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Identification of chlorophyll transformation products in a lake sediment by combined liquid chromatography–mass spectrometry

C. B. ECKARDT, B. J. KEELY and J. R. MAXWELL*
School of Chemistry, Organic Geochemistry Unit, University of Bristol, Cantock's Close, Bristol BS8 1TS (UK)

ABSTRACT

Negative-ion liquid chromatographic–mass spectrometric analysis of the pigment composition of the bottom sediment (15–20 cm) of a eutrophic lake revealed the presence of a novel series of chlorophyll transformation products, in which a series of C_{27}–C_{30} sterols and stanols are esterified to a pyrophaeophorbide *a* nucleus of algal origin. The major components are algal-derived chlorophyll *a* and *b* degradation products, and the presence of a bacteriochlorophyll-related phaeophytin indicates the presence of anoxic conditions extending into the photic zone when the sediment was laid down.

INTRODUCTION

Since the first report of the high-performance liquid chromatographic (HPLC) separation of chlorophyll transformation products [1], the technique has been developed further and been widely applied in the analysis of chlorophyll pigments, their degradation products and co-occurring carotenoid pigments. For example, the approach has been used to separate higher plant chlorophylls and carotenoids [2], bacteriochlorophylls of green photosynthetic bacteria [3], algal pigments [4–6] and sedimentary chlorins [7,8] and carotenoids [9]. The most widely used conditions have typically involved reversed-phase columns employing mixtures of aqueous and organic mobile phases, and using an ion-pair or buffer system to effect the separation of free acid pigments [5,6].

These studies have relied on a variety of approaches for the assignment of components, *inter alia* (i) retention time comparison with standards or with known distributions of components in selected organisms, (ii) single- [2] or dual-wavelength [4] detection by UV–VIS spectrophotometry, fluorescence detection [7,8], or a combination of both [10], (iii) stop-flow scanning [5] or diode-array detection [11]. Such approaches have proved adequate for pigment identification when the distributions are relatively simple, *e.g.*, from a single organism, or when the interest lies only in the assignment of the major components. In other circumstances, they may, however, be insufficiently precise for compound identification, as follows: (i) the mixtures are

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complex, *e.g.*, in surface sediments where the chlorophylls, their degradation products and the carotenoids have arisen from a variety of organisms and transformation pathways [8,12]; (ii) minor key components are of interest; (iii) novel compounds are present, *e.g.*, the recently identified bacteriochlorophyll *e* [13] or chlorophyll c_3 [14]. In these circumstances, the direct coupling of liquid chromatography with mass spectrometry (LC–MS), without a significant loss of chromatographic resolution, offers a means of obtaining structural information from consideration of the mass spectra of the individual compounds, and of detecting co-elution of components. Recently, we have demonstrated [15] that such an approach had the potential for application in the investigation of the transformations of chlorophylls in aquatic environments and for recognizing in bottom sediments components which could be markers for inputs from particular classes of organisms in the primary producer community. For example, bacteriochlorophyll *c*, *d* and *e* and their transformation products in sediments would be markers for the occurrence of anaerobic photosynthetic bacteria in the water column, and would, therefore, provide indirect evidence for the extension of anoxic conditions into the photic zone. Likewise, the occurrence of bacteriochlorophyll *e* in the water column of the Black Sea provides direct molecular evidence for such conditions [13].

In this study we describe the application of LC–MS to a further investigation of the tetrapyrrole assemblage of a Recent lake sediment which has previously been shown to contain a number of chlorophyll *a* and *b* transformation products [16,17]. Priest Pot (Cumbria, UK) is a small eutrophic lake [18,19] which becomes stratified owing to the development of a thermocline during the summer months. The phytoplankton periodicity is complex although, in general, species of Chlorophyta (green algae) dominate. Blooms of diatoms and of chlorococcales (green algae) and of volvocales (green algae) occur in the spring and summer, respectively, and blue-green algae are common. In addition, the presence of photosynthetic bacteria (Chlorobiacea e) has been reported [18,19].

EXPERIMENTAL

Sample extraction and fractionation

A frozen sediment sample (section 15–20 cm), obtained from a 1-m core of the bottom sediment from Priest Pot Lake [20], was allowed to thaw and excess water was removed by centrifugation (MSE, Crawley, UK; Centaur 1 with four-place swing-out No. 43124-126; 10 min at 3000 rpm). The sediment was extracted by sonication in acetone (*ca.* 1.5 ml g^{-1} sediment). Following centrifugation (as above) the supernatant was decanted and the extraction procedure repeated twice. The combined extracts were filtered and an aliquot for LC–MS analysis (Fig. 1a) was methylated using diazomethane. The remainder of the extract was fractionated by gel permeation chromatography (GPC) on a Polymer Labs. (Church Stretton, UK) PL-Gel (50 Å) column (600 mm × 7.5 mm I.D.) using a Spectra-Physics (Hemel Hempsted, UK) SP8000 ternary delivery HPLC system, fitted with a Rheodyne (Cotati, CA, USA) Model 7125 injection valve. HPLC chromatograms were obtained by monitoring the absorbance at 400 nm. Using methanol–dichloromethane (51:49, v/v) at a flow-rate of 1.5 ml min^{-1}, six fractions were collected (1–6 in Fig. 1b). Each was analysed by UV–VIS spectrophotometry (see below) and aliquots for LC–MS analysis were methylated using diazomethane.

Preparation of standards

Tetrapyrrole standards (see below) were prepared from chlorophyll *a* and *b*, according to previously published methods [21–23], and, after purification by reversed-phase HPLC, were characterized using fast atom bombardment (FAB) MS and ^{1}H NMR spectroscopic techniques [20]. Carotenoid standards were obtained Dr. H. Kjøsen (University of Trondheim, Norway) (isorenieratene **1**) and Dr. G. Britton (Biogeochemical Department, University of Liverpool, UK) (zeaxanthin, **2**). Standards used for relative retention time and mass spectral comparisons are listed in Table I.

To determine their detection limits in LC–MS analysis, injected quantities of pyrophaeophorbide *a* methyl ester (**4**) and phaeophytin *a* (**7**) were calculated by measuring the absorbance in acetone at 662 nm and using molar absorptivities of 0.146 and 0.061 l mol^{-1} cm^{-1}, respectively [20].

LC–MS

Instrumentation. LC–MS coupling was carried out using a Waters Assoc. (Watford, UK) MS 600 Silk quaternary delivery HPLC system and a Finnigan MAT (Hemel Hempsted, UK) TSQ 70 quadrupole mass spectrometer, linked via a Finnigan MAT TSP-2 thermospray interface [15]. Prior to entering the ion source, the HPLC effluent was passed through a variable-wavelength absorbance detector (Waters Model 484), to allow monitoring of the chromatographic separation. Sample injection was performed using a Rheodyne model 7125 injection valve, equipped with a 20-μl injection loop. The injector and the detector cell were chosen to withstand back-pressures up to 4000 p.s.i. The detector was linked to a VG (Altringham, UK) Minichrom data acquisition system, to allow UV–VIS-monitored chromatograms to be obtained.

HPLC conditions. Analyses were carried out under reversed-phase conditions, using two Waters Nova-Pak C_{18} radial compression cartridges (each 100 mm × 5 mm I.D.) in-line with a precolumn containing the same phase (Waters Guard-Pak C_{18}; 10 mm × 5 mm I.D.) and operated at a flow-rate of 1.0 ml min^{-1}, using a linear gradient

TABLE I

STANDARDS USED FOR RELATIVE RETENTION TIME AND MASS SPECTRAL COMPARISONS

Standard (structure)[a]	t_R (min)[b]
Phaeophorbide *a* methyl ester (**3**)	20.3
Zeaxanthin (**2**)	20.7
Pyrophaeophorbide a methyl ester (**4**)	23.1
Isorenieratene (**1**)	39.3
Phaeophytin *b* (**5**)	40.5
Pyrophaeophytin *b* (**6**)	43.5
Phaeophytin *a* (**7**)	44.0
Pyrophaeophytin *a* (**8**)	47.2

[a] For structures see Fig. 6.

[b] Reversed-phase HPLC (see Experimental).

TABLE II

SOLVENT ELUTION PROGRAMME FOR HPLC

Time (min)	Acetone (%)	Methanol (%)	Water (%)
0	0	90	10
5	0	90	10
15	70	15	15
40	90	5	5
75	90	5	5
85	0	90	10
95	0	90	10

programme (Table II). Typical back-pressures using the HPLC system off-line were *ca.* 1600 psi at 1.0 ml min^{-1} methanol–water (90:10, v/v). Conventional chromatograms were obtained by monitoring the absorbance at 400 nm. Prior to injection, samples were filtered through Millex GV_{13} 0.22-μm membrane filters (Millipore, Watford, UK).

Interface and MS conditions. The LC effluent was ionized in the discharge ionization mode, using the following source conditions: discharge voltage, 1200 V; vaporizer temperature, 65°C; source temperature, 250°C; repeller electrode, 0 V. Typical back-pressures were *ca.* 1900 p.s.i. with methanol–water (90:10, v/v) at 1.0 ml min^{-1}. Mass spectral information was obtained in the negative-ion mode, with scanning from m/z 300 to 1000 in 1.5 s.

UV–VIS spectrophotometry

Spectra (750–350 nm) were recorded in acetone (1 cm path length) using a Shimadzu (Duisburg, Germany) UV-180 spectrophotometer at a scan speed of 120 nm min^{-1} and a slit width of 2 nm.

Solvents

In general, doubly distilled solvents were used. For HPLC and LC–MS analyses HPLC-grade solvents, filtered through 0.2-μm membrane filters, were used.

RESULTS AND DISCUSSION

Fractionation of total extract

In earlier studies of the pigments of the surface sediment (0–5 cm) of Priest Pot [20], GPC was used to separate the carotenoid pigments (early eluting fraction) from the chlorophylls and their degradation products using dichloromethane as eluent, according to the method of Repeta [24]. Under these conditions, phaeophytins (*e.g.*, phaeophytin *a*, **7**) were separated (as a discrete fraction) from phaeophorbide methyl esters (*e.g.*, phaeophorbide *a*, **3**), and there was some separation within the latter fraction [20]. However, HPLC analysis of the tetrapyrroles revealed the presence of a complex mixture of components, particularly within the phaeophorbide-containing fraction.

In the present LC–MS study of the pigments from 15–20 cm depth, it was decided to use dichloromethane–methanol as GPC eluent, in an attempt (i) to improve

the separation within the phaeophorbide to allow more detailed fractionation and (ii) to allow a search for minor porphyrin transformation products from the chlorophylls *c*, which would be markers for the presence of diatoms and/or dinoflagellates in the water column when the sediment was laid down *ca*, 25–35 years ago. Under these conditions, however, true size-exclusion behaviour was not observed within the tetrapyrroles. Hence, the phaeophorbide methyl esters eluted before the phaeophytins and novel high-molecular-weight pyrophaeophorbide esters (see below). On the other hand, the conditions did allow these minor high-molecular-weight chlorins to be concentrated within one fraction, six fractions being collected (Fig. 1b). Assignment of

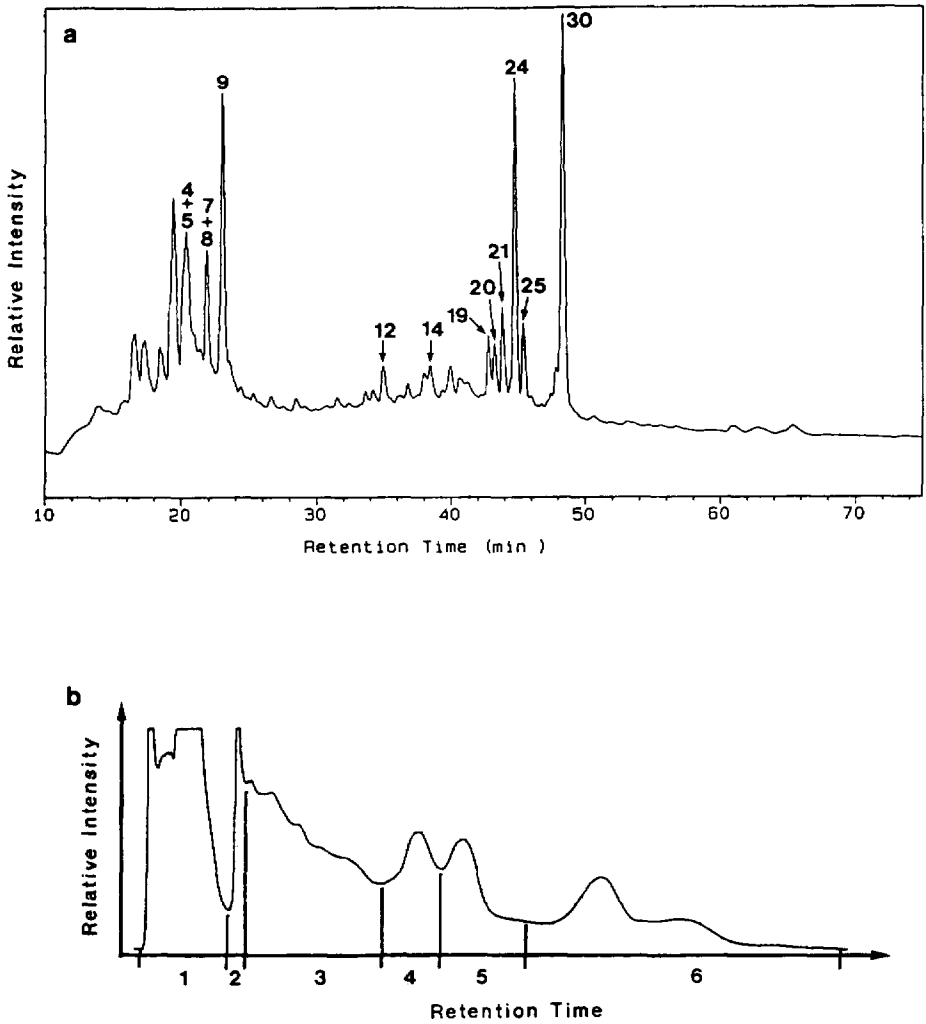

Fig. 1. (a) UV–VIS-detected chromatogram (400 nm) from the LC–MS analysis of the total methylated extract from the Priest Pot lake bottom sediment (15–20 cm). For peak identification see Table III; for HPLC and MS conditions see Experimental. (b) Gel permeation chromatogram (UV–VIS, 400 nm) from the separation of the total extract into GPC fractions 1–6. For conditions see Experimental.

components in the fractions was based on comparison of their negative-ion mass spectra and retention times with those of standards, or by mass spectral interpretation based on the fragmentation behaviour of the standards.

LC-MS spectra of phaeophytins and phaeophorbide methyl esters

As observed previously [15] for a number of chlorophyll *a*-derived standards, the chlorophyll *b*-derived standards used here show abundant $M^{-\cdot}$ species (Fig. 2). Also,

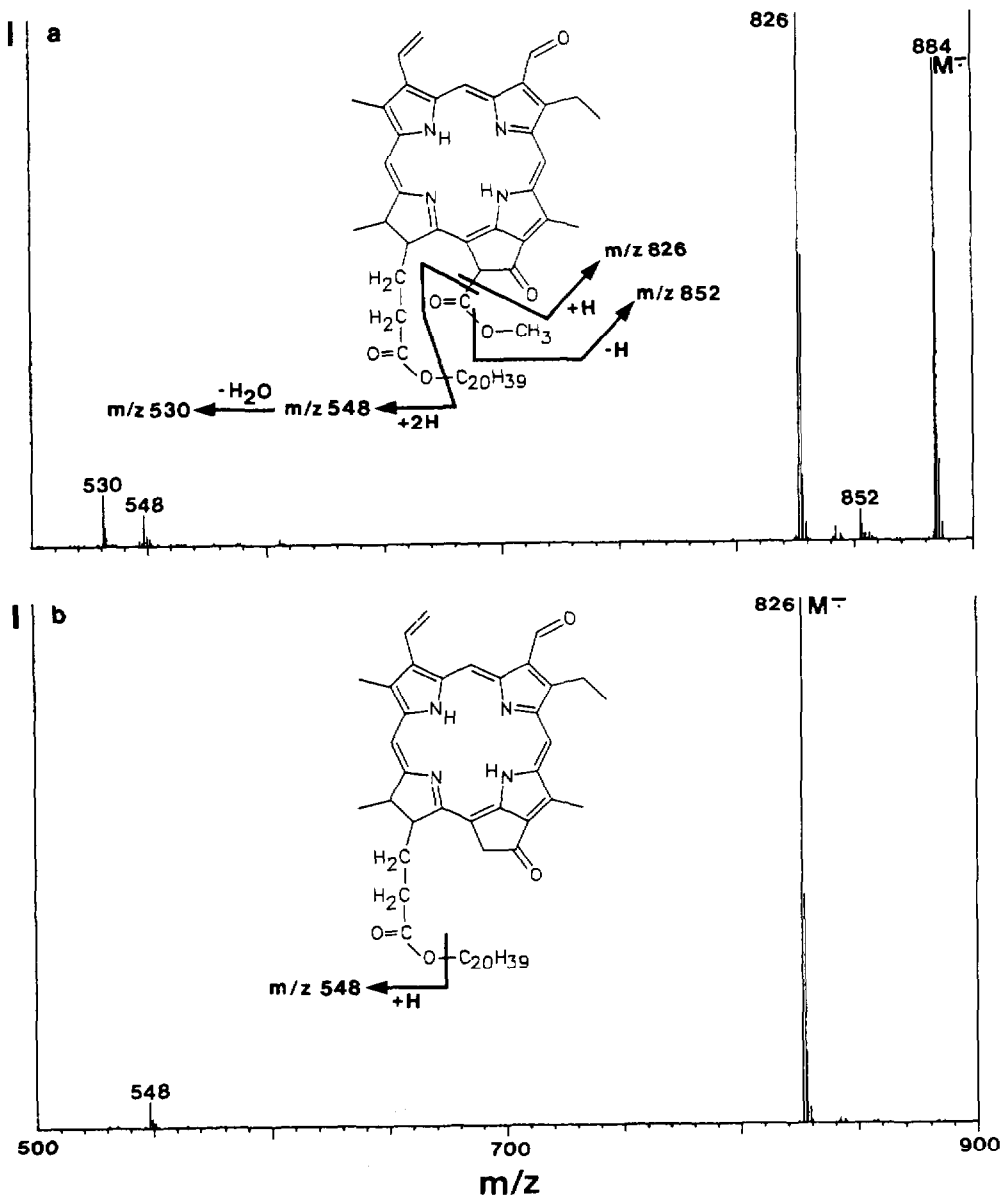

Fig. 2.

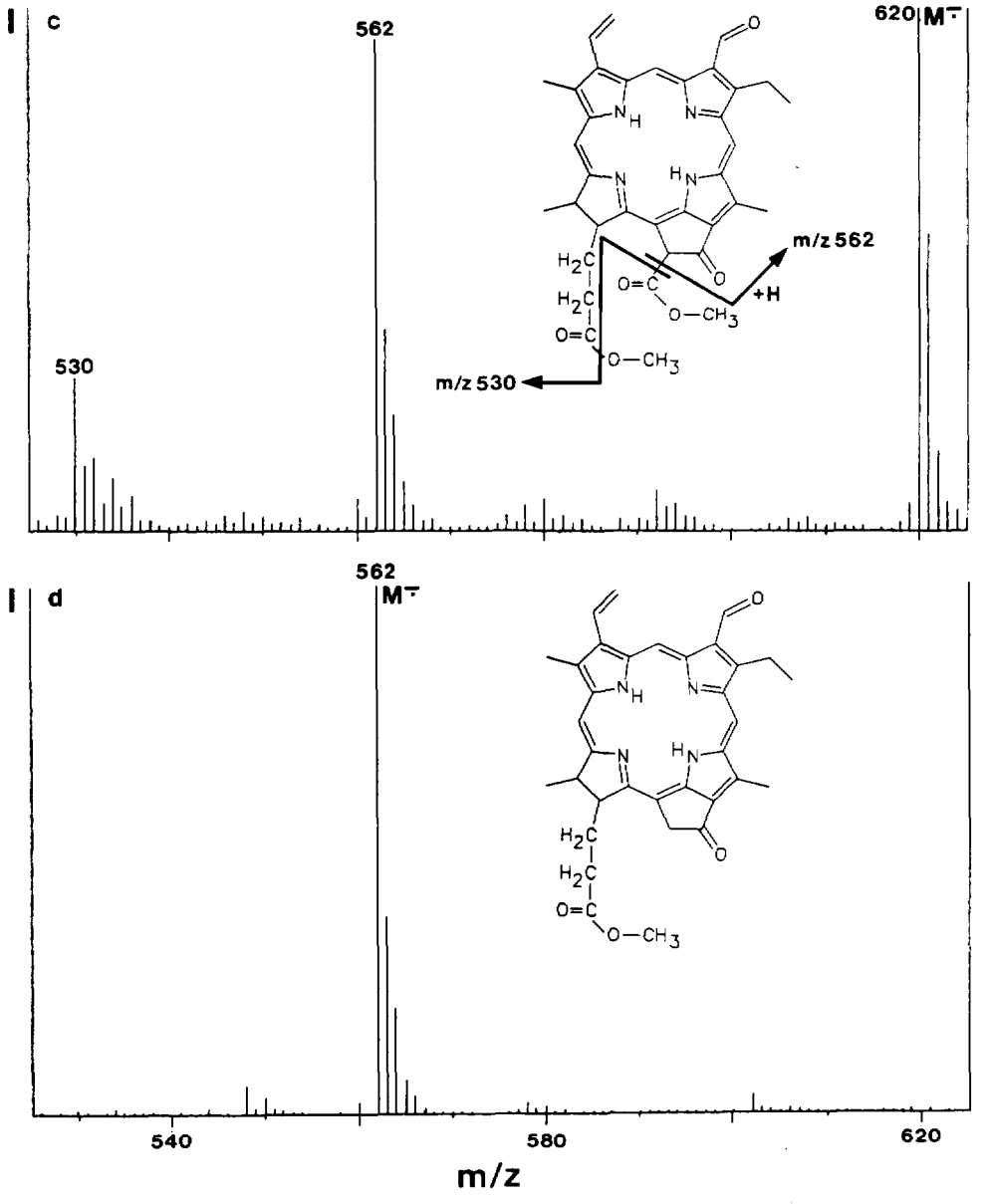

Fig. 2. Mass spectra of standards of (a) phaeophytin *b*, (b) pyrophaeophytin *b* and (c) phaeophorbide *b* methyl ester and (d) of peak 8 (*cf.*, Table III) in GPC fraction 3, obtained by negative-ion LC–MS with discharge ionization. Source conditions: source temperature, 250°C; discharge, 1200 V; vaporizer temperature, 65°C; repeller, 0 V. For HPLC conditions see Experimental.

the fragmentation (where observed) is explicable in terms of that seen under positive-ion FAB-MS conditions [25]. Hence, the spectrum of phaeophytin *b* (**5**), apart from $M^{-\cdot}$ at *m/z* 884 (Fig. 2a) and a minor ion at *m/z* 852, from loss of CH_3OH from

the C-13[2] carbomethoxy substituent, is dominated by *m/z* 826, corresponding to loss of the whole C-13[2] substituent with hydrogen transfer. The ion at *m/z* 548 results from additional loss of the phytyl side-chain (as $C_{20}H_{38}$), with *m/z* 530 representing additional loss of H_2O. In the spectrum of pyrophaeophytin *b* (Fig. 2b), lacking the C-13[2] carbomethoxy substituent, the only fragment ion occurs at *m/z* 548, again from loss of the phytyl substituent as $C_{20}H_{38}$. Perhaps surprisingly, no additional loss of H_2O was observed to give *m/z* 530, although this fragmentation is seen in the negative-ion mass spectrum of pyrophaeophytin *a* (**8**) [15]. The presence of the C-13[2]

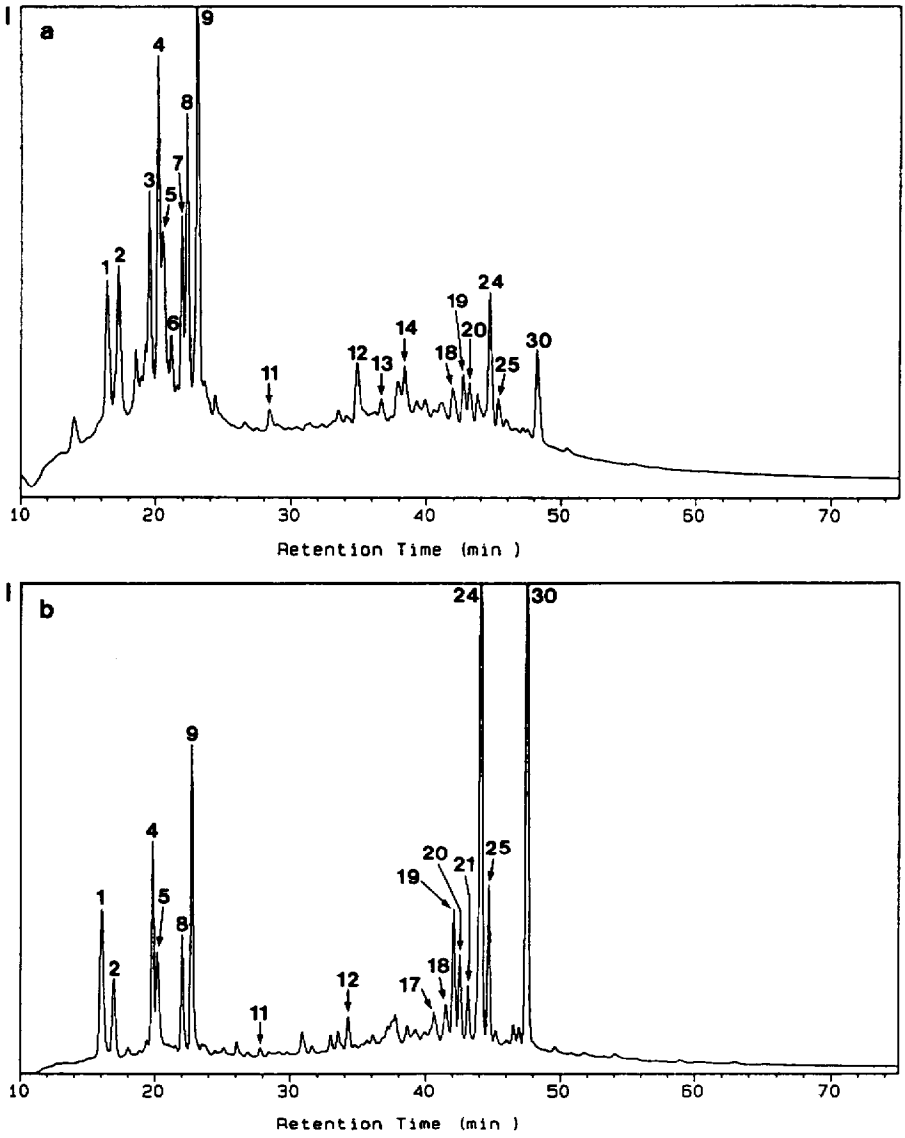

Fig. 3.

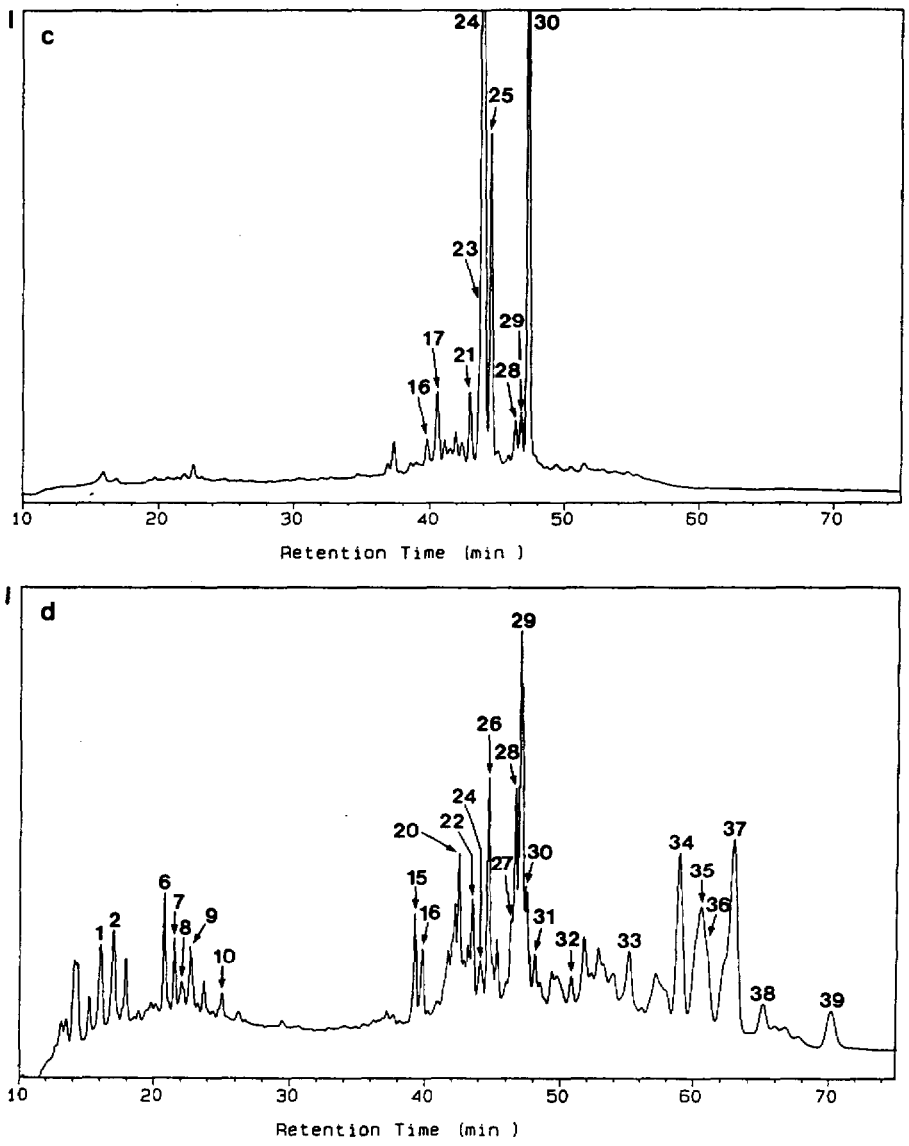

Fig. 3. UV–VIS-detected chromatograms (400 nm) from the LC–MS analyses of (a) GPC fraction 1, (b) GPC fraction 3, (c) GPC fraction 4 and (d) GPC fraction 6. For peak identification see Table III. For HPLC and MS conditions see Experimental.

carbomethoxy substituent in the spectrum of phaeophorbide *b* methyl ester (**9**, Fig. 2c) is readily observed by the presence of an abundant ion at *m/z* 562 (*cf.*, Fig. 2a, *m/z* 826), with the ion at *m/z* 530 corresponding to additional loss of CH_3OH within the C-17 propionic substituent. A standard of pyrophaeophorbide *b* methyl ester (**10**) was not available. The spectrum of peak 8 in gel fraction 1 (Fig. 3a) is shown in Fig. 2d. By

TABLE III
CHLOROPHYLL DEGRADATION PRODUCTS IN PRIEST POT SEDIMENT[a]

Peak	Fig.	$M^{-\cdot}$[b] (m/z)	Prominent ions[c] (m/z)	Assignment of major component(s)	Esterifying alcohol	Structure[d]
1[e]	3, 5		532, 516	Unidentified carotenoid		
2[e]	3, 5	622	532, 516	Unidentified carotenoid		
3[e]	3	564	562	Unidentified carotenoid		
4	1, 3, 5	606	566, *548*, *516*	Phaeophorbide *a*[f]	Methanol[g]	**3**
5[e]	1, 3, 5	606	582, 566, 562, *548*,	Phaeophorbide *a* epimer	Methanol[g]	**3**
		568	532, 518, *516*	Zeaxanthin[f]		**2**
6	3	564		Unidentified carotenoid		
7	1, 3	564		Unidentified carotenoid		
8	1, 3, 5	562		Pyrophaeophorbide *b*[h]	Methanol[g]	**10**
9[e]	3, 5	548	*516*	Pyrophaeophorbide *a*[f]	Methanol[g]	**4**
10	3		530, 516	Unidentified carotenoid(s)		
11	3, 5	770	566, 548, 516	Bacteriophaeophytin[h,i]	Farnesol	**11**
12[e]	1, 3, 5	788	594	Unknown phaeophytin		
		762		Unknown phaeophytin		
13[e]	3	802		Unknown phaeophytin		
		776		Unknown phaeophytin		
14[e]	1, 3	854	816, 564, 562	Unknown phaeophytin		
15	3	528		Isorenieratene[f]		**1**
16	3	528		Renieratene[h,i]		**16**
17[e]	3, 5	884	*826*, 814, *548*, *530*, 516	Phaeophytin *b*[f]	Phytol	**5**
		872		Pyrophaeophytin *b* methoxylactone[h]	Phytol	**14**
18[e]	3, 5	884	*826*, 814, *548*, *530*, 516	Phaeophytin *b* epimer	Phytol	**5**
		872		Pyrophaeophytin *b* methoxylactone epimer[h]	Phytol	**14**
19[e]	1, 3, 5	886	816, 758, 740, 552,	Phaeophytin *a* allomer[j]	Phytol	
			534, 530, 518, 516			
20	1, 3, 5	566		Unidentified carotenoid		
21[e]	1, 3, 5	916	886, 828, 812, 530, 516	Phaeophytin *a* methoxylactone[h]	Phytol	**15**
22[e]	3	598	534, 516	Unidentified carotenoid		
		566		Unidentified carotenoid		
23	3	826	812, 730, 548, *530*, 516	Pyrophaeophytin *b*[f]	Phytol	**6**
24	1, 3, 5	870	838, 826, 814, *812*,	Phaeophytin *a*[f]	Phytol	**7**
			794, *534*, *516*			

25	3, 5	870	838, 814, *812*, 794, 548, *534, 516*	Phaeophytin *a* epimer	Phytol	7
26	3	598	516	Unidentified carotenoid		
27	3	536	516	Carotenes[h,i]		
28	3	536	516	Carotenes[h,i]		
29	3	536	516	Carotenes[h,i]		
30[e]	1, 3, 5	812	*534, 516*	Pyrophaeophytin *a*[f]	Phytol	8
31[e]	3	868	812, 580, 536, 516	Unknown		
32[e]	3	902	716, 536, 516	Unknown		
		812		Unknown		
33[e]	3	944	812, 534[k], 530, 516	Pyrophaeophorbide *a* ester	C_{30} sterol *l*	
		930		Pyrophaeophorbide *a* ester	C_{29} sterol *l*	
		914		Pyrophaeophorbide *a* ester	C_{28} sterol *m*	
		900		Pyrophaeophorbide *a* ester	C_{27} sterol *m*	
34[e]	3	928	534[k], 516	Pyrophaeophorbide *a* ester	C_{29} sterol *m*	
		916		Pyrophaeophorbide *a* ester	C_{28} sterol *l*	
		902		Pyrophaeophorbide *a* ester	C_{27} sterol *l*	
35[e]	3	928	534[k], 516	Pyrophaeophorbide *a* ester	C_{29} sterol *m*	
		916		Pyrophaeophorbide *a* ester	C_{28} sterol *l*	
36[e]	3	930	534[k], 516	Pyrophaeophorbide *a* ester	C_{29} sterol *l*	
		928		Pyrophaeophorbide *a* ester	C_{29} sterol *m*	
		916		Pyrophaeophorbide *a* ester	C_{28} sterol *l*	
37[e]	3	930	534[k], 516	Pyrophaeophorbide *a* ester	C_{29} sterol *l*	
38[e]	3	930	788, 534[k], 516	Pyrophaeophorbide *a* ester	C_{29} sterol *l*	
		918		Pyrophaeophorbide *a* ester	C_{28} stanol	
39[e]	3	946	866, 534[k], 516	Pyrophaeophorbide *a* ester	C_{30} stanol	

[a] Based on spectral interpretation or comparison with spectra and relative retention times of standards.
[b] Molecular ion of major component(s).
[c] Other ions in mass spectrum. Fragment ions in spectra of standards given in italics.
[d] See Fig. 6.
[e] Co-elution.
[f] t_R and mass spectrum compared with authentic standard.
[g] Due to esterification prior to analysis.
[h] Tentative assignment.
[i] One of several possible structural isomers.
[j] Single-oxygen allomer.
[k] Fragment ion assigned to macrocycle.
[l] One double bond.
[m] Two double bonds.

comparison with the spectra in Fig. 2a–c, peak 8 was assigned as this component (Table III), although no ion at *m/z* 530 was apparent (*cf.*, Fig. 2c). The assignment remains, however, tentative, as the spectrum (not shown) of pyrophaeophorbide *a* methyl ester (**4**) does contain a low-intensity ion at *m/z* 516, corresponding to loss of CH_3OH from $M^{-\cdot}$.

Consideration of the spectra in Fig. 2 and of those of their chlorophyll *a*-derived counterparts allowed the assignment of several phaeophytins and phaeophorbide methyl esters on the gel fractions.

Gel fractions 1 and 2

The UV–VIS spectra (not shown) of fraction 1 (major, Fig. 3a) and fraction 2 (minor) showed them to be dominated by carotenoids. Hence, the Soret band of free base chlorins (*ca.* 400 nm) was obscured and any absorbance bands in the region of 664 nm, which would be typical of functionalized chlorins, were present only at very low relative intensities. The absorbance chromatograms obtained during LC–MS analysis showed, however, similar distributions of components at the wavelength monitored (400 nm), particularly within the tetrapyrroles. The spectrum of peak 5 indicated the presence of co-eluting components. One of these was shown to be the bicyclic carotenoid diol zeaxanthin (**2**), from comparison with the spectrum of a standard ($M^{-\cdot}$ = *m/z* 568 and little fragmentation) and with its retention time. A co-eluting tetrapyrrole was assigned as the C-13^2 epimer of phaeophorbide *a* methyl ester (**3**), based on retention time and mass spectral comparison with the epimer present in the standard of phaeophorbide *a* (*cf.*, peak 4 in Table III). The other tetrapyrroles assigned in fractions 1 and 2 are discussed in relation to fractions 3 and 4 where they are present in greater abundance.

Gel fraction 3

All of the phaeophorbide methyl esters in fractions 1 and 2 were present in fraction 3, the UV–VIS spectrum showing a dominance of chlorin components (λ_{max} 664 nm). Apart from the epimeric phaeophorbide *a* methyl esters (**3**, peaks 4 and 5), and pyrophaeophorbide *b* methyl ester (**10**, peak 8), the only other phaeophorbide was pyrophaeophorbide *a* methyl ester (**4**, peak 9).

The spectrum of a minor component (peak 11) showed $M^{-\cdot}$ at *m/z* 770 (Fig. 4a) and was tentatively assigned as a bacteriophaeophytin (**11**) derived from one of the bacteriochlorophylls *c* (**12**) or two of the bacteriochlorophylls *d* (**13**) from spectral interpretation. The absence of an abundant ion at *m/z* 712 confirms the absence of a C-13^2 carbomethoxy substituent (*cf.*, Fig. 2a and b). From comparison of the spectra of phaeophytin *a* (**7**) the ion at *m/z* 566 would correspond to loss of $C_{15}H_{24}$, indicating that the esterifying alcohol is farnesol, which would be lost as the tetraene (*cf.*, phytyl chain as phytadiene [15]). Similarly, *m/z* 548 is explicable in terms of additional loss of H_2O, as observed in the spectrum of phaeopthytin *a* [15]. The ions at *m/z* 516 and 530 are more difficult to explain in the absence of an appropriate standard. They may indicate the presence of a co-eluting tetrapyrrole component, although *m/z* 530 could result from an additional loss of H_2O from the C-3 secondary alcohol substituent (Fig. 4a). The presence of a bacteriochlorophyll transformation product, albeit in very low relative abundance, provides molecular evidence for the presence of anaerobic photosynthetic bacteria (see above) in the water column when the sediment was laid

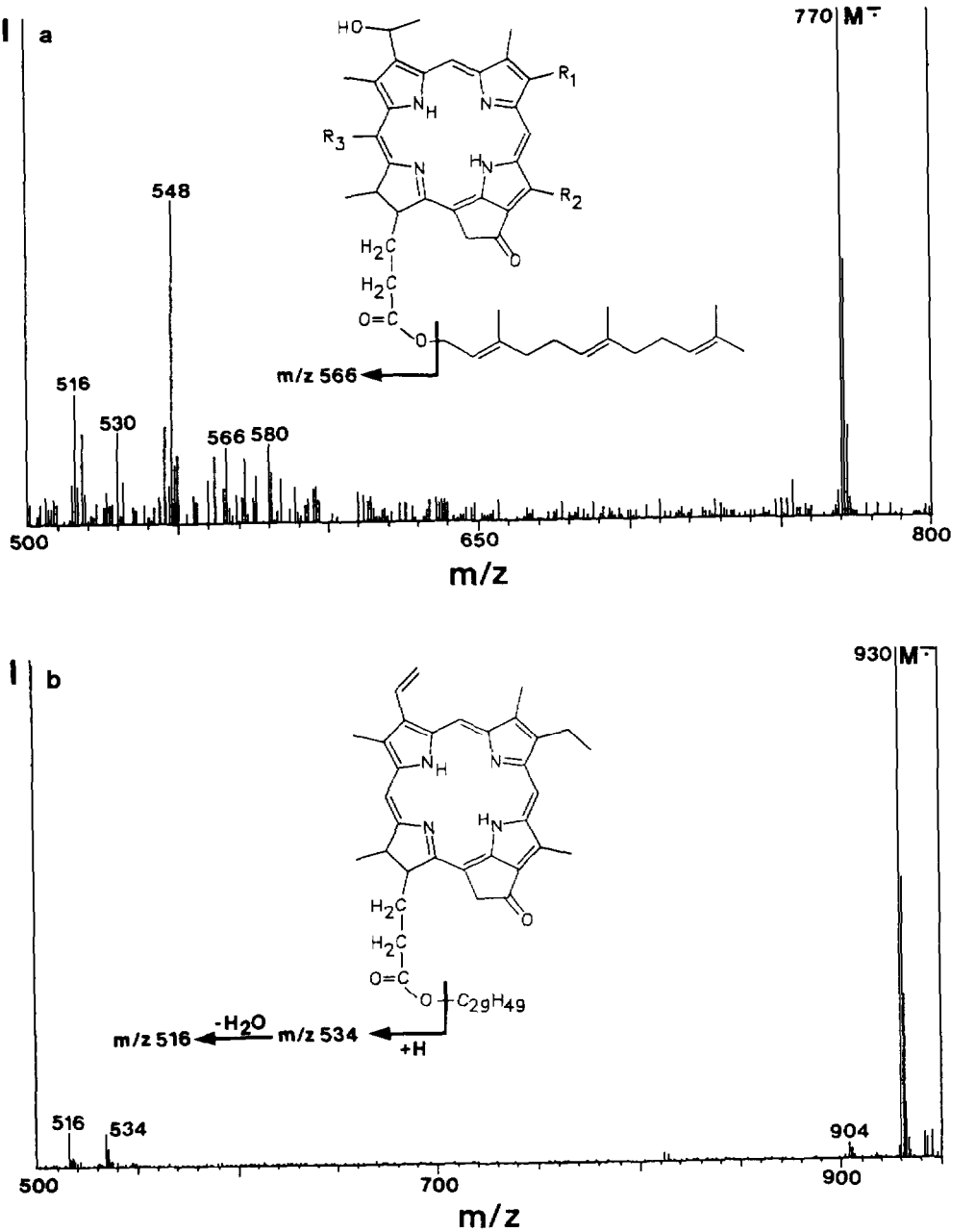

Fig. 4. Mass spectra of (a) peak 11 (*cf.*, Table III) and (b) major component in peak 37 (*cf.*, Table III), obtained by negative-ion LC–MS. Source conditions as in Fig. 2. For HPLC conditions see Experimental.

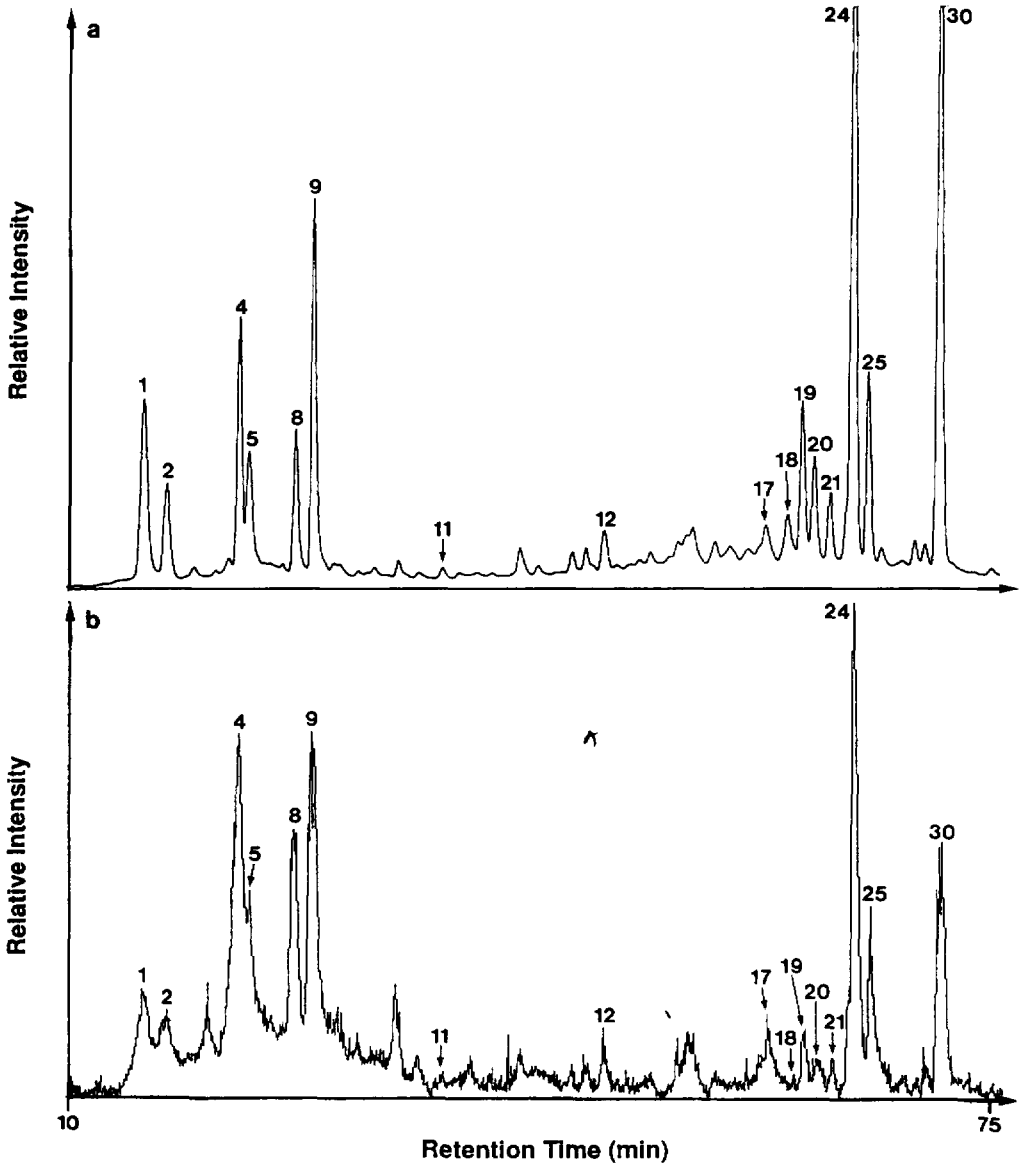

Fig. 5. Partial (10–75 min) (a) UV–VIS and (b) RIC chromatograms from negative-ion LC–MS analysis of GPC fraction 3. For peak identification see Table III. For HPLC and MS conditions see Experimental.

down *ca.* 25–35 years ago, although the dominance of chlorophyll *a*- and *b*-derived products in the sediment indicates that the major tetrapyrrole input was algal in origin.

Peak 12 appears to contain two other co-eluting phaeophytins ($M^{-\cdot}$ = *m/z* 788 and 762, Table III) with major components (peaks 24, 25 and 30) being the phaeophytin *a* epimers (**7**, $M^{-\cdot}$ = *m/z* 870) and pyrophaeophytin *a* (**8**, $M^{-\cdot}$ = *m/z* 812), as shown by comparison with standards.

Peak 17, as well as containing phaeophytin *b* (**5**), showed an abundant $M^{-\cdot}$ at *m/z* 872, which, based on its retention position, could correspond to the methoxylactone of pyrophaeophytin *b* (**14**), an autoxidation product formed in the presence of a nucleophilic solvent, *e.g.*, methanol [26]. Likewise, peak 18 appears to contain the C-13[2] epimer of this component, co-eluting with the phaeophytin *b* epimer (**5**). Similarly, peak 21, with $M^{-\cdot}$ at *m/z* 916, appears to contain the methoxylactone autoxidation product of phaeophytin *a* (**15**).

A comparison between the UV–VIS -monitored chromatogram of fraction 3 and the recontructed ion current chromatogram (RIC) is shown in Fig. 5, indicating only a slight loss of chromatographic resolution between the HPLC detector and the ion source. The differences in the relative abundances of the components are thought to result from differences in (i) absorption at 400 nm and (ii) ionization efficiencies [15]. From injection of a known amount (see Experimental) of pyrophaeophorbide *a* methyl ester (**4**) and of phaeophytin *a* (**7**), peaks 9 and 24 correspond to *ca.* 560 nmol and 880 nmol of these components. The detection limits of **4** and **7** to give an acceptable mass spectrum and a 3:1 signal-to-noise ratio in the mass chromatograms of *m/z* 548 (for **4**) and 870 (for **7**) were 15–25 nmol (10–20 μg).

Gel fractions 4 and 5

The UV–VIS spectra of fractions 4 (Fig. 3c) and 5 (minor) indicated them to contain predominantly tetrapyrrole pigments. The tetrapyrrole distributions are very similar and are dominated by phaeophytins (peaks 23–25 and 30, Table III) of the chlorophyll *a* and *b* series.

Gel fraction 6

This minor fraction showed a number of components found in earlier fractions, presumably as a result of "tailing" in the gel permeation chromatogram. Peaks 15 and 16 (Table III) were assigned as the aromatic carotenoid hydrocarbons isorenieratene (**1**, peak 15) and renieratene (**16**) or renierapurpurin (**17**) from comparison with a standard of isorenieratene. The occurrence of isorenieratene provides further molecular evidence for the presence of anaerobic photosynthetic bacteria in the water column when the sediment was laid down, as it has been reported as a major carotenoid in some species of the Chlorobiaceae [27], such bacteria having been reported to occur in the water column of Priest Pot (see above).

Peaks 33–39 showed a series of $M^{-\cdot}$ species which had unexpectedly high *m/z* values in comparison with the other tetrapyrroles observed. The spectrum of the major component in peak 37 (Fig. 4b) showed significant fragment ions at *m/z* 534 and 516. From direct comparison with the spectrum of pyrophaeophytin *a* [15], *m/z* 534 would indicate loss of the esterifying side-chain as $C_{29}H_{48}$ by way of a rearrangement with hydrogen transfer, *i.e.*, that the esterifying alcohol is a C_{29} monounsaturated sterol, as $C_{29}H_{48}$ corresponds formally to a C_{29} steroid diene. The ion at *m/z* 516 corresponds to an additional loss of H_2O [15]. The spectra from peaks 33–36 and 38–39 all showed similar characteristics, with *m/z* 534 and 516 as significant fragment ions (Table III), the differences being attributable to different C_{27}–C_{30} sterol or stanol esterifying moieties. On this basis, the chlorin nucleus appears to be the same as in pyrophacophytin *a* (**8**). In this context, the two major esterified chlorins in an ancient lacustrine sediment of Miocene age, which were characterized after isolation of the

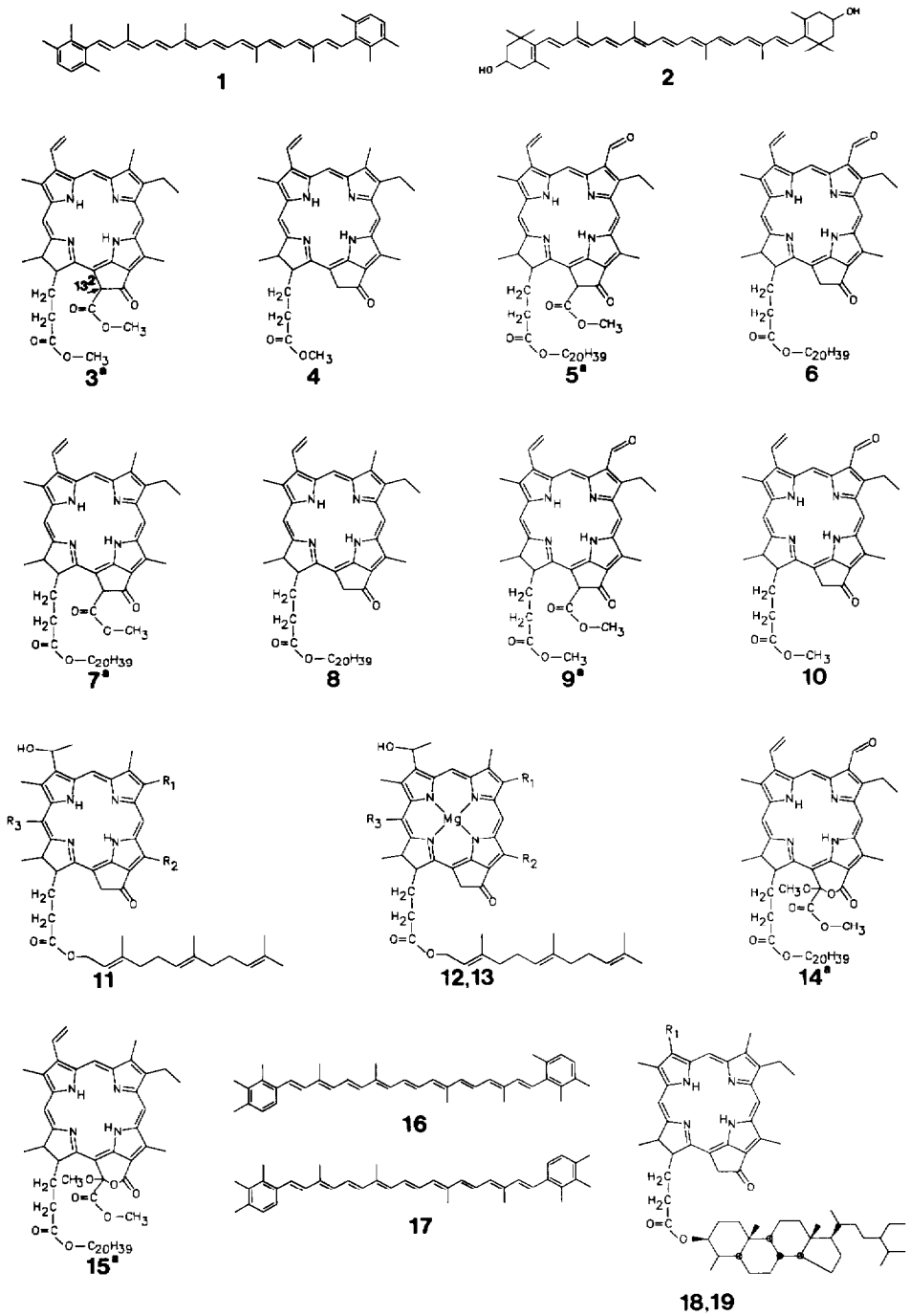

Fig. 6. Structures of compounds. **1** = Isorenieratene; **2** = zeaxanthin; **3**[a] = phaeophorbide *a* methyl ester; **4** = pyrophaeophorbide *a* methyl ester; **5**[a] = phaeophytin *b*; **6** = pyrophaeophytin *b*; **7** = phacophytin *a*; **8** = pyrophaeophytin *a*; **9**[a] = phaeophorbide *b* methyl ester; **10** = pyrophaeophorbide *b* methyl ester; **11** = major component in peak 11 (*cf.*, Fig. 3a and b), R_1 = Et, $R_{2,3}$ = Me or R_1 = *n*-Pr, R_2 = Me, R_3 = H or $R_{1,2}$ = Et, R_3 = H; **12** = bacteriochlorophyll *c*, R_1 = Et, $R_{2,3}$ = Me; **13** = bacteriochlorophyll *d*, R_1 = *n*-Pr, R_2 = Me, R_3 = H or $R_{1,2}$ = Et, R_3 = H; **14**[a] = phaeophytin *b* methoxylactone; **15**[a] = phaeophytin *a* methoxylactone; **16** = renieratene; **17** = renierapurpurin; **18** = pyrophaeophorbide *a* sterol ester (*cf.*, [28]), R_1 = vinyl; **19** = mesopyrophaeophorbide *a* sterol ester (*cf.*, [28]), R_1 = Et. [Me = methyl, Et = ethyl, Pr = propyl. [a] Epimeric at C-13^2 (*cf.*, **3**)].

individual components, are the novel sterol esters **18** and **19** [28]. In that case, since no chlorophylls with sterol side-chains are known, it was concluded that the components were of algal origin, with the esterification being a biological reaction occurring at the time of sediment deposition. This study provides further evidence for this hypothesis, as components of this type are present in a bottom sediment from a present-day lake, and suggests that esterification of phaeophorbides with algal sterols is a novel and widespread transformation of chlorophyll degradation products in the aquatic environment. Further studies involving full identification of the Priest Pot components are in progress.

CONCLUSIONS

Negative-ion LC–MS provides a valuable approach in the investigation of the complex degradative pathways of chlorophylls in aquatic environments, can assist in providing structural information about novel transformation products and readily reveals the presence of co-eluting components.

The major chlorins of the bottom sediment (15–20 cm) of Priest Pot lake are transformation products of chlorophylls *a* and *b* of algal origin. Despite the incomplete separation on 50 Å Styragel of the pigment classes, resulting from a change in elution solvent from dichloromethane to dichloromethane–methanol, LC–MS analysis of the fractions has revealed the presence of (i) marker compounds (**1** and **11**) indicative of the presence of photosynthetic bacteria in the water column when the sediment was laid down *ca.* 25–35 years ago, and (ii) an unexpected suite of chlorins esterified with a range of C_{27}–C_{30} sterols and stanols.

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