mechanism suggested by Hynninen.[26]

Stage 2 of this mechanism (Fig. 2) shows that strict regiospecific control is imposed on incoming nucleophiles. Nucleophiles are incorporated at the $C(13^1)$ position in the purpurin allomers and at the $C(15^1)$ position of the lactone allomers. Our assignments of the OCD_3-lactone and the OCD_3-purpurin allomers are consistent with the regiospecific control suggested in the Hynninen[26] mechanism. That is to say that the OCD_3-purpurin allomer is found to have the CO_2CD_3 group at the $C(13^1)$ position and the OCD_3-lactone allomer the OCD_3 group at the $C(15^1)$ position.

The allomerization of bchl *a*

Previous studies of the allomerization of bchl *a* (**X**; Fig. 6) have been far fewer in number than those of chl *a*.[11] This is mainly due to the relative instability of bchl *a,* which is highlighted particularly by the formation of bacterioviridin (bvir) from bchl *a via* dehydrogenation at C(7)–C(8) (Fig. 6). In the presence of light and oxygen bvir is a troublesome impurity that has affected many studies of bchl *a*[29,39–41] and consequently few conclusions have been reached concerning the allomerization



X XI XII XIII

Fig. 6 Structural formulae of bchl *a* (**X**) and the allomers of bchl *a* (**XI**–**XIII**); for brevity only ring E is shown for the allomers. Bacterioviridin is formed by the introduction of a double bond between C(7)–C(8).

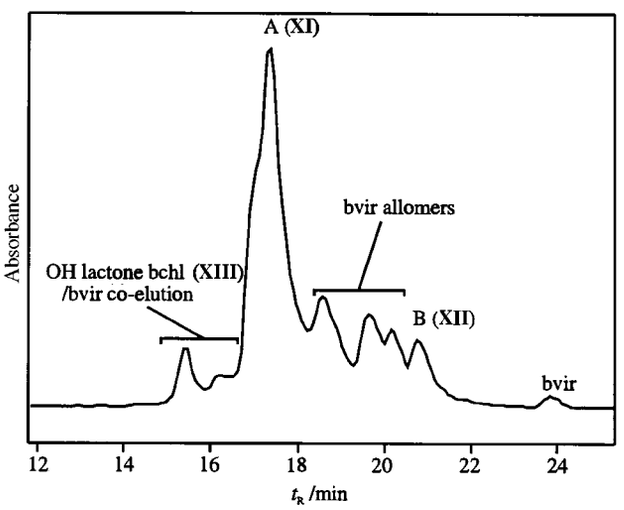


Fig. 7 Partial chromatogram, monitored at 360 nm, from the RPHPLC analysis of the products of allomerization of bchl *a*. A and B indicate components discussed in the text.

of bchl *a*. Schaber *et al.*[11] concluded that the sole product of the allomerization of bchl *a* is C(13^2)-OH bchl *a* (**XI**; Fig. 6). Raser *et al.*[40] studied the stability of bchl *a* monolayers using HPLC, UV–VIS absorption spectroscopy and SERRS and also identified C(13^2)-OH bchl *a* as a major oxidation product. The structure of this allomer has been confirmed by Brereton *et al.*[41] by use of in-beam electron ionization MS.

The allomerization of bchl *a* under the same conditions as those described above for chl *a* produced a complex mixture of products which could only be partially resolved using RPHPLC (Fig. 7). In accordance with previous studies, the formation of bvir and its allomers in addition to the allomers of bchl *a* was a significant problem, leading to an increase in the number of components in the mixture (Fig. 7). Formation of these products caused problems in the separation of the allomers using RPHPLC due to co-elution of many of the species. Nevertheless, we have been able to identify the structures of three bchl *a* allomers formed by reaction in undried methanol. FABMS of the mixture of products displayed major ions ([M + H]$^+$) at *m*/*z* 927, 943 and 957. By comparison with the products of the allomerization of chl *a* these products correspond to C(13^2)-OH bchl *a* (**XI**), C(15^1)-OMe-lactone bchl *a* (**XII**) and C(15^1)-OH lactone bchl *a* (**XIII**, Fig. 6), respectively. This FABMS analysis also revealed the major product of the reaction as C(13^2)-OH bchl *a*. In agreement with the results of Schaber *et al.*,[11] the two other allomers were formed in minor amounts.

Isolation of the allomers and their subsequent study using off-line spectroscopic techniques can provide structural assignments. However, because of the problems caused by the co-

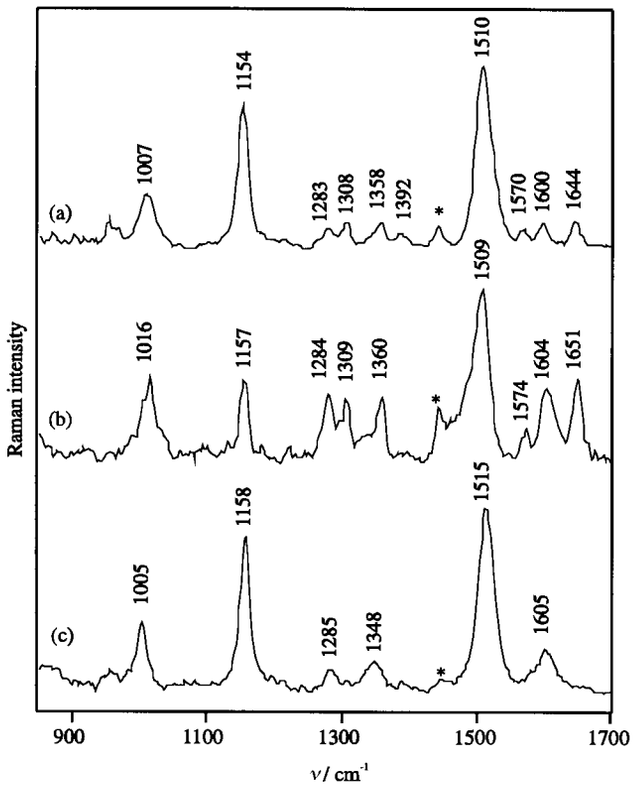


Fig. 8 PSERR spectra of (a) bchl *a*, (b) C(13^2)-OH bchl *a*, and (c) C(15^1)-OMe lactone bchl *a*. λ_{ex} 514.5 nm, 40 mW. Bacteriochlorophyll concentration *ca.* 5×10^{-6} mol dm^{-3}. Dioxane solvent bands are denoted by *.

eluting bvir-type impurities, only two of the bchl *a* allomers (labelled A and B in Fig. 7) could be isolated in a pure form. Fortunately, however, the fact that the allomers of bchl *a* are expected to elute in the same order as those of chl *a* assists in their structural assignment. Comparing the elution order of the bchl *a* allomers with those of chl *a*, the second to elute, A, t_R 17.1 min, is expected to be C(13^2)-OH bchl *a* (**XI**; Fig. 6). The FABMS of component A displayed a protonated molecule at *m*/*z* 927, consistent with the incorporation of a single oxygen atom into bchl *a*. The position of insertion was confirmed to be at C(13^2) by comparing the PSERR spectrum of A with that of bchl *a* (Fig. 8). The band positions in these spectra are largely very similar although there is an upshift of the ν C(13^1)=O band[23] from 1644 cm^{-1} in the spectrum of bchl *a* to 1651 cm^{-1} in that of A (Fig. 8). The wavenumber upshift is characteristic of increased strain in ring E caused by incorporation of groups other than H (in this case -OH) at the C(13^2) position.[23] The HPLC analysis shows the OH allomer to be the major product of bchl *a* allomerization (Fig. 7).

The second bchl *a* allomer isolated, B, t_R 20.1 min, gave rise to a protonated molecule at *m*/*z* 957. This mass increase of 47 Da compared to bchl *a* is consistent with B being either the bchl *a* OMe-lactone or purpurin allomer (Fig. 6). The evidence from RPHPLC (Fig. 7) is that B is C(15^1)-OMe lactone bchl *a* (**XII**; Fig. 6) since, as observed for chl *a*, this lactone is expected to be the last of the allomers to elute. This is further supported by the PSERR spectrum of B which lacks the strong ν C(13^1)=O band. In our previous PSERR study of the allomers of chl *a*,[23] the spectra of lactone allomers were seen to lack the ν C(13^1)=O band, whereas for the purpurin allomers the band was clearly observed.

The identification of the C(15^1)-OH lactone bchl *a* (**XIII**; Fig. 6) is tentative, coming only from the observation of an ion at *m*/*z* 943 in the FABMS of the product mixture. There is, however, a bchl *a*-type pigment, t_R 15.8 min, which elutes prior to the other products of allomerization and on the basis of the

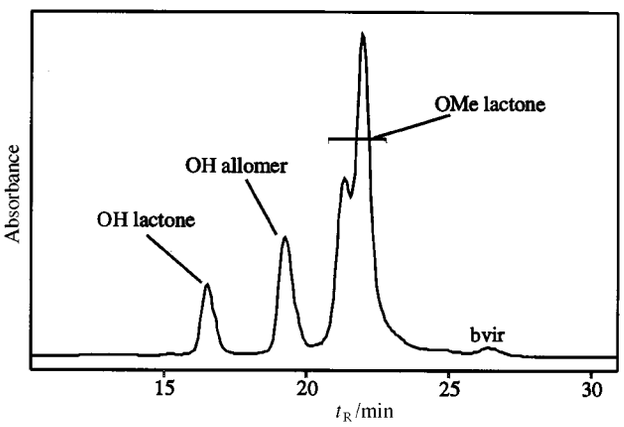


Fig. 9 Partial chromatogram, monitored at 420 nm, from the RPHPLC analysis of the products of allomerization of bvir

elution order observed for chl *a*, is likely to be the OH-lactone allomer (Fig. 7).

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The evidence above clearly demonstrates that the major product of bchl *a* allomerization is the OH allomer. This contrasts with the reaction of chl *a*, in which the OMe-lactone allomer is the major product. This observation is surprising as the ring E structures of bchl *a* and chl *a* are identical. In fact, bchl *a* differs from chl *a* only in the nature of the C(3) substituent (acetyl in bchl *a*, vinyl in chl *a*) and the C(7)–C(8) bonding (Figs. 1 and 6). In order to study which of these structural differences exerts the major influence on the reactivity of ring E we examined the allomerization of bvir (Fig. 6). Bvir provides a convenient link in this comparative study of bchl *a* and chl *a* as it has structural features of both species; the macrocycle is a chlorin [*i.e.* the C(7)–C(8) position contains a double bond] but the C(3) substituent is acetyl (Fig. 6).

RPHPLC analysis of the mixture of bvir allomers formed on reaction in methanol indicates the formation of three major allomer products (Fig. 9) which were identified using the RPHPLC elution order established for chlorophyll allomers and from analysis of the FABMS of the product mixture which exhibited ions at *m/z* 925, 941 and 955. These analyses indicate the most abundant allomer to be the OMe-lactone (*m/z* 955), t_R 21.9 min (Fig. 9). The OH (*m/z* 925), t_R 19.2 min and OH-lactone (*m/z* 941), t_R 19.2 min, allomers are formed in relatively low yields. Therefore, this product distribution is similar to that observed for chl *a* and contrasts with that of bchl *a*.

Previously, it has been demonstrated that the nature of the C(3) substituent influences the properties of ring E, particularly the extent of enolization, by a so-called long-range effect.[42] However, the product distributions observed for the allomerization of bchl *a*, bvir and chl *a* clearly demonstrate that it is the nature of bonding between C(7) and C(8) that influences the reactivity of ring E in the case of allomerization.

The nature of the C(7)–C(8) bonding has a large effect on the macrocyclic conformation of chlorophylls.[43] In bchl *a*, the bacteriochlorin macrocycle is less conjugated and significantly less planar than the chlorin macrocycle of chl *a*.[43] It is possible that the differences in macrocyclic conformation and conjugation affect the reactivity of ring E. For instance, the difference in product distributions observed for bchl *a* may be due to a steric effect. The difference in conformation may bring the ring E and C(17) substituents into close proximity, resulting in the incorporation of more bulky groups such as OMe being unfavourable. Alternatively, puckering of the bchl *a* macrocycle might lead to differences between its orbital overlap with ring E of bchl *a* and of chl *a*. Clearly, further investigations are required for a better understanding of this effect.

Conclusions

The combined spectroscopic and chromatographic approach employed here has facilitated the identification of all the products of chl *a* allomerization under normal conditions and enabled their elution order during RPHPLC analysis to be established. The elution order and spectroscopic data have been used for the identification of the distribution of chl *a* allomers formed under a variety of reaction conditions. The product distributions formed under these conditions have allowed us to draw conclusions regarding the mechanism of allomerization.

Water has been shown to be the source of hydroxyl in the reaction; in the absence of water hydroxylated allomers are not formed. Reaction in CD_3OD yields the hydroxylated allomers and two deuteriated allomers which are formed with strict regiospecific control, giving experimental support in favour of the allomerization mechanism suggested by Hynninen.[26] It should be noted, however, that hydroxyl and methoxyl radicals, suggested by Hynninen to be involved in the reaction[26] and represented in Fig. 2, may not be the active species in the allomerization reaction. Studies are underway to identify the nature of the radical or nucleophile species involved. Studies of the allomerization of C(13^2)-OH chl *a* and pyrochl *a* have enabled us to confirm that a C(13^2)-CO_2Me group is required for allomerization, thereby demonstrating clearly that the allomerization pathway suggested by Brereton *et al.*[16] is incorrect.

We have used a similar approach to identify the products of allomerization of bchl *a* as C(13^2)-OH bchl *a*, C(15^1)-OMe lactone bchl *a* and C(15^1)-OH lactone bchl *a*, where the C(13^2)-OH bchl *a* is the principal product. This product distribution differs from that observed for the reactions of bvir and chl *a,* where the OMe-lactone allomers were the major products. The nature of the C(7)–C(8) bonding is the major influence on the products formed.

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