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Characterisation of Naturally Occurring Steryl Esters derived from Chlorophyll *a*

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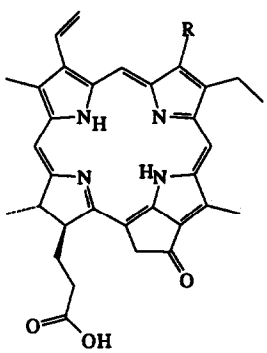
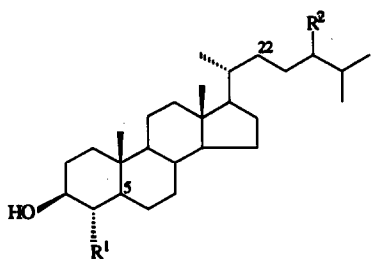
Key words: pyrophaeophorbide *a*, pyrophaeophorbide *b*, chlorophyll *a*, steryl esters of pyrophaeophorbide *a*, LC-MS, negative ion mass spectra.

Abstract: *A suite of steryl esters of pyrophaeophorbide* a *(1a), a water column degradation product of phytoplankton chlorophyll* a, *has been prepared in good yield using dicyclohexylcarbodiimide as dehydrating agent. Comparison and co-injection of these standards with the complex mixtures of steryl chlorin esters in various recent sediments, using negative ion HPLC-MS, has confirmed the structures of a number of the natural components.*

The recent discovery in an ancient lake sediment[1] and a recent marine sediment[2] of components comprising a chlorophyll *a* derived macrocycle (pyrophaeophorbide *a*, **1a**) esterified to a variety of sterols revealed the operation of an unexpected transformation pathway, involving hydrolysis and esterification, or transesterification, of the phytyl ester group originally present in chlorophyll *a*. That the pathway is ubiquitous is suggested by the further discovery of complex mixtures of these significant components (up to 20% of total chlorins) in a variety of recent sediments from different locations[3,4]. Their presence in a phytoplankton sample (containing > 50% dead and senescent cells) from the Baltic Sea, but not in actively growing cultures, indicates that the transformation reactions occur in the water column and are probably enzymatically mediated as a result of cellular disruption[4].



1a R = Me **1b** R = CHO



2a R^1 = Me, R^2 = Et; 5α(H)
b $R^1 = R^2$ = H; 5α(H)
c $R^1 = R^2$ = H; Δ^5
d R^1 = H, R^2 = Me; Δ^5
e R^1 = H, R^2 = Et; 5α(H)
f R^1 = H, R^2 = Et; $\Delta^{5,22}$

In one sample the component mixture was assigned by hydrolysis of the total steryl chlorin ester fraction, followed by GC-MS and HPLC analysis of the resultant sterols and chlorin nucleus **1a**, respectively[2]. In another case one isolated component was identified by partial assignment of the ^{1}H NMR spectrum and by GC-MS analysis of the sterol **2a** from hydrolysis[1]. To confirm the structures of a number of the compounds we have synthesised several standards for HPLC-MS comparison and co-injection studies.

The compounds were prepared from **1a** and the appropriate sterol **2b-f** by a mild esterification technique using dicyclohexylcarbodiimide (DCC) as dehydrating agent. The use of DCC has been reported previously[5] in the preparation of dimeric chlorophyll *a* derivatives linked via an ethylene glycol bridge. Initial attempts using this method afforded low yields, mainly as a result of significant formation of an unwanted N-acyl dicyclohexylurea derivative of pyrophaeophorbide *a*. This problem has been described previously[6], it being proposed that the N-acyl urea is more stable than the corresponding O-acyl urea that is an intermediate in the reaction. Holmberg and Hansen[6] resolved the problem by the addition of a small amount of strong acid, for example *p*-toluenesulphonic acid (*p*TSA), resulting in inhibition of N-acyl urea formation. Hence **1a** (typically 5mg, 9.3 μmol), the sterol (*ca.* 20 μmol), DCC (2.8 mg, 14 μmol) and *p*TSA (0.2 mg, 1μmol) were stirred in the dark under nitrogen (10 min.). Removal of the pyridine under reduced pressure and flash chromatography on silica (10% acetone in hexane), followed by reversed phase, semi-preparative HPLC afforded the ester (yield *ca.* 45 - 60%).

HPLC-MS analysis of the standards under reversed phase conditions with on line photodiode array (PDA) detection, as described previously[3], afforded negative ion mass spectra with the same characteristics as those of the natural compounds. The spectra all show the expected molecular ion, a fragment ion at *m/z* 534 corresponding to the pyrophaeophorbide *a* nucleus after loss of the steryl side chain (as the sterene) and a further peak at *m/z* 516 due to loss of water from the pyrophaeophorbide *a* nucleus (Fig. 1). The fragmentation pattern is analagous to that of pyrophaeophytin *a*, in which the base peak is the molecular ion at *m/z* 812 and the major fragment ions are also at *m/z* 534 and *m/z* 516.

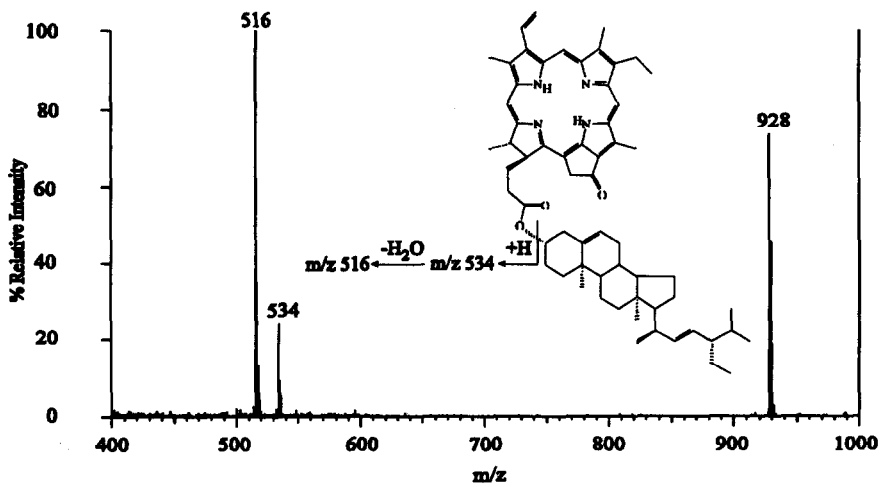


Fig 1: Negative-ion mass spectrum of stigmasteryl pyrophaeophorbide *a* ester.

The on line UV/VIS spectra (λ_{max}; 412, 508, 538, 610, 666 nm; in 90% acetone, 5% methanol, 5% water) are identical to those of the natural compounds. In the ^1H NMR spectra (400 Mhz, d^6 acetone) all the chemical shifts attributable to the chlorin acid moiety are virtually identical (± 0.04 ppm, except the propionic side chain protons, ± 0.18 ppm) to the corresponding ones in the spectrum of pyrophaeophytin *a* (phytyl ester of **1a**)[7]. The C-3 proton in the steryl moiety occurs as a multiplet at δ 4.15 ± 0.04 ppm and the compounds with C-5 unsaturation show a doublet at δ 5.11 ± 0.01 ppm for the C-6 proton. The NMR spectrum of stigmasteryl pyrophaeophorbide *a* ester exhibits a multiplet centered on δ 5.10 ppm due to the C-22 and C-23 alkenic protons.

Although there is extensive coelution within the complex natural mixtures, co-chromatography of the appropriate component with the standard could be readily discerned in each case from enhancement of the molecular ion. Where co-injections were not carried out components could still be assigned by comparison of retention times and mass spectra with those of the standards (Table 1). In all of the samples the cholesteryl ester of **1a** is a major component and it can be seen that the steryl esters synthesised are present in most of the sediments examined.

Samples	Setting	Sediment Depth	Water Depth	Esterifying sterol				
				2b	2c	2d	2e	2f
Priest Pot lake Cumbria, U.K.	lacustrine eutrophic	20-55 cm	*ca.* 4 m	*	✓	*	*	*
Lake Valencia Venezuela	lacustrine eutrophic	30-42 cm	*ca.* 40 m	✓	✓	*	x	✓
Black Sea 1 41°58' N, 35°30.3'E	marine anoxic	0-2 cm	*ca.* 160 m	*	✓	*	*	*
Black Sea 2 42°17' N, 35°22' E	marine anoxic	26-29 cm	*ca.* 1925 m	✓	✓	✓	o	*
Baltic Sea	marine	0-5 cm	*ca.* 97 m	o	✓	x	o	*

Table 1: Identification of sedimentary steryl esters of pyrophaeophorbide *a*.
Key: ✓ = co-chromatography * = component present (from t_R and MS data)
x = no co-chromatography o = component absent (from t_R and MS data)

As expected, the mass spectra of all of the significant components in the sediment fractions are consistent with a pyrophaeophorbide *a* nucleus, chlorophyll *a* being the major phytoplankton chlorophyll. Methanolysis (MeOH, 2% H_2SO_4, 1.5 hours, N_2, in dark) of the steryl chlorin ester fractions from Priest Pot and Lake Valencia bottom sediments was then carried out and the products were analysed by HPLC-MS. This revealed the presence of low relative abundances of pyrophaeophorbide *b* (**1b**), as can be seen from the

mass spectrum (Fig. 2). The identity was confirmed by co-injection of the methanolysis product with a standard of the methyl ester of pyrophaeophorbide *b*. Hence the steryl chlorin esters are not formed solely from chlorophyll *a*.

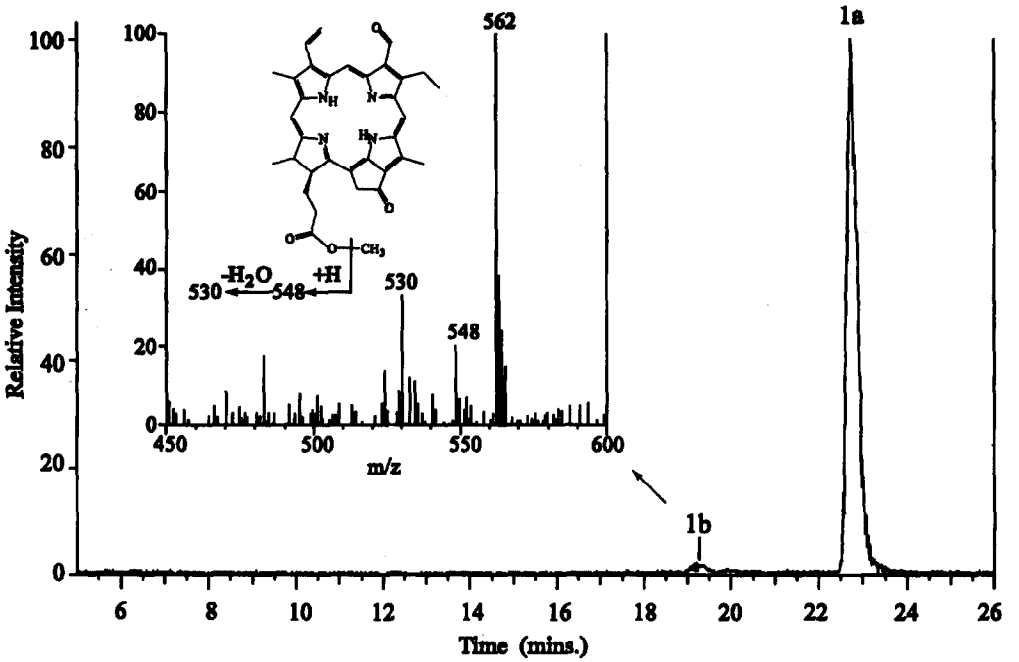


Fig 2: HPLC-MS chromatogram of methanolysis products from steryl chlorin ester fraction from Lake Valencia sediment. Inset shows negative ion mass spectrum of the methyl ester of pyrophaeophorbide *b*.

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