Surface-enhanced resonance Raman spectroscopic identification of chlorophyll *a* allomers

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Protected surface-enhanced resonance Raman spectroscopy (PSERRS) has been used to study the chlorophyll *a* allomers, enabling both Soret- and Q_y-resonant spectra to be acquired with good analytical sensitivity. Spectral assignments have been made which provide a two-dimensional 'fingerprint' and detailed structural information concerning each allomer.

Introduction

We recently described a novel method of obtaining surface-enhanced resonance Raman (SERR) spectra of water-insoluble porphyrins and chlorins using silver hydrosols with a dioxane molecular spacer.[1] The method, which affords considerable sensitization of Raman scattering and efficiently quenches fluorescence, employs conditions under which the adsorbate structure is protected from perturbation by surface effects. The use of a molecular spacer ensures that spectral differences between compounds studied do not arise from artefacts resulting from direct adsorption to silver, but fluorescence quenching remains effective for adsorbates distanced from the surface by the molecular spacer.[2] We shall refer to this as the 'protected SERRS' (PSERRS) method for the purpose of the following discussion. Fluorescence quenching extends the range of excitation wavelengths that can be used to generate SERR spectra of chlorophyll (chl) from the red region of the spectrum to the blue. It has been suggested that acquisition of SERR spectra with both red and blue excitation may facilitate complete characterization of the complicated vibrational spectra of these large pigment structures.[3,4]

We present here an application of this method which reveals good sensitivity of the technique to minor structural differences between closely related members of a family of compounds, the chl *a* allomers (Fig. 1). Allomers are formed when chl *a* is exposed to air in polar solvents[5] and during natural transformation pathways; for example, during Autumn leaf senescence[6] and in the early stages of chl diagenesis.[7,8] The structural differences between allomers permit their separation by reversed-phase high performance liquid chromatography (RP-HPLC).[9] However, because the modified positions are at peripheral sites around the macrocycle, the electronic absorption spectra of allomers are largely indistinguishable. Accordingly, the last two–three years have seen a growth of interest in the isolation and subsequent characterization of allomers using NMR spectroscopy[10] and mass spectrometry (MS),[11] including LC–MS.[12] Here we demonstrate the value of the recently developed PSERR spectroscopic technique in obtaining, in a matter of minutes, compound identification and structural information from nanogram quantities of chl *a* allomers.

Experimental

Chl *a* was isolated from spinach leaves and purified by chromatography on sucrose according to methods described previously.[13] The number of chromatographic separations required was reduced by performing an additional purification step involving precipitation of chlorophylls from their crude acetone extract using dioxane–water.[14]

Allomerization reactions were performed by stirring a methanolic solution of chl *a* in the dark in contact with air. Volumes were typically 5 cm^3 and the chl *a* concentration *ca.* 10^{-3} mol dm^{-3}. Samples of each allomer were collected for PSERRS using semi-preparative RP-HPLC as described below. The purity of samples prepared for PSERRS was typically ⩾95%, as monitored by fast atom bombardment FABMS and analytical RP-HPLC.

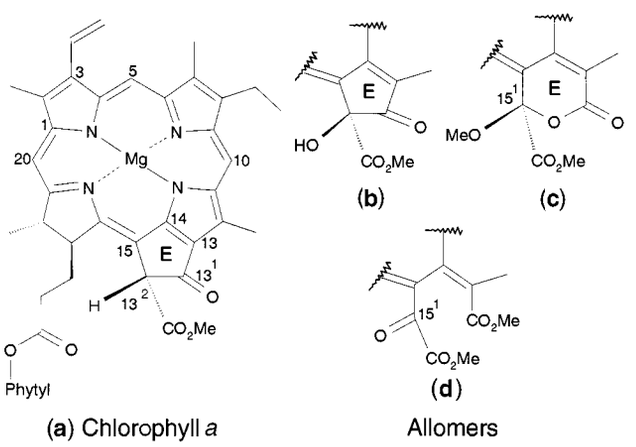

Fig. 1 Structural formulae of (**a**) chl *a*, (**b**) C(13^2)–OH chl *a*, (**c**) C(15^1)–OMe lactone chl *a* and (**d**) Mg-purpurin-7-dimethylphytyl ester. For brevity, only ring E is shown for allomers.

FABMS was performed on a Micromass Autospec instrument in positive ion mode. The FAB matrix has been developed to give a significant amount of the $M^{\cdot+}$ species and consists of 9 nitrobenzyl alcohol : 1 2,2′-thiodiethanol [bis(2-hydroxyethyl) sulfide] (v/v).[15] A Micromass FAB gun operating at 20 keV and 2 μA provided a beam of fast moving Cs^+ ions for sample bombardment which was incident at an angle of 30°.

Analytical RP-HPLC 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 C-18 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 min^{-1} and was eluted with a mobile phase consisting of mixtures of acetonitrile, ethyl acetate, methanol and water using a gradient programme.[16] Semi-preparative RP-HPLC 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 0.7 cm^3 min^{-1}. The mobile phase consisted of 86% methanol, 11% acetone and 3% water (v/v).

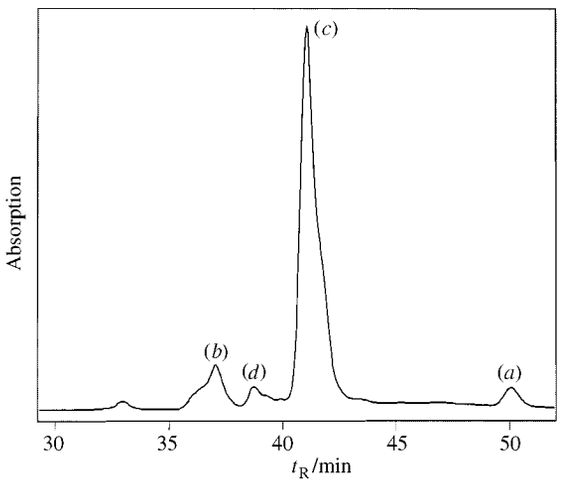

Fig. 2 Partial chromatogram, monitored at 425 nm, from preparative RP-HPLC analysis of chl *a* allomerization products. Peaks labelled (*a*)–(*d*) correspond to those structures described in Fig. 1. Unlabelled shoulders correspond to the diastereoisomers of structures (**a**)–(**c**) (see text).

The silver substrate used for PSERRS was a silver hydrosol prepared by standard borohydride reduction methods.[1,17] AR grade $AgNO_3$, $NaBH_4$ and NaCl were used as received from Fisons. The resulting hydrosols were aged for 1–2 days before use; at this time they exhibited a symmetrical absorption band centred at 395 nm, indicative of hydrosol particles in the range 10–20 nm diameter.[18,19] SERRS active solutions were produced by mixing 0.01 cm^3 of *ca.* 5×10^{-4} mol dm^{-3} dioxane solution of the adsorbate with 1 cm^3 of hydrosol. Preparation in this manner prevents structural perturbation of the adsorbate as dioxane acts as a molecular spacer.[1] Raman excitation was provided by a Spectra Physics 2025 argon ion (457.9 nm) or a Coherent Innova 90 krypton ion laser (647.1 nm); the laser power at the sample was *ca.* 40 mW. Raman scattering was collected at 90° to the exciting laser beam and analysed by a SPEX 1403 double monochromator coupled to a Wright Instruments CCD detector.[20]

Results and discussion

Because the structural differences between allomers influence their polarity, RP-HPLC affords good separation of the complex mixture of products formed on reaction of chl *a* in methanol. Fig. 2 displays a typical chromatogram, which compares well with those published previously.[9,12] Identification of the allomers prior to PSERR spectroscopy was performed by comparison of retention characteristics with those published previously, by examination of on-line UV–VIS absorption spectra and by FABMS of isolated allomers. The first major component of the allomers to elute, with retention time (t_R) 37.0 min, displayed a UV–VIS absorption spectrum which is almost identical to that of chl *a*. By contrast, the FABMS of the isolated compound is quite different from that of chl *a* (chl *a* $M^{\bullet+}$ *m*/*z* 892) and displays a molecular ion of *m*/*z* 908. By comparison with the results of Schaber *et al.*,[9] who identified a compound with similar retention characteristics and identical molecular mass, we assign this structure as C(13^2)–OH chl *a* (**b**). The next two major components to elute, at t_R 38.7 and 41.4 min, respectively, both give FABMS spectra which display molecular ions at *m*/*z* 938. These spectra exhibit quite different fragmentation patterns, which facilitate structural identification of the two compounds as Mg-purpurin-7-dimethylphytyl ester (**d**) (t_R 38.7 min) and C(15^1)–OMe lactone chl *a* (**c**) (t_R 41.4 min), respectively.[12b] The assignment of the lactone (**c**) was confirmed by comparison of ^{1}H NMR spectroscopic data with that published previously.[10] This lactone allomer (**c**) was found to be the most abundant product of the reaction, consistent with results from other studies of the methanolic allomerization reaction of chl *a*.[9,10,12] The asymmetry of this peak in the HPLC chromatogram is attributed to near-coincident elution of the diastereoisomer of the lactone allomer (**c**); shoulders on the other peaks indicate that other allomers also have diastereoisomers which elute close to one another. No attempt was made to separate the diastereoisomers from one another as PSERRS appears to be insensitive to differences in chirality between chl allomers.

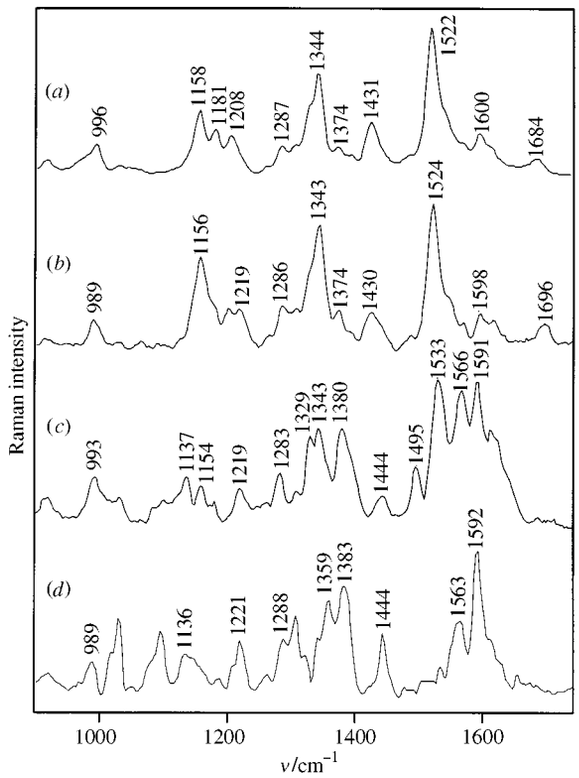

Fig. 3 PSERR spectra of (*a*) chl *a*, (*b*) C(13^2)–OH chl *a*, (*c*) C(15^1)–OMe lactone chl *a* and (*d*) Mg-purpurin-7-dimethylphytyl ester. Adsorbate concentration *ca.* 5×10^{-6} mol dm^{-3}. λ_{ex} 457.9 nm, 50 mW. Spectral resolution 5 cm^{-1}. Collection time 5 min.

Acquisition of PSERR spectra of chl *a* and its allomers using Soret-resonant blue and Q_y-resonant red excitation yields two very different series of spectra as there are different electronic states involved in the RR effect.[21] Within each series of spectra there are a number of significant differences both in band intensities and positions as discussed below. For the allomers it is seen that there are large changes in the blue-excited PSERR spectra compared with that of chl *a* (Fig. 3), whereas the red-excited spectra (Fig. 4) show more subtle changes. The two sets of spectra obtained using blue and red excitation wavelengths have largely been assigned by comparison with the normal coordinate analysis of chl *a* carried out by Boldt *et al.*[3a] (Table 1) which enables us to rationalize the differences between the spectra of chl *a* and its allomers in terms of structural differences. For instance, the band centred at *ca.* 990 cm^{-1} in both the blue and red excited spectra, which is assigned mainly to out-of-plane C–C–N deformations, remains constant throughout. Similarly, the band at 1325 cm^{-1} in the red-excited spectra (Fig. 4), which we assign to scissoring of the C(3) vinyl group, does not shift. By contrast, the vibrations of the chlorin skeleton around 1520 cm^{-1} in the blue-excited spectrum of chl *a* show large variations between the allomers.

The red-excited PSERR spectra of chl *a* and C(13^2)–OH chl *a* (**b**) are virtually identical (Fig. 4), presumably because the structural difference between the two is small and on the periphery of the chlorin macrocycle. However, the Soret-resonant PSERR spectra of the same compounds, although very similar, exhibit significant differences. In particular, the wavenumber of the ν C(13^1)=O band exhibits an upshift from 1684 cm^{-1} in the

Table 1 Wavenumbers of chl *a* bands from the blue- and red-excited PSERR spectra and band assignments

ν/cm^{-1}		
457.9 nm	647.1 nm	Assignment[a]
1684		ν C(13^1)=O
	1667	ν [C(14)–C(15) + C(15)–C(16)] + ν C(13^1)=O
1616		
1600	1600	ν [C(4)–C(5) + C(5)–C(6)] + ν [C(9)–C(10) + C(10)–C(11)]
1570		
1544	1547	ν [C(11)–C(12) + C(13)–C(14)] + ν C(2)–C(3)
1522	1523	ν C(2)–C(3) + ν [C(11)–C(12) + C(13)–C(14)]
1486	1483	ν C(1)–C(20) + ν [C(1)–C(2) + C(3)–C(4)]
1431	1433	ν [C(14)–C(15) + C(15)–C(16)] + ν [C(1)–C(20) + C(19)–C(20)] + ν [C(1)–C(2) + C(3)–C(4) + C(6)–C(7) + C(8)–C(9) + C(11)–C(12) + C(13)–C(14)]
1394	1390	ν [C(6)–C(7) + C(8)–C(9)] + ν [C(4)–C(5) + C(5)–C(6) + C(1)–C(20) + C(19)–C(20)]
1374		ν [C(16)–N + C(19)–N] + ν [C(1)–C(2) + C(3)–C(4) + C(11)–C(12) + C(13)–C(14)]
1344		ν [C(16)–N + C(19)–N] + ν [C(1)–N + C(4)–N + C(11)–N + C(14)–N]
	1325	δ C(3)H$_2$ vinyl scissors
1287	1287	δ [C(5)–H + C(10)–H] + ν [C(6)–N + C(9)–N + C(16)–N + C(19)–N]
	1261	
	1223	γ C(18)–H + δ C(20)–H
1208		δ C(18)–H + ν [C(16)–N + C(19)–N]
1181	1180	ν C(13^2)–C(13^1) + γC(18)–H
1158		ν [C(16)–N + C(19)–N] + δ [C(4)–C(5)–C(6) + C(9)–C(10)–C(11) + C(14)–C(15)–C(16) + C(19)–C(20)–C(1)]
	1147	ν [C(6)–N + C(9)–N] + δ C(1)–N–C(4)
	1119	ν [C(16)–C(17) + C(18)–C(19)] + ν C-peripheral substituent
996	991	δ [C(14)–C(15)–N + C(10)–C(11)–N] + ν C(13^1)–C(13^2)
917	913	δ [C(7)–C(8)–ethyl + C(8)–C(9)–ethyl]
797		δ [C(7)–C(8)–ethyl + C(8)–C(9)–ethyl]
	743	δ [C(7)–C(8)–ethyl + C(8)–C(9)–ethyl]

[a] Assignments based on those of Boldt *et al.*[3a] and Thomas *et al.*[21] Mode assignments are ν = stretch, δ = in-plane deformation and γ = out-of-plane deformation. Figures in parentheses refer to the macrocyclic positions in Fig. 1.

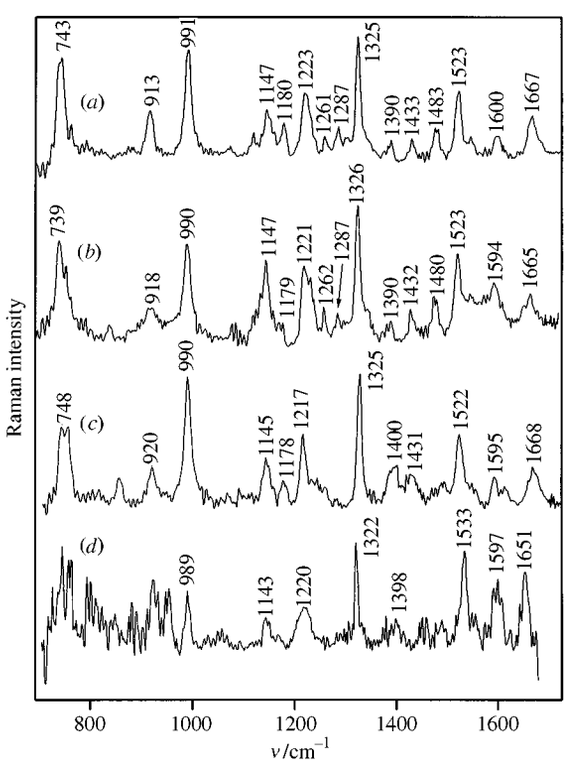

Fig. 4 PSERR spectra of (*a*) chl *a*, (*b*) C(13^2)–OH chl *a*, (*c*) C(15^1)–OMe lactone chl *a* and (*d*) Mg-purpurin-7-dimethylphytyl ester. Adsorbate concentration *ca.* 5 × 10^{-6} mol dm^{-3}. λ_{ex} 647.1 nm, 40 mW. Spectral resolution 3 cm^{-1}. Collection time 10 min.

spectrum of chl *a* to 1696 cm^{-1} in that of the C(13^2)–OH allomer (**b**) (Fig. 3). This can be understood in terms of the differences in molecular strain associated with ring E. X-Ray crystallographic data from a chl model complex show that the presence of ring E causes widespread molecular strain.[22] We attribute this strain to the location of the C(13^2) sp^3 hybridized carbon among four ring E sp^2 hybridized carbons. Due to this combination of hybrid states, none of the ring E carbon atoms exists in its favoured geometry and ring E is distorted from the plane of the macrocycle. Despite this distortion, the C(13^1)=O group is conjugated to the rest of the macrocycle. Ring E distortion is increased in allomer (**b**) by incorporation of the OH-group in the C(13^2) position. As a consequence, ring E is less well conjugated to the rest of the macrocycle than in chl *a*. The observed wavenumber upshift of the ν C(13^1)=O band in the PSERR spectrum of allomer (**b**) by comparison with that of chl *a* is attributed to this loss of conjugation. Electrochemical investigations of chl derivatives modified in ring E suggest that changes in the C(13^2) position have little effect on the electronic properties of the molecule as a whole[23,24] and this is consistent with the lack of sensitivity to such changes shown by the UV–VIS spectra.

Conversely, the spectra of C(15^1)–OMe lactone chl *a* (**c**) and Mg-purpurin-7-dimethylphytyl ester (**d**) show the effects of reduction in molecular strain compared with chl *a*. In the lactone allomer (**c**) replacement of the five-membered exocyclic ring E by the six-membered lactone ring reduces molecular strain. This is apparent in the blue-excited PSERR spectrum of this lactone (Fig. 3) where the bands assigned to modes involving ν C(13)–C(14) are positioned at 1531 and 1566 cm^{-1}, upshifted from 1522 and 1540 cm^{-1}, respectively, in the spectrum of chl *a*. This shift indicates that the C(13)–C(14) bond, which forms part of ring E, increases in strength as a result of the decrease in strain. The absence of the characteristic ν C(13^1)=O band (*ca.* 1690 cm^{-1}) in the spectrum of the lactone allomer (**c**) (Fig. 3) is attributed to increased flexibility of the six-membered ring compared with that of the five-membered ring in chl *a*. The consequent loss of conjugation of C(13^1)=O with the rest of the macrocycle, leading to a loss of RR enhancement for the ν C(13^1)=O mode, results in the absence of the characteristic carbonyl band from the PSERR spectrum.

The purpurin allomer (**d**) results from cleavage of ring E. This structural change makes spectral comparisons between the blue-excited spectra of chl *a* and this allomer difficult. There is,

however, the expected upshift of the ν C(14)–C(15) band from 1431 cm^{-1} in the spectrum of chl *a* to 1444 cm^{-1} in that of the purpurin allomer (**d**) (Fig. 3). The red-excited spectra show more subtle changes (Fig. 4); here the upshift of the chl *a* ν C(13)–C(14) band is observed from 1523 to 1533 cm^{-1}, again reflecting the increased bond order of the C(13)–C(14) bond. Ring E opening causes large changes in the ν C=O region of the spectrum due to the change in chemical environment. The $C(13^1)$=O group of chl *a* is absent from the purpurin allomer; instead, there is a highly conjugated C=O group in the $C(15^1)$ position. This is reflected by the presence of a band at 1651 cm^{-1} in the red-excited PSERR spectrum of the purpurin allomer (**d**) rather than that at 1667 cm^{-1} in the spectrum of chl *a*.

Conclusions

Despite the recent advances in LC–MS,[11,25,26] the identification of chl allomers still largely depends on their spectroscopic characterization following isolation.[8–10] Here we have shown that vibrational spectroscopy, in the form of the PSERR technique, can be used for this purpose. PSERR spectroscopy with dioxane-coated silver hydrosols yields spectra of chl *a* and its allomer derivatives essentially free from fluorescence interference. Availability of the spectra provided by Soret- and Q_y-resonant excitation provides a two-dimensional structural 'fingerprint' which affords excellent structure sensitivity as the PSERR spectra of the allomers can be interpreted by comparison with the RR spectra of chl *a* and their well-established band assignments. For the chl *a* allomers, the red-excited spectra are less sensitive to changes in ring E than are the blue-excited spectra. Far from being a deficiency in the technique, this is particularly useful where the spectral changes are large, making comparison between the blue-excited spectra difficult. The spectra shown here were acquired from nanogram quantities of adsorbate, demonstrating the good analytical sensitivity of this technique. The PSERR technique evidently is one of general applicability to the study of chlorins and porphyrins. It thus has great potential for the study of environmental samples where the amounts of material available for analysis may be very small, such as its application to study the structures of chlorophyll degradation products present in sedimentary deposits in the natural environment.

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