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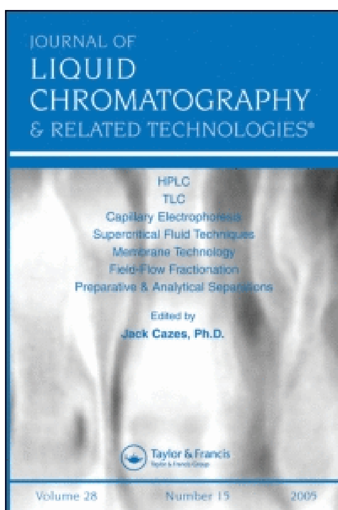

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Polystyrene-Divinylbenzene (PS-DVB), a Mild Stationary Phase for the Chromatographic Purification of the Unstable 13^2, 17^3-Cyclopheophorbide *a*-Enol

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Polystyrene-Divinylbenzene (PS-DVB), a Mild Stationary Phase for the Chromatographic Purification of the Unstable 13^2, 17^3-Cyclopheophorbide *a*-Enol

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Abstract: One of the most abundant pigments in certain recent sediments (Black Sea, Mediterranean Sea, Peru margin, Florida Bay) is an unstable derivative of chlorophyll-a known as 13^2, 17^3-cyclopheophorbide *a*-enol (aka CPP, CYCLO, CPP516). To date, the purification of synthetic or natural samples of this compound has always been challenging, since it is readily converted to a ∼1:1 mixture of (13^2 *R/S*) chlorophyllones *a* (Chlone), 15^1- hydroxychlorophyllonelactone-*a*, and/or other derivatives when chromatographed over silica gel or alumina supports in normal or reversed phase modes. In this study, Polystyrene-Divinylbenzene (PS-DVB) support (aka Polymeric Reversed Phase, or PRP) is introduced as an efficient stationary phase for the flash column chromatographic purification of CPP with little oxidation/conversion to artifacts. Similarly, PRP-1 in analytical sized columns is shown to be highly useful for purity verification prior to UV/Vis, FTIR, and NMR characterizations.

Keywords: Cyclopheophorbide, Unstable pigments, Chlorophyll derivatives, Flash chromatography, Polymeric RP-HPLC, PS-DVB, PRP

INTRODUCTION

13^2, 17^3-Cyclopheophorbide *a*-enol (CPP, alternately CYCLO, Phorbide 686.5 or CPP516) has been synthesized by both Dieckmann[1,2] or

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"Claisen-type intramolecular"[3] routes and possesses an UV/Vis absorption spectrum unique amongst tetrapyrrole pigments. The synthesis of CPP, as well as other unusual dihydroporphyrins, has been reviewed.[4] CPP has been isolated from many natural sources including a sponge,[5] Pleistocene sediments from the Guaymas Basin in the Gulf of California,[6,7] the Black Sea,[8] and the highly sulfidic carbonate marls of Florida Bay.[9] In the later case, geochemical conversion of pyropheophorbide-*a* into CPP was traced downhole, mimicking the *in vitro* Dieckmann reaction.

CPP is so easily oxidized and rearranged that it must be considered as an extremely powerful antioxidant. Its extreme instability would, however, be a drawback for therapeutic uses in which the antioxidant nature could be of use. However, several closely related pigments have been investigated for use in photodynamic therapy (PDT) of certain cancers.[10]

13^2, 17^3-Cyclopheophorbide-*a*-enol (CPP) is so unstable that, when it is chromatographed over normal phase silica gel or alumina or even silica based C_{18} HPLC, it is converted partially or totally to other chlorophyll related artifacts. Often, silica based C_{18} HPLC leads to the partial oxidation of CPP resulting in a ~1:1 mixture of (13^2 *R*/*S*) chlorophyllones *a* (Chlone).[1] Purification of individual chlorins from sediments by means of size exclusion chromatography has also been reported.[2] In their report, Sachs and Repeta[2] first fractionated pigments, including CPP (aka Chl686[2]), over flash silica in a solid phase extraction method. However, prior to application of the pigments to their silica column, about 1 L of 7% MeOH/ Me_2Cl_2 was run through the column. The fact that (some/majority?) of the CPP went through their column, may be due to a "methanol deactivation" of silica akin to the technique reported by Purcell.[11] However, in our hands, MeOH deactivated silica, while passing some CPP in an unaltered state, always led to more artifacts than starting material (CPP) being eluted.

Alumina ($Al_2O_3 \cdot nH_2O$) is a relatively strong Lewis acid, while silica gel and even 'end capped' reversed phase silicas have silanol groups capable of acting as proton donors and/or strong hydrogen bonding sites.

Previously, our group has isolated CPP from deep sea sediments,[7] though misidentified at the time,[6,7] using increasing percentages of acetone in petroleum ether (br 30–60°C) to fractionate pigments in columns of microcrystalline cellulose. It was and remains unknown as to how many of the 'unknown' chlorins[7] in that sample were actually generated from the CPP (aka phorbide-686.5) during chromatography.

Attempts at the purification of CPP on silica gel thin layer chromatography (TLC) failed due to decomposition of the pigment.[3] In our report on the geochemical transformation of pyropheophorbide-*a* into CPP with depth in the sulfidic carbonate marls of Florida Bay,[9] we separated CPP from sedimentary bitumen (lipid extractables) using highly end capped C_{18} silica based (Waters Nova Pak, 4 μm, 3.9 × 150 mm) reversed phase high performance liquid chromatography (RP-HPLC). However, several unknown chlorins with pheophorbide-*a*-like spectra were always noted and

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these may have derived from CPP upon exposure to the silica based packing. After comparison to presently known pigments, 2 of the unknowns in those samples were indeed the (R/S) chlorophyllones-*a*. Similarly, several reports exist in the literature of the successful isolation/identification of CPP by RP-HPLC.[12,13] However, it is usually also noted that the R/S chlorophyllones were also present/produced. The fact remains that, in extracts of natural sediments it appears easier to have at least some amount of CPP traverses the C_{18} silica based media without total alteration. It may be that the partially coeluting non-pigment lipids in some manner partially retard the conversion of CPP into other derivatives. Chlorophyllones-*a* apparently were also formed during the freeze drying processing[14] of sediment samples from which CPP had previously been isolated.[9]

To date, purification of hemisynthetic CPP has relied upon normal phase silica chromatography and crystallization techniques using CH_2Cl_2/hexane under nitrogen.[1–3]

In order to obtain optimized purity for CPP, or other labile chlorophyll derivatives or isolates, this study investigated polystyrene-divinylbenzene (PS-DVB, aka PRP for polymeric reversed phase) as a potentially suitable stationary phase for fast column chromatography (viz. flash LC). There are several advantages of using PS-DVB polymers over traditional reversed phase silica phases. First, there are obviously no silanol groups, free or end-capped. Second, this polymer is also quite stable toward most organic solvents, mobile phase additives as well as pH over the range of 1 to 13. Third, it can also be used at any temperature between 20 and 85°C.[15] Our studies were all at "room" temperature (~23–24°C).

As will be shown, CPP is easily purified by flash chromatography over PRP (PRP-1) without crystallization.

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EXPERIMENTAL

13^2,17^3-cyclopheophorbide-*a*-enol (CPP, 2), was prepared from pyropheophorbide-*a* methyl ester (**1**) following published methods.[1–4] The structural comparison of the starting material (**1**) and the product (**2**) is shown as Figure 1.

The starting material, pyropheophorbide *a* methyl ester (pPBIDa-ME), was prepared from chlorophyll-*a* by standard procedures[16,17] or most often purchased from Frontier Scientific (Logan, Utah, USA).

Sodium bis(trimethylsilyl) amide in THF (Aldrich Chemical Co.; 1.0 mL, 1.0 mmol, 1.0 M in THF) was added to a solution of pyropheophorbide *a* methyl ester (54.6 mg, 0.1 mmol) in anhydrous THF (6 mL) under an atmosphere of argon and stirred at room temperature for about 10 minutes. The reaction mixture was then poured on to a deoxygenated (Ar) mixture of CH_2Cl_2 (80 mL), saturated NaH_2PO_4 (20 mL), and ice (20 g) made from degassed deionized water. After solvent extraction, the organic layer was dried over sodium sulfate, filtered, and evaporated *in vacuo*.

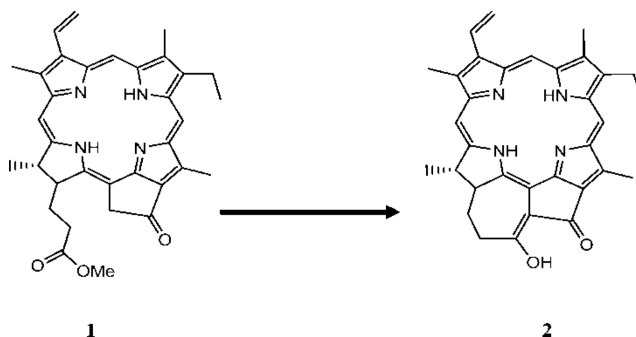

Figure 1. Cyclization reaction of pyropheophorbide-*a* (**1**) yielding CPP (**2**).

The preparative polymeric reversed phase material (PRP-1^{TM}; 50–75 μm particle and 100 Å pore sizes; P/N 79583) and the PRP-1^{TM} analytical column (4.1 × 250 mm, 5 μm particle and 100 Å pore sizes; P/N 79820) were purchased from Hamilton Inc. (Reno, Nevada, USA).

Additionally, standard RP-HPLC was performed using a Waters NovaPak C_{18} silica based column (3.9 × 300 mm, spherical 4 μm particle and 60 Å pore sizes, fully end-capped, 7.3% C load; P/N WAT011695), using a ternary gradient as given previously.[9]

Flash chromatography was performed using a 25 × 203 mm flash column (ACE Glass, # 5872-64) packed with preparative PRP-1 and isocratic elution with 90% acetonitrile. Pressure ($\leq$7 psi) was applied using UHP-grade argon. Eluates were evaporated *in vacuo* on a rotary evaporator.

Ultraviolet-visible (UV/Vis) spectra were recorded using a Perkin Elmer Model Lambda- 2 Spectrometer routinely quality assured versus holmium oxide.

HPLC was performed using a 100 μL loop with a Rheodyne 7125 injector, a Thermo Separation Products ConstaMetric 4100 series Quaternary solvent delivery system pump, a RP-HPLC column described above, with detection, spectral characterization and data manipulation using a Waters 990 Photodiode Array Detector (PDA) with Waters 990 software. A binary linear solvent gradient program at 1 $mL \cdot min^{-1}$ flow rate was used (Table 1). Columns were stored in 85% aqueous methanol. All solvents were HPLC grade.

All procedures were performed either in the dark or subdued yellow light and solutions were kept cold/frozen and under argon whenever possible.

Mass spectra were obtained using a Thermo Electron LCQ Deca (San Jose, CA) ion trap mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. Data acquisition was performed using Xcalibur software running on windows PC. Direct infusion introduced the sample to the APCI chamber using a ternary solvent system of acetonitrile/water/acetone (85:10:5, v/v/v) at a flow rate of 5 μL/min. The vaporizer and the transfer capillary temperature were set at 450°C and

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Table 1. Solvent gradient used for RP-HPLC with PRP-1

Time (min)	% A 90% Acetonitrile	% B 100% Ethyl acetate
0	75	25
15	75	25
20	50	50
45	50	50
50	0	100
55	0	100
59	75	25

250°C, respectively. The corona discharge current was set to 5 μA. Mass spectra were obtained in the positive mode, scanning a range of 100 to 1000 m/z in 1 second.

Nuclear magnetic resonance (NMR) structural confirmation experiments were performed on a 400 MHz Varian Mercury-plus™ in 1D (^{1}H, ^{13}C) and 2D (COSY, NOESY, HSQC) modes.

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RESULTS AND DISCUSSION

The crude product (CPP, 2) was flash chromatographed over the polymeric reversed phase (PRP-1™) using 90% acetonitrile and 100% ethyl acetate as solvents. Major (polar) impurities eluted with 90% acetonitrile. CPP was collected as the heart cut of a bright green band and the solvent evaporated *in vacuo*. The leading and tailing portions of the major (CPP) band were pooled and rechromatographed to recover CPP not in the first batch, if necessary. That is, if only the rapid purification of CPP directly from the reaction mixture is desired, only flash chromatography is required. If, on the other hand, maximization of overall yield is a consideration, then rechromatography of the non-heart cut materials, with or without crystallization, can be added to the procedure.

The UV spectrum of pure CPP, shown as Figure 2, exhibits maxima at 361, 427, and 687 nm (CH_2Cl_2), and matches the literature.[6] Second derivative chromatography was found to be extremely helpful in screening CPP fractions for the presence of alteration products. That is, the unique chromophore and band I absorption of CPP ($\lambda = 687$ nm) sets it apart from the majority of its alteration products, which revert to a pheophorbide-*a* or chlorin-like (band I $\lambda \sim 662-668$ nm) spectra.

The APCI-MS of CPP (Calculated MW = 516) shows base peak at 517.4 m/z in the positive mode, corresponding to the pseudomolecular ion ($[M + H]^+$) of the target compound.

NMR data is not presented here, but all modes confirm the structure of CPP.[3] These data will be published elsewhere during presentation of

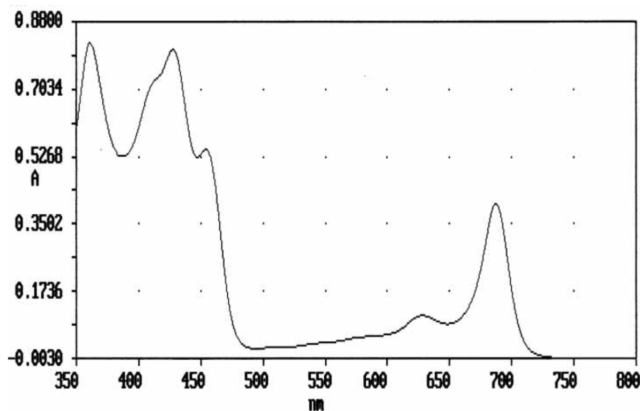

Figure 2. UV spectrum of pure 13^2, 17^3-cyclopheophorbide *a* enol in CH_2Cl_2.

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the hemi-synthesis and structural characterization of a related compound, *meso*CPP.

Figure 3 is the chromatogram of the purified CPP (eluting at ~27.1–27.5 min) at 410 nm. As it shows, CPP is oxidized over the silica column to form mainly R/S chlorophyllones (17–19 min.) plus a few more minor unidentified products (~16.5, 21, 24, 26, 31.2 min.). The instability of Chlorin 686 during chromatographic conditions with silica gel, alumina, and in some cases on silica based RP-C_{18} HPLC has been confirmed previously.[1]

Since column chromatography of Chlorin 686 over PRP-1 was a new and successful approach for purification, an analytical PRP-1 HPLC column was utilized to obtain a chromatogram which proves the purity of CPP. The solvent gradient was given above as Table 1.

Figure 4a is the chromatogram of purified CPP as determined by RP-HPLC using a PRP-1™ column. The PDA UV spectrum of the small hump after 53 minutes confirmed the absence of any type of pigment, and is due to minor column bleed resulting from flushing the column with 100% ethyl acetate.

The two small peaks that follow the solvent front were assumed to be formed over the column. To confirm this hypothesis, the CPP elute was collected within 23–28 minutes and another RP-HPLC was performed on that heart cut. The two early eluting peaks reappeared in the chromatogram (Figure 4b). This would only be possible if they were generated from CPP immediately upon injection and initial adsorption onto the PRP-1 stationary phase. However, having shown that these peaks are indeed minor artifacts of the analytical RP-HPLC column, verification of CPP purity can be reasonably performed. That is, calculated percentages for these 2 peaks and CPP average 3, 10. and 87%, respectively. Calculations were made using $\epsilon_{mM} = 52.76$[17] and 33.4[3] for the unknowns and CPP, respectively. The identities of the 2 early eluting unknowns were not pursued at this time.

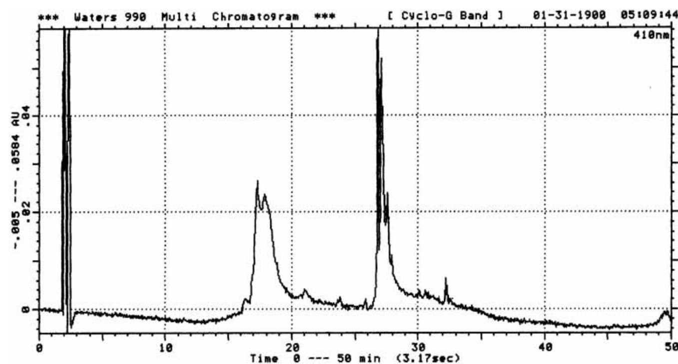

Figure 3. HPLC chromatogram (410 nm) of purified 13^2 17^3-cyclopheophorbide-*a*-enol (C_{18} column). The two peaks at 17.23 and 17.86 min. correspond to the chlorophyllones.

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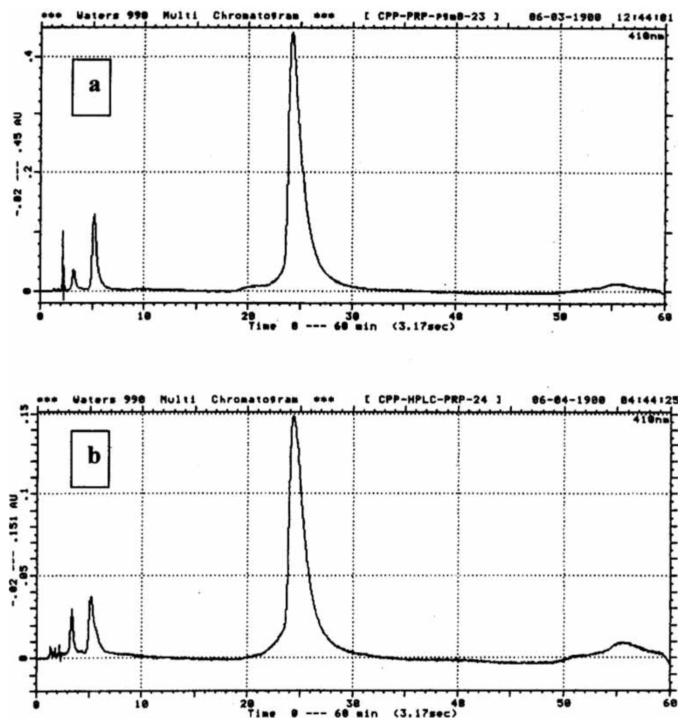

Figure 4. RP-HPLC analytical (PRP-1^{TM}) chromatograms ($\lambda = 410$ nm) of CPP purified by flash chromatography (see text). (a) Analytical check of CPP purity. (b) Reinjection of the heart cut (22–28 min.) of CPP from Figure 4a.

CONCLUSIONS

13^2, 17^3-cyclopheophorbide-*a*-enol has gained the reputation of being a very unstable compound that is easily altered during chromatography on most commonly used stationary phases. Polystyrene-Divinylbenzene (PS-DVB; aka PRP-1^{TM}), as a stationary phase for reversed phase column chromatography, is shown herein to be a simple and reproducible way to isolate and purify the unstable 13^2, 17^3-cyclopheophorbide *a*-enol (CPP aka Chl686, Phorbide686.5). Moreover, the analytical PRP-1 HPLC column can be used to characterize and confirm the purity of these series of the compounds.

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