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Isolation and structure determination of the unstable ^{13}C , ^{17}C -Cyclophosphoride *a* enol from recent sediments

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Abstract—We observed that the unstable ^{13}C , ^{17}C -cyclophosphoride *a* enol is one of the most abundant pigments in recent sediments (Black Sea, Mediterranean Sea, Peru margin) and here we report its isolation and structure determination (high-performance liquid chromatography, ultraviolet-visible spectrometry, mass spectrometry, and nuclear magnetic resonance). This enol has been considered as the precursor of sedimentary chlorins and porphyrins bearing either a seven + five- or a seven-membered ring system. Analysis of sediment trap samples collected from the Peru Upwelling area revealed relatively high concentrations of ^{13}C , ^{17}C -cyclophosphoride *a* enol in trap samples containing high concentrations of metabolically derived carotenoid transformation products. The abundance of ^{13}C , ^{17}C -cyclophosphoride *a* enol in sediment trap samples and recent sediments suggests that formation of an exocyclic seven-membered ring is a major diagenetic pathway for chlorophylls. Biologically mediated transformations of chlorophylls can therefore yield products not included in the classical Triebis degradation scheme for cyclic tetrapyrroles. Copyright © 1999 Elsevier Science Ltd

1. INTRODUCTION

Chlorins and porphyrins **1–6** bearing either a seven + five- or a seven-membered ring system have been isolated from sedimentary organic matter (Fookes, 1983; Wolff et al., 1983; Ocampo et al., 1984; Chicarelli and Maxwell, 1986; Prowse et al., 1987; Keely and Maxwell, 1989; Keely et al., 1994; Verne-Mismer et al., 1990; Chillier et al., 1993; Sundararaman et al., 1994; Harris et al., 1995; Schaeffer et al., 1993,1994). Despite their instability, tetrapyrroles (chlorins and porphyrins) **1–5**, bearing a seven + five-membered exocyclic ring system, are often abundant in ancient and recent sediments. These compounds have been described as free tetrapyrroles **1–4** (Chicarelli and Maxwell, 1986; Prowse et al., 1987; Keely and Maxwell, 1989; Keely et al., 1994; Verne-Mismer et al., 1990; Chillier et al., 1993; Sundararaman et al., 1994; Harris et al., 1995) as well as tetrapyrroles **4,5**, bound to a macromolecular network *via* sulphide bridges (Schaeffer et al., 1993,1994). Their preservation has been attributed to depositional conditions (Verne-Mismer et al., 1990; Sundararaman et al., 1994) and to a very early, selective incorporation into a protective macromolecular environment (Schaeffer et al., 1993,1994). Chlorin **7** or its enolized forms **8** or **9** (see Fig. 1) have been proposed as a potential biological precursor of chlorins and porphyrins **1–7**. From a suite of recent sediments (Black Sea, Mediterranean Sea, and Peru margin), we have isolated and characterized the unstable ^{13}C , ^{17}C -cyclophosphoride *a* enol **8** (Ocampo et al., 1997).

2. EXPERIMENTAL PROCEDURES

2.1. Samples

Peru surface sediments were collected in a productive coastal upwelling system characterized by organic rich sediments (4–9% TOC)

underlying an intense oxygen minimum zone; samples were collected (July, 1987) by an MK-III box core device on *R/V* Moana Wave 8708 at 14°59.93' S, 75°50.53' W in 285 m water depth. Surface sediments (0–10 cm) from the Black Sea were collected (May, 1988) at 42°50' N, 32°00' E in 2129 m water depth (*R/V* Knorr 134–9) with the same coring device. Samples of Unit II sapropel were collected (March–April, 1995) at a nearby station 42°58' N, 31°25' E (*R/V* Knorr 134–8) in 2066 m water depth. The Black Sea is currently an oligotrophic system characterized by relative low productivity, but with anoxic intermediate and deep waters below depths of 100–200 m. Samples of Mediterranean Sea sapropel S7 were collected by the Ocean Drilling Program Leg 160, Hole 969C, that cored at 33°50.323' N, 24°53.005' E on the Mediterranean Ridge, approximately 160 Km south of Crete. Sapropel S7 was sampled from Core 1H, section 02, interval 76–82 cm, and was deposited approximately 193 Kyr before present.

2.2. General

Ultraviolet-visible (UV-vis) spectra were recorded on a Hewlett-Packard HP8452-A spectrophotometer in CH_2Cl_2 and acetone. High-performance liquid chromatography (HPLC) separations were performed using a Waters 600 E multisolvent delivery system (Rheodyne 7125 syringe loading sample injector) with a Waters 990-photodiode array detector and software. Separation conditions were as follows: Alltech adsorbosphere HS RP-18, 3 μm (4.6×150 mm), 1.5 mL/min using a gradient from 100% A to 100% B in 30 min, followed by a 30-min isocratic elution of B. Eluent A is a 20% (0.5 N) ammonium acetate in MeOH, and eluent B is acetone-MeOH (1/4). A Showa Denko K.K. Shodex GPC K-801, exclusion limit of 1500 Dalton (8×300 mm) and fitted with a Shodex K-G guard column (8×100 mm, 1 mL/min CH_2Cl_2) was also used. Mass spectra were obtained on VG AutoSpecQ (CH_4 as ionization gas, 35 eV) and Finnigan Mat TSQ 70 (direct insertion probe, 16 eV) spectrometers. Elemental analysis and stable isotopic measurements were performed at the Marine Biological Laboratory in Woods Hole using a Heracus CHN Rapid Elemental Analyzer coupled to a Finnigan Mat Delta S IRMS. All solvents were HPLC-grade; glassware was cleaned with HPLC-grade solvents. Pasteur pipettes, Na_2SO_4 , glass vials, glass fiber filters, sand, aluminum foil, and glass wool were precombusted at 450°C for 8 h. Teflon cap liners, cotton and boiling chips were Soxhlet extracted in MeOH- CH_2Cl_2 (7/93). Silica gel plates G/UV₂₅₄ 0.25 mm and alumina IB (0.25 mm) and PR-18 MKC-18F (0.2 mm) plates were used for thin layer chromatography.

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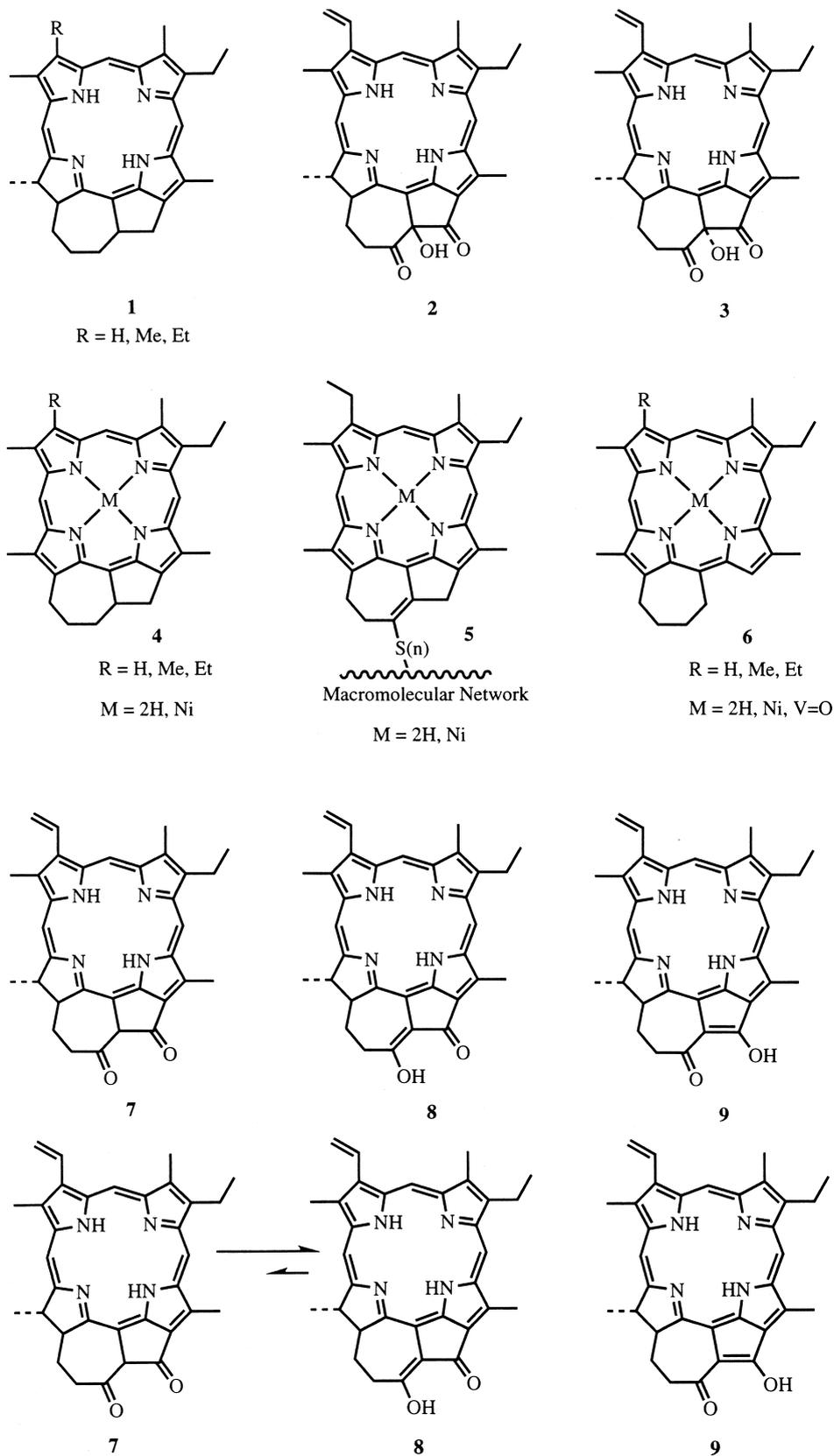


Fig. 1. Chlorin 7 and its enolized forms 8 and 9. This compound exists only as enolic form 8 in solution and in the crystalline state. The five-carbon exocyclic ring of enol 9 bears an additional double bond and is far more strained than enol 8. Thus enol 9 is sterically unfavorable compared to enol 8.

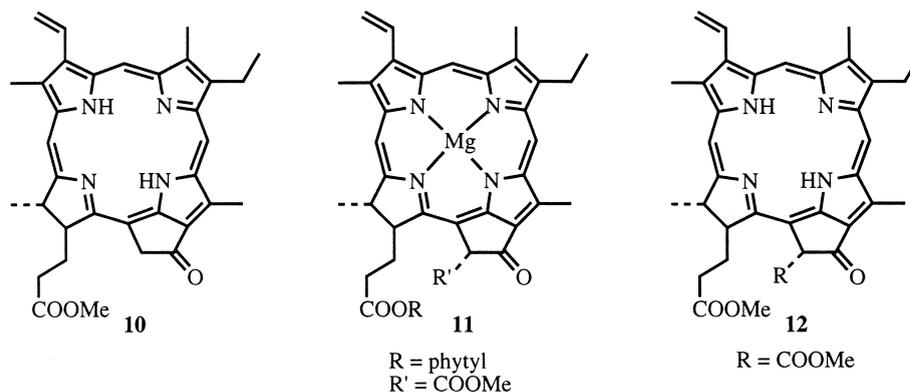


Fig. 1. (Continued)

2.3. Hemisyntheses

2.3.1. Methyl pyropheophorbide *a* (10)

Pyropheophorbide *a* methyl ester **10** was prepared from chlorophyll *a* **11** (extracted from stinging nettles) after demetallation to pheophytin (HCl 2N), transesterification (MeOH + 5% H_2SO_4), chromatographic separation of methyl pheophorbide *b* (silica gel; diethylether) and finally refluxing on collidine (Fischer and Orth, 1937; Jeandon et al., 1993).

2.3.2. 13^2 , 17^3 -Cyclophenophorbide *a* enol (8)

To a solution of pyropheophorbide methyl ester (**10**; 109.2 mg, 0.2 mmol) in THF (12 mL), under an atmosphere of argon, was added $(\text{TMS})_2\text{-NNA}$ (1.4 mL, 1.4 mmol, 1.0 M in THF). The yellow solution was stirred at room temperature for 3 min and then poured on to a desoxygenated (Ar) mixture of CH_2Cl_2 (160 mL), saturated NaH_2PO_4 (40 mL), and ice (40 g). The mixture was shaken until the yellow color turned to bright green. After the aqueous phase was separated, the organic phase was dried over Na_2SO_4 , filtered and evaporated in vacuo. The product was crystallized from CH_2Cl_2 -hexane under argon, giving the title product (**8**; 84 mg, 85%). The product was recrystallized three times from CH_2Cl_2 -hexane under argon.

UV-vis λ_{max} (CH_2Cl_2) 362, 418, 508, 536, 630, 686 nm (rel. int. 0.95, 1.0, 0.08, 0.08, 0.15, 0.44); EIMS m/z 516 (M^+); $^1\text{H NMR}$ (500 MHz, 12 mg/mL C_6D_6) δ 13.96 (s, OH), 8.95 (s, 5-H), 8.56 (s, 10-H), 7.75 (dd, $J = 18.0, 11.8$ Hz, 3'-H), 7.40 (s, 20-H), 6.07 (dd, $J = 18.0, 1.5$ Hz, 3''a-H), 5.87 (dd, $J = 11.8, 1.5$ Hz, 3''b-H), 3.29 (q, $J = 8.0$ Hz, 8'-2H), 2.98 (s, 12'-3H), 2.90 (s, 7'-3H), 2.87 (s, 3'-3H), 2.65 (m, 18-H), 2.32 (m, 17'-Ha), 2.2 (2m, 17-H, 17'-Hb), 1.53 (t, $J = 8.0$ Hz, 8''-3H), 1.20 (m, 17''-Ha), 1.19 (d, $J = 7.0$ Hz, 18''-3H), 1.10 (m, 17''-Hb), 0.64 (b s, NH), -1.32 (b s, NH).

(Falk et al., 1975): UV-vis λ_{max} (CH_2Cl_2) 361, 429, 455, 629, 688 nm. $^1\text{H NMR}$ (100 MHz, 20.6 mg/mL CDCl_3) δ 12.93 (s, OH), 8.33 (s, 10-H), 8.06 (s, 5-H), 7.6–7.3 (dd, 3'-H), 6.74 (s, 20-H), 5.80 (dd, 3''-2H), under 3.2 (17-H, 18-H), 3.13 (q, $J = 7.5$ Hz, 8'-2H), 2.80/2.74 (3s, 2', 3H, 7'-3H, 12'-3H), 2.2–1.7 (m, 17'-2H, 17''-2H), 1.42 (t, $J = 7.5$ Hz, 8''-3H), 0.84 (d, $J = 7.5$ Hz, 18'-3H), 0.6 (br s, NH), -2.38 (br s, NH).

(Ma and Dolphin, 1996): UV-vis λ_{max} (CH_2Cl_2) 364, 430, 456, 592, 630, 690 nm. $^1\text{H NMR}$ (400 MHz, 2.5 mg/mL CDCl_3) δ 13.24 (s, 1H), 8.64 (s, 1H), 8.43 (s, 1H), 7.38 (s, 1H), 7.70 (dd, $J = 18.0, 11.6$ Hz, 1H), 6.12 (dd, $J = 18.0, 1.6$ Hz, 1H), 6.04 (d, $J = 11.6, 1.6$ Hz, 1H), 3.31 (q, $J = 7.9$ Hz, 2H), 3.08 (s, 3H), 3.02 (s, 3H), 2.94 (s, 3H), 2.93 (q, $J = 7.2$ Hz, 1H), 2.58 (m, 1H), 2.45 (t, 2H), 1.71 (m, 2H), 1.80 (d, $J = 7.1$ Hz, 3H), 1.52 (t, $J = 7.9$ Hz, 3H), 0.30 (br s, 1H), -1.72 (br s, 1H).

3. RESULTS AND DISCUSSION

3.1. Extraction and detection of pigments

When solvent extracts from sediments of the Black Sea, Unit I (surface) and Unit II (3–5 kyr b.p.), Eastern Mediterranean Sea, sapropel S7 (193 kyr b.p.) and Peru margin (surface), were applied to gel permeation column chromatography (GPC) we

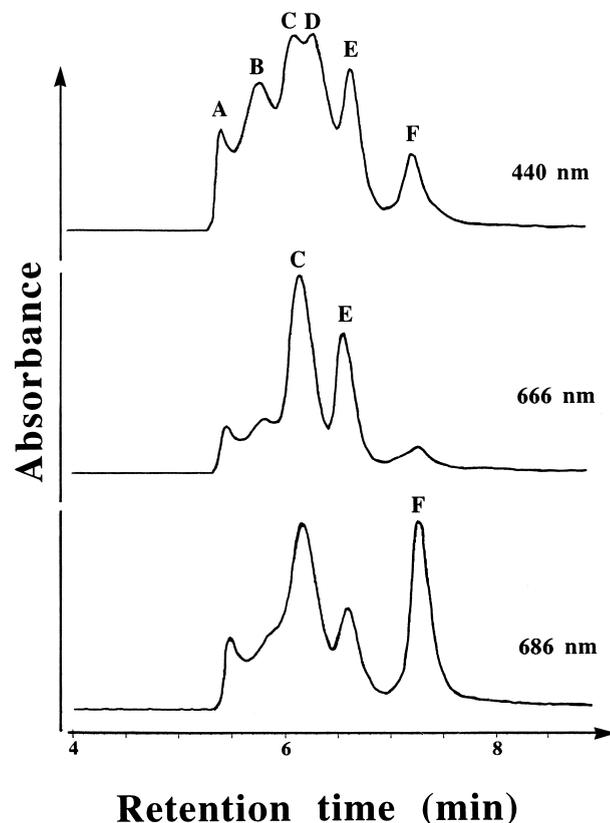


Fig. 2. Gel permeation chromatography of a Peru Margin sediment extract at 440 nm, 666 nm, and 686 nm. **A** = high molecular weight chlorins; **B** = chlorin steryl esters; **C** = pheophytin; **D** = carotenoids; **E** = pheophorbides; **F** = Chlorin 686. Conditions: Shodex K-801 column (8 x 300 mm) eluted with methylene chloride at 1 mL/min.

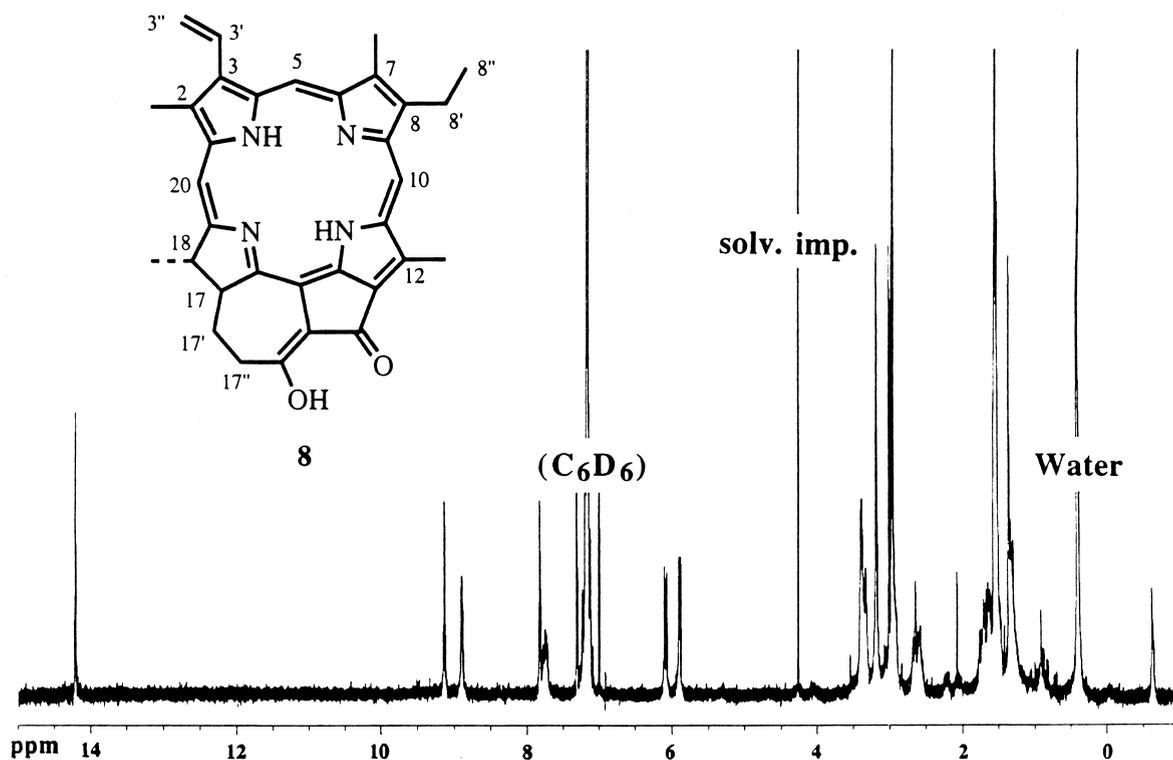


Fig. 3. ^1H NMR (500 MHz, C_6D_6) spectrum of Chlorin 686.

observed, among the classical chlorophyll degradation products detected at 440 and 666 nm (Fig. 2), the presence of an unknown chlorin-like compound with λ_{max} 686 nm. Chlorin 686 is of interest because of its high abundance in all the samples (it seemed to be the most abundant chlorin in the Black Sea samples). We estimated the percentage of Chlorin 686 relative to total chlorins based on UV-vis spectra; Peru margin = 11%, Peru sediment trap 6 = 5%, Peru sediment trap 14 = 12%, Mediterranean Sea sapropel (S7) = 3.1%, Black Sea Unit I = 50%, and Black Sea Unit II = 21%. This compound has an unusual UV-vis spectrum with a broad and split Soret band: λ_{max} (CH_2Cl_2) = 362, 418, 456, 508, 536, 630, 686 nm (rel. int. 0.95, 1.0, 0.57, 0.08, 0.08, 0.15, 0.44); in acetone, λ_{max} = 412 instead of 418 nm. It is more strongly retained by GPC than other chlorins, eluting after methyl pheophorbide *a* (**12**) (Fig. 2).

Compounds with an absorption at 686 nm have occasionally been detected in extracts of sedimentary organic matter. Such a type of compound was alluded to in an early report addressing pigments in a Black Sea surface sediment (Peake et al., 1974), and was attributed to an "aberrant type of chlorophyll." A compound called "phorbide-686.5," was reported in sediments from a sub-bottom depth of 31–35 m in the Gulf of California (Baker and Louda, 1982; Louda and Baker, 1986). Finally, upon re-inspection of chromatograms of Black Sea surface sediment extracts (King, 1993) we found it was also present in these samples.

3.2. Isolation of individual pigments

To study Chlorin 686 at the molecular level, we isolated it from the Unit I Black Sea surficial sediment by GPC. It was

then purified by RP-18 HPLC. It is a relatively non-polar chlorin, eluting just before pheophytin *a* on RP-18 HPLC. Chlorin 686 is very unstable and is destroyed under chromatographic conditions such as silica gel, alumina, and in some cases on RP-18 HPLC. Destruction of Chlorin 686 occurs even when antioxidants (ascorbic acid) as well as buffer or basic solutions (sodium phosphate, *t*-butylamine, etc.) were added to the mobile phase during elution. Chlorin 686 was purified by recrystallization from CH_2Cl_2 -hexane to conduct further studies by MS and NMR.

3.3. Structure elucidation

Chlorin 686 has major ions at m/z 413 (100%), 429 (76%), 465 (21%), and 517 (20%) on CH_4 CIMS and m/z 516 (M^+) on EIMS. The C/N ratio is 12.4, intermediate between those of pyropheophytin *a* (13.25) and methyl pyropheophorbide *a* (8.5). In Black Sea sediments chlorin 686 has an $^{14}\text{N}/^{15}\text{N}$ isotopic composition ($\delta^{15}\text{N} = -4.9\%$) identical to pyropheophytin *a* (-4.8%) but their $^{12}\text{C}/^{13}\text{C}$ isotopic compositions are slightly different ($\delta^{13}\text{C} = -23.1$ and -24.9% , respectively) (Sachs, 1997). ^1H NMR (500 MHz, C_6D_6) studies (Fig. 3) indicated that Chlorin 686 has a "northern half" moiety very similar to that of pheophorbide *a* and related chlorins (meso protons at 9.13, 8.89, and 7.83; vinyl protons at 7.75, 6.08, and 5.99; methyls at 3.18, 2.97, 2.96, and 1.54; ethyl at 3.39 and 1.55 ppm, etc.). Further ^1H NMR studies demonstrated the presence of an enol proton at 14.20 ppm and suggested that Chlorin 686 may be the unstable 13^2 , 17^3 -cyclophorbide *a* enol **8** (Fig. 3). Chlorin 686 ^1H NMR (500 MHz, C_6D_6) δ 14.20 (s, OH), 9.13 (s, 10-H), 8.89 (5-H), 7.83 (s, 20-H), 7.75 (dd,

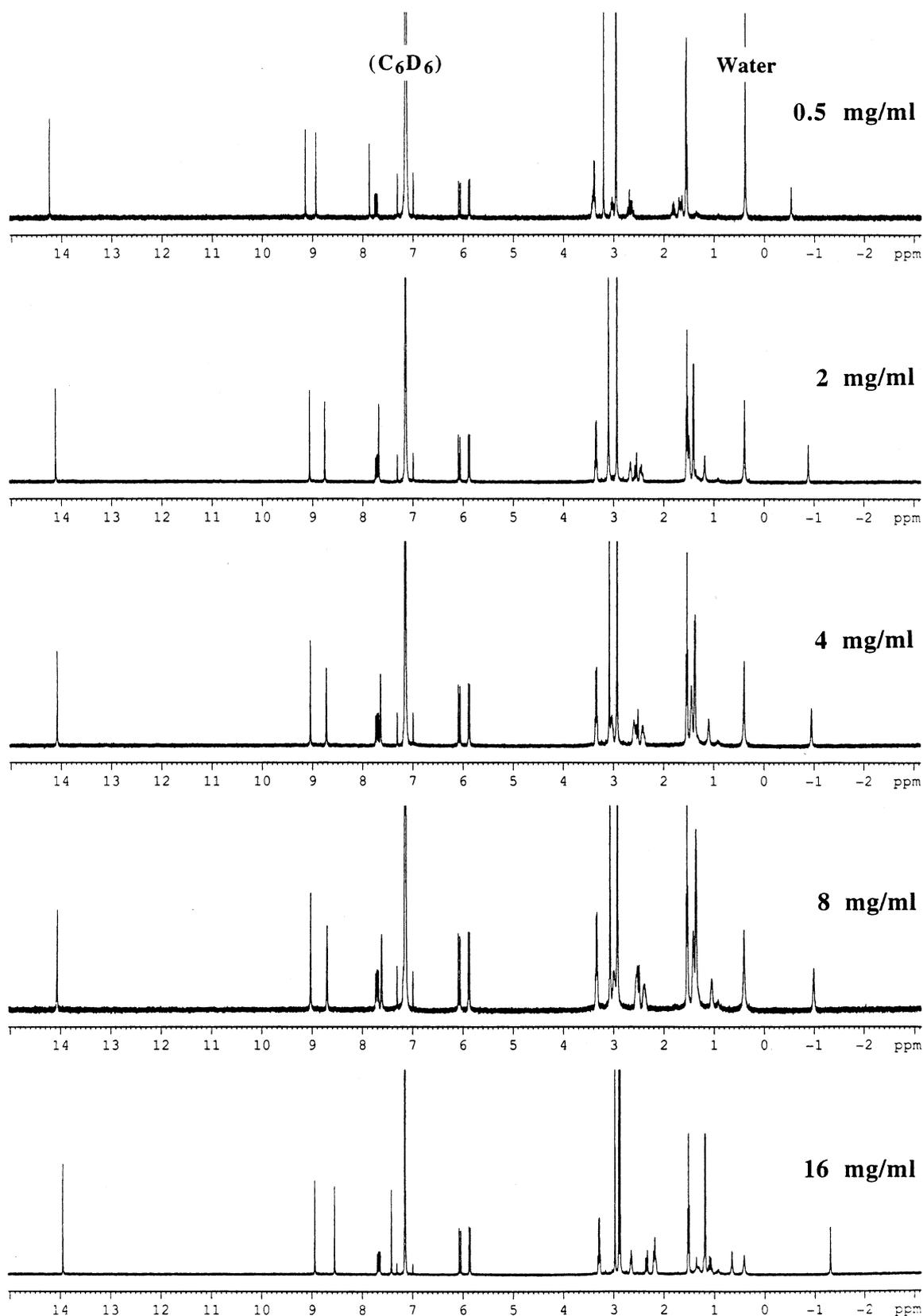


Fig. 4. ^1H NMR (500 MHz, C_6D_6) spectra of hemisynthetic ^{13}C , ^{17}O -cyclophenophorbide *a* 8 at different concentrations (from 0.5 mg/mL to 16 mg/mL).

$J = 18.0, 11.8$ Hz, 3'-H), 6.08 (dd, $J = 18.0, 1.5$ Hz, 3''Ha), 5.89 (dd, $J = 11.8, 1.5$ Hz, 3''Hb), 3.39 (q, $J = 8.0$ Hz, 8'-2H), 3.38 (m, 18-H), 3.18, 2.97, 2.96 (3s, 12'-3H, 7'-3H, 2'-3H), 2.92 (m, 17'-H), 2.65, 2.59 (2m, 17-H, 17'H), 1.69, 1.67 (2m, 17''-Ha, 17''-Hb), 1.55 (t, $J = 8.0$ Hz, 8''-3H), 1.54 (d, $J = 7.0$ Hz, 18''-3H), 1.33 (b s, NH), -0.60 (b s, NH). To confirm this hypothesis, we prepared a synthetic standard of **8**, from Me pyropheophorbide **a** **10**, according to the procedure of Falk et al., 1975 and modified by Ma and Dolphin, 1996. We observed a strong aggregation effect for different protons, changes in chemical shifts can reach up to 0.8 ppm (Abraham et al., 1979; Krane et al., 1983; Ocampo et al., 1985), when we run ^1H NMR (500 MHz, C_6D_6) experiments at different concentrations (from 0.5 mg/mL up to 16 mg/mL) (Fig. 4). We compared, our hemisynthetic and sedimentary samples, by UV-visible, GPC, RP-18, MS, and ^1H (500 MHz) NMR (at similar dilutions close to 0.5 mg/mL) (Figs. 3 and 4a). The chromatographic and spectroscopic behavior of both samples (Chlorin 686 and hemisynthetic 13², 17³-cyclophorbide **a** enol **8**) were virtually identical.

Chlorins **2,3,7-9** occur as metabolites of chlorophyll *a* in sponges and clams (Karuso et al., 1986; Sakata et al., 1990; Yamamoto et al., 1992; Watanabe et al., 1993), but have not been reported in marine algae or in phytodetritus collected by sediment traps. We found no evidence of enol **8** upon a re-examination of pigment data for Black Sea sediment traps, even though this compound is very abundant in the underlying sediments. However, re-analysis of sediment trap samples (24–70 h deployments, 20–40 m) collected from the Peru Upwelling area revealed relatively high concentrations of enol **8** in samples with high concentrations of metabolically derived carotenoid transformation products (Table 5 and samples 3, 6, 12, and 14 in Repeta and Gagosian, 1984). Sediment trap samples with pigment distributions dominated by unaltered carotenoids (Table 5 and samples 1, 2, 4 in Repeta and Gagosian, 1984) contain little enol **8**. Enol **8** is occasionally observed in laboratory grazing experiments (Goericke et al., 1999), suggesting that zooplankton and other microherbivores do have the ability to alter chlorophylls to give the seven + five-membered exocyclic ring system. In the sea, production during grazing in the water column and removal on large rapidly sinking particles is therefore one route by which enol **8** is added to sediments. However, the high abundance of enol **8** in the Black Sea sediment samples suggests either transformation during water column grazing is episodic and restricted to specific classes of herbivores, or that chemical or microbiologically mediated post depositional transformation processes may also be an important route for enol **8** formation.

Chlorins **2,3,7-9** share a similar structural framework pattern to a series of fossil chlorins and porphyrins **1-5** bearing a seven + five-membered ring system, and have been considered to be their diagenetic precursors. Chlorins and porphyrins **1-5** have been isolated, sometimes as major components, from sedimentary organic matter and described as free tetrapyrroles **1-4** (Chicarelli and Maxwell, 1986; Prowse et al., 1987; Keely and Maxwell, 1989; Keely et al., 1994; Verne-Mismer et al., 1990; Chillier et al., 1993; Sundararaman et al., 1994; Harris et al., 1995) as well as tetrapyrroles bound **5**, *via* sulphide bridges, to the macromolecular network of a sulfur-rich marl (Schaeffer et al., 1993,1994). The abundance of chlorins and porphyrins **1-4**

in some sediments contrasts with their high instability and may be linked to special depositional conditions (Verne-Mismer et al., 1990; Sundararaman et al., 1994) or, in the case of **5**, to a selective incorporation into a protective macromolecular environment (Schaeffer et al., 1993,1994). Another series of geoporphyrins that could be fossils of chlorins **2,3,7-9** is that of porphyrins **6** bearing a seven-membered ring system (Fookes, 1983; Wolff et al., 1983; Ocampo et al., 1984; Verne-Mismer et al., 1990). The abundance of enol **8** in our samples suggests an important contribution of chlorins **2,3,7-9** as well as other chlorophyll degradation products to the formation of fossil chlorins and porphyrins **1-5,6**. The presence and relative abundance of compound **8** in sediments reinforce the plausibility of a major degradation pathway leading chlorophyll degradation products through fossilization processes to the formation of fossil tetrapyrroles (chlorins and porphyrins) **1-6** bearing either a seven + five or a seven-membered ring system.

4. CONCLUSIONS

The presence of 13², 17³-cyclophorbide *a* enol **8** in the samples studied provides an explanation why sedimentary chlorins and porphyrins **1-5** bearing a seven + five-membered ring system are, in some cases, very abundant in geological samples. Our results confirm that 13², 17³-cyclophorbide *a* enol **8** is a potential precursor of these tetrapyrrole fossils **1-5** as well as porphyrins **6**. Available data from water column suspended particulate matter and sediment trap pigment analyses suggest that enol **8** is formed largely by biologically mediated processes and attest to a major chlorophyll diagenetic pathway not included in the classical Treibs degradation scheme.

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