ORGANIC LETTERS

2013
Vol. 15, No. 17
4430–4433

Chlorophyll c_{CS-170} Isolated from *Ostreococcus sp.* Is [7-Methoxycarbonyl-8-vinyl]protochlorophyllide *a*

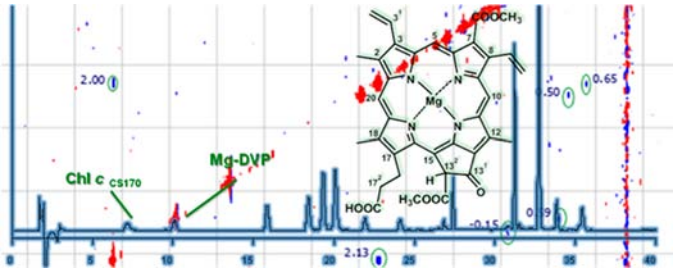
Susana Álvarez,[†] Francisco Rodríguez,[‡] Pilar Riobó,[§] José L. Garrido,*[,§] and Belén Vaz*[,∥]

IBIV Instituto de Investigación Biomédica de Vigo, Universidade de Vigo, 36310 Vigo, Spain, Instituto Español de Oceanografía, Subida a Radio Faro, 50. 36390 Vigo, Spain, Instituto de Investigacións Mariñas (CSIC), Av. Eduardo Cabello 6, 36208, Vigo, Spain, and Departamento de Química Orgánica, Facultade de Química, Universidade de Vigo, 36310 Vigo, Spain

garrido@iim.csic.es; belenvaz@uvigo.es

Received July 15, 2013

ABSTRACT



The controversial molecular identification of the so-called chlorophyll c_{CS-170} has been settled. Despite its relevance as a potential biomarker in the study of eukaryotic picophytoplankton, the structure of this chlorophyll remained so far uncertain. A full characterization by NMR, UV−vis, and ESI-MS is reported, revealing this chlorophyll as [7-methoxycarbonyl-8-vinyl]-protochlorophyllide *a*.

The term picophytoplankton designates the photosynthetic unicellular organisms in the 0.2−2 μm size range, encompassing both prokaryotes and eukaryotic algae, ubiquitous in the oceans. The enormous importance of these small cells lies in their elemental position at the base of food webs and nutrient recycling as well as their crucial role in biogeochemical cycles. The class *Mamiellophyceae*,[1] one of the ecologically most important groups of photosynthetic picoeukaryotes in the marine environment and recently differentiated within the Chlorophyta,[2] accounts for a significant amount of picophytoplankton biomass and photosynthesis.[3] Their miniaturized cell composition features a single mitochondria and one chloroplast.[4] The pigment composition of their photosynthetic apparatus is also very characteristic,[5] with chlorophylls *a* and *b* and the carotenoids prasinoxanthin, neoxanthin, violaxanthin, and micromonal as main constituents. Together with these pigments, the chlorophyll biosynthetic intermediate [8-vinyl]-Pchlide *a* (known also as Mg-divinyl phaeoporphyrin a_5, Mg-DVP) was detected in significant amounts. More importantly, an additional chlorophyll pigment appeared in certain mamiellophyceans and in one strain of *Prasinococcus* (*Prasinophyceae* genus) isolated in deep waters near the bottom of the photic zone.[6] Due to its

[†] IBIV Instituto de Investigación Biomédica de Vigo, Universidade de Vigo.
[‡] Instituto Español de Oceanografía.
[§] Instituto de Investigacións Mariñas (CSIC).
[∥] Departamento de Química Orgánica, Facultade de Química, Universidade de Vigo.

(1) Šlapeta, J.; López-García, P.; Moreira, D. *Mol. Biol. Evol.* **2006**, *23*, 23.
(2) Marin, B.; Melkonian, M. *Protist* **2010**, *161*, 304.
(3) Vaulot, D.; Eikrem, W.; Viprey, M.; Moreau, H. *FEMS Microbiol. Rev.* **2008**, *32*, 795.
(4) Piganeau, G.; Grimsley, N.; Moreau, H. *Res. Microbiol.* **2011**, *162*, 570.
(5) (a) Latasa, M.; Scharek, R.; Gall, F. L.; Guillou, L. *J. Phycol.* **2004**, *40*, 1149. (b) Jeffrey, S. W.; Wright, S. W.; Zapata, M. In *Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography*; Roy, S., Llewellyn, C. A., Egeland, E. S., Johnsen, G., Eds.; Cambridge University Press: Cambridge, U.K., 2011, p 3.
(6) (a) Worden, A. Z.; Not, F. In *Microbial Ecology of the Oceans*, 2nd ed.; Kirchman, D. L., Ed.; Wiley: NJ, 2008, p 159. (b) Rodríguez, F.; Derelle, E.; Guillou, L.; Le Gall, F.; Vaulot, D.; Moreau, H. *Environ. Microbiol.* **2005**, *7*, 853.

10.1021/ol4019826
Published on Web 08/28/2013

precise localization, this pigment was regarded as a potential biomarker to identify low light ecotypes. Despite its importance, the structure of this chlorophyll remains uncertain.

This pigment was first detected in 1989 in a tropical strain of *Micromonas pusilla* (CS-170),[7] being termed since then chl $c_{CS\text{-}170}$. A tentative identification as "a propionate derivative of chl c_3" was published in 1997 (Figure 1) together with the visible spectrum in diethyl ether of the isolated pigment,[8] but neither isolation procedures nor spectral evidence were indicated. Since then, chl $c_{CS\text{-}170}$ has been reported in natural samples and culture studies on picoeukaryotes,[5,6,9] but no other contributions to its molecular structure have been published. The suggested structure is isomeric to the [8-ethyl]-analogue of chl c_3, and due to their similarity in chromatographic properties, UV−vis spectra and mass spectra, both pigments were claimed to be actually the same compound.[10]

The absorbance profile of chl $c_{CS\text{-}170}$ features a main absorption maximum at 449 and two other maxima at 579 and 621 nm (Figure S2 in the Supporting Information), values that are closely related to those previously reported for Pchlide *b*: Soret band at 448 nm, Qx band at 578 nm, and Qy band at 622 nm.[11] In addition, this UV spectrum shows higher absorbance of the Qx band in comparison to the Qy band, which is characteristic of both the Pchlide *b* and the chl c_3 families. This spectral similarity suggested that chl $c_{CS\text{-}170}$ could actually be Pchlide *b*. Hence, certain mamiellophyceans could accumulate this pigment as a chl *b* biosynthetic intermediate, as it has been described for [8-vinyl]-Pchlide *a*.

Pigment	R^1	R^2	R^3
Chl $c_{CS\text{-}170}$	CO_2Me	$CH=CH_2$	$CH_2\text{-}CH_2\text{-}COOH$
Chl c_3	CO_2Me	$CH=CH_2$	$CH=CH\text{-}COOH$
[8-Ethyl]-Chl c_3	CO_2Me	$CH_2\text{-}CH_3$	$CH=CH\text{-}COOH$
Pchlide *b*	CHO	$CH_2\text{-}CH_3$	$CH_2\text{-}CH_2\text{-}COOH$
[8-Vinyl]-Pchlide *a*	Me	$CH=CH_2$	$CH_2\text{-}CH_2\text{-}COOH$

Figure 1. Comparison of the tentative structure proposed for chlorophyll c_{CS-170} and structurally related chlorophylls.

To prove the identity of the isolated chlorophyll (chl $c_{CS\text{-}170}$) a careful NMR characterization was mandatory in order to unequivocally elucidate the structure of this pigment, by comparison with that available for [8-ethyl]-chl c_3, recently characterized in the haptophyte *Emiliania huxleyi*,[12] and of Pchlide *b*,[13] the structure of which could be compatible with chl $c_{CS\text{-}170}$. In the present study we isolated chl $c_{CS\text{-}170}$ from the mamiellophycean *Ostreococcus sp.* and report its molecular structure as [7-methoxy carbonyl-8-vinyl]-Pchlide *a* using UV−vis, ESI-MS, and NMR spectroscopy techniques.

Ostreococcus sp. (RCC 788) was obtained from the Roscoff Culture Collection (Roscoff, France), and it was cultured in light/dark cycles and harvested soon into the light phase in order to maximize the production of the chlorophyll under study (see below). Special care was essential for a successful pigment extraction process, since important pigment degradation was observed at the high centrifugation fields necessary for good biomass recovery. Alternatively, cultures were thoroughly filtered in small aliquots and extracted with 90% aqueous acetone. A detailed description of the extraction and purification process is described as Supporting Information.

After HPLC purification, the sample was dissolved in THF-d_8, the same deuterated solvent used for [8-ethyl]-chl c_3, to allow a direct comparison between the two closely related pigments. In order to minimize aerobic oxidation of the pigment, the solution was degassed through freeze−thaw cycles and kept under an argon atmosphere.

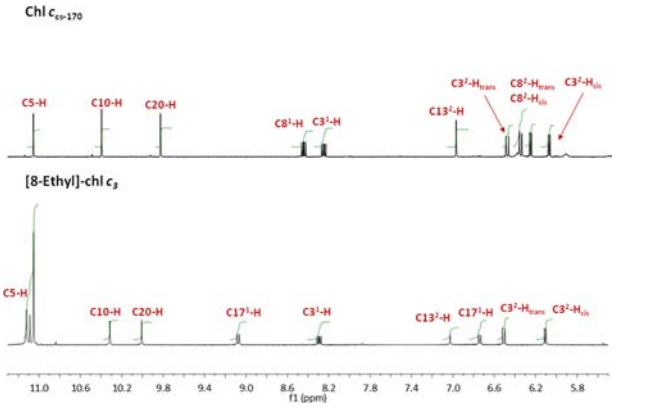


Figure 2. Comparison of low-field ^{1}H NMR spectra of chlorophyll $c_{CS\text{-}170}$ (upper trace) and the closely related [8-ethyl]-chl c_3 (lower trace).

The ^{1}H NMR spectrum of our sample at 750 MHz (Varian Inova 750 spectrometer) showed three signals in the lowest field, between δ ~11.5 and δ ~9.5 ppm, corresponding to the meso-CH protons at positions C5, C10, and C20 (Figure 2). In the proximal downfield region, two independent spin systems featuring three protons each were identified and assigned to two peripheral vinyl moieties at positions C3 and C8, by comparison with the

(7) Jeffrey, S. W. In *The Chromophyte Algae: Problems and Perspectives*; Green, J. C., Leadbeater, B. S. C., Diver, W. L., Eds.; Clarendon Press: Oxford, 1989; p 13.
(8) Jeffrey, S. W.; Mantoura, R. F. C.; Bjornland, T. In *Phytoplankton Pigments in Oceanography*; Jeffrey, S. W., Mantoura, R. F. C., Wright, S. W., Eds.; UNESCO: Paris, 1997; p 449.
(9) Goutx, M.; Guigue, C.; D. Aritio, D.; Ghiglione, J. F.; Pujo-Pay, M.; Raybaud, V.; Duflos, M.; Prieur, L. *Biogeosciences* **2009**, *6*, 1229.
(10) Goericke, R.; Olson, R. J.; Shalapyonok, A. *Deep-Sea Res. Pt. I* **2000**, *47*, 1183.
(11) Reinbothe, S.; Pollmann, S.; Reinbothe, C. *J. Biol. Chem.* **2003**, *278*, 800.
(12) Alvarez, S.; Zapata, M.; Garrido, J. L.; Vaz, B. *Chem. Commun.* **2012**, *48*, 5500.
(13) Shedbalkar, V. P.; Ioannides, I. M.; Rebeiz, C. A. *J. Biol. Chem.* **1991**, *266*, 17151.

reported data for chl c_3.[12] Unlike the closely related members of the chl c_3 family, the vinyl signals corresponding to the acrylic acid at C17 are absent in this spectrum. Instead, two methylene units were observed at high field as an AA′BC-type spin system [δ = 4.10 ppm (m, 2H), 3.06 ppm (ddd, J = 17.0, 9.3, 6.8 Hz, 1H) and 2.85 ppm (m, ddd, J = 17.0, 9.6, 7.0 Hz 1H)] as a result of the electron-withdrawing effect of the close carboxylic acid and the ring current of the aromatic porphyrin system. COSY and TOCSY correlations are clearly observed between the three signals (Figures S5 and S6 in the Supporting Information). Worth noting is the marked splitting observed for both methylene groups, probably due to the chiral environment given by the neighboring stereogenic center at C13^2.[14] Additionally, the chemical shifts for the methyl groups at high field are clearly visible, two of which correspond to the methyl esters [δ = 4.32 ppm (s, 3H, OCH_3) and 3.74 ppm (s, 3H, OCH_3)] and three to those attached to the aromatic macrocycle [δ = 3.82 ppm (s, 3H, CH_3); 3.65 ppm (s, 3H, CH_3) and 3.57 ppm (s, 3H, CH_3)]. The chemical shift values observed are compatible with those reported previously for chl c_3 and the corresponding monovinyl derivative studied before. Due to sample limitations, acquisition of a ^{13}C NMR spectrum was not feasible. Instead, analysis of the short-range ^{1}H−^{13}C correlations (HSQC, Figure S7 in the Supporting Information) led us to assign most of the nonquaternary carbons of the chlorophyll skeleton (Table S1 in the Supporting Information), showing a good agreement with previous data for related chlorophylls c.[12] Remarkable are the values obtained for the ^{13}C of the methylenes in the propionic acid moiety [δ = 23.2 ppm (C17^2-H) and 36.2 ppm (C17^1-H)] as well as the differentiated values observed for the methyl esters and the methyl groups attached to the porphyrin skeleton (δ ~50 and δ ~12 ppm respectively), which assisted in the signal assignments.

The spin systems of the sample were clearly determined by TOCSY and COSY spectra, as depicted in Figure 3. An AA′BC and two ABX coupling patterns were identified and assigned to the methylene groups of the propionic acid and the vinyl substituents at C3 and C8, respectively. In addition, 2D-ROESY correlations confirmed the resonance assignments of the ^{1}H NMR spectrum, revealing the spatial proximity of the substituents in the [7-methoxycarbonyl-8-vinyl]-protochlorophyllide *a* structure and the connectivities between the substituents around the porphyrin skeleton as shown in Figure 3.

Probably due to the low concentration of our sample, ROESY correlations were not detected in the region of the methoxycarbonyl substituent at C7. Therefore, a direct comparison of the ^{1}H NMR data of the sample under study and the data available for chl c_3 for the upper part of the macrocycle (from C2 to C8) was performed. The coincidence of data with a maximum difference of δ ~0.01 ppm allowed the structural confirmation of the chl c_3-like substitution profile featuring a methyl ester at C7.

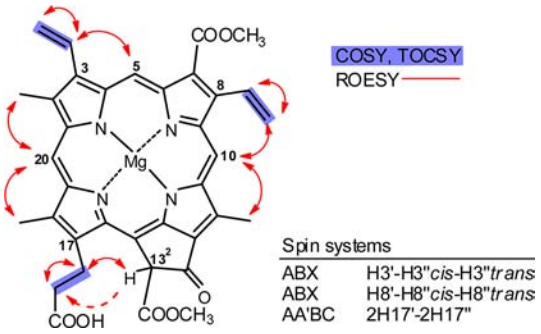


Figure 3. Spin systems identified in chl c_{CS-170} and ROE correlations.

This fact also rules out the possibility of having a formyl group at this position, which is characteristic of the Pchlide *b* structure, as the stronger withdrawing effect of this functionality would clearly affect the chemical shift values of the vicinal protons. Furthermore, a fourth signal in the lowest field region around δ ~11 ppm expected for the CHO group[13] is not present in our sample as well as the corresponding low field value (aprox. 190 ppm) of the ^{13}C at the formyl group, which finally discards the structural coincidence of Pchlide *b* and our sample. With all these results taken into account, a systematic name is proposed for the so far known chl c_{CS170} as [7-methoxycarbonyl-8-vinyl]-Pchlide *a*.

MS analysis was also conducted reproducing the conditions described before[12] for electrospray ionization in negative mode, and the ESI-MS spectrum showed a molecular ion peak at 654.0 m/z consistent with the formula $C_{36}H_{30}MgN_4O_7$. A major fragment ion is observed at 610 m/z $[M\text{-}H{-}CO_2]^-$, probably due to the loss of the carboxylic acid present in the structure of the chlorophyll. An additional high resolution MS analysis employing ESI in the positive mode showed ions consistent with $[M+Na]^+$, 677.1488 and with $[M+H]^+$ 655.2029 m/z. Fragment ions at m/z 623.1784 ($[M+H\text{-}32.0245]^+$) and 595.1826 ($[M+H\text{-}60.0203]^+$) were interpreted as due to loss of CH_3OH and $HCOOCH_3$ fragments from a methoxycarbonyl substituent.[15]

In terms of activity, chl *c* pigments in general have been found in the antenna complexes of Chromophyte algae,[16] where they act transferring energy efficiently to chl *a*.[17] The exact role that [7-methoxycarbonyl-8-vinyl]-Pchlide *a* might play in the photosynthetic apparatus is unknown, but a light harvesting function seems likely. The closely related chl c_3, a pigment bearing also a methoxycarbonyl substituent at C7 (but an acrylic side chain at C17), was found in

(14) Helfrich, M.; Ross, A.; King, G. C.; Turner, A. G.; Larkum, A. W. D. *Biochim. Biophys. Acta, Bioenerg.* **1999**, *1410*, 262.

(15) Airs, R. L.; Garrido, J. L. In *Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography*; Roy, S., Llewellyn, C. A., Egeland, E. S., Johnsen, G., Eds.; Cambridge University Press: Cambridge, U.K., 2011; p 314.

(16) Zapata, M.; Garrido, J. L.; Jeffrey, S. W. In *Chlorophylls and Bacteriocyhlorophylls. Biochemistry, Biophysics, Functions and Applications*; Grimm, B., Porra, R. J., Rüdiger, W., Scheer, H., Eds.; Springer: Dordrecht, The Netherlands, 2006; p 39.

(17) Büchel, C. *Biochemistry* **2003**, *42*, 13027.

the light-harvesting pigment–protein complexes in the flagellate alga *Prymnesium parvum*.[18] A similar role has also been shown for [8-vinyl]-Pchlide *a* (with a propionic acid substituent at C17) in certain Prasinophytes[19] (where it was misidentified as a chl *c*) even before its structure was elucidated.[14] [7-Methoxycarbonyl-8-vinyl]-Pchlide *a* showed variable ratios to chl *a* depending on light conditions, consistent with previous studies on the ratio of [8-vinyl]-Pchlide *a* to chl *a* carried out in Prasinophyceae (*Pseudoscourfieldia marina*) and Mamiellophyceae (*Bathycoccus prasinos*[20] and two *Ostreococcus* strains[21]). We observed that in *Ostreococcus* sp. RCC 788 these ratios were dependent both on light intensity and length of the irradiation period. HPLC analyses (data not shown) showed that the ratios of both Pchlides to chl *a* lowered with increasing irradiation times. When monitoring pigment diurnal variations, maximum [7-methoxycarbonyl-8-vinyl]-Pchlide *a* values were detected at the end of the dark period, whereas its ratio to chl *a* progressively diminished during the day. The pigment practically disappeared when the culture was maintained under a continuous light regime (115 μmol photons m^{-2} s^{-1}). The observed accumulation of [7-methoxycarbonyl-8-vinyl]-Pchlide *a* in the dark or at low light conditions and the fact that it occurs typically in deep water isolates suggest its implication in light harvesting especially in low light environments.

In summary, a structural study based on NMR spectroscopy of the so far generically denominated chl $c_{CS\text{-}170}$ was conducted in order to shed light on the actual structure of this pigment. Our results confirmed the structure tentatively proposed[8] and provided unambiguous evidence for the presence of two vinyl groups at C3 and C8 as well as a propionic acid at C17. We propose therefore the use of [7-methoxycarbonyl-8-vinyl]-Pchlide *a* to refer properly to this chlorophyll. From a biosynthetic point of view, referencing this compound as "a propionate derivative of chl c_3" no longer makes sense, as it is actually a Pchlide with a propionic acid rather than an acrylic residue, and occurs in species that accumulate [8-vinyl]-Pchlide *a* (Mg-DVP). Thus, the generation of [7-methoxycarbonyl-8-vinyl]-Pchlide *a* seems plausible from [8-vinyl]-Pchlide *a* through the methoxycarbonylation at C7. This oxidized functional group would arise from the C7-*gem*-diol, via consecutive steps of oxidation at $C7^1$, analogously to the biogenesis of the 7-formyl group in chl *b*. Further dehydration of the diol group toward the formation of a 7-carboxylate derivative and subsequent methylation would form the final methyl ester moiety.[22] Concerning the absolute configuration at $C13^2$, a tentative assignment can be proposed as *R*, based on the results reported by Mizoguchi et al. for the biosynthetically close related chlorophylls *c*.[23]

Acknowledgment. The authors wish to acknowledge the financial support from the Isidro Parga Pondal Program (Xunta de Galicia, Spain) to B.V. and the funding from the European Union Seventh Framework Programme [FP7/REGPOT-2012-2013.1] under Grant Agreement No. 316265, BIOCAPS. This work was partly supported by Xunta de Galicia through project Incite09 402 253PR. The technical help of Noelia Sanz and Javier Tamame (Instituto de Investigacións Mariñas, CSIC, Vigo, Spain) is gratefully acknowledged. We thank Thermo Fisher for allowing the use of the Exactive Orbitrap Mass Spectrometer and Dr. Ángel R. de Lera and Dr. Rosana Álvarez (Departamento de Química Orgánica, Universidade de Vigo, Spain) for helpful discussions. We dedicate this work to the memory of our friend and colleague M. Zapata, whose input and dedication were of great value.

Supporting Information Available. UV–vis absorption, ESI and NMR spectra, and detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

(18) Wilhelm, C.; Wiedemann, I. *Photosynthetica* **1991**, *25*, 249.
(19) (a) Rhiel, E.; Mörschel, E. *Mol. Gen. Genet*. **1993**, *240*, 403. (b) Wilhelm, C. *Botanica Acta* **1988**, *101*, 14. (c) Wilhelm, C.; Lenartz-Weiler, I.; Wiedemann, I.; Wild, A. *Phycologia* **1986**, *25*, 404.
(20) Rodríguez, F.; Chauton, M.; Johnsen, G.; Andresen, K.; Olsen, L. M.; Zapata, M. *Mar. Biol*. **2006**, *148*, 963.
(21) Six, C.; Finkel, Z. V.; Rodríguez, F.; Marie, D.; Partensky, F.; Campbell, D. A. *Limnol. Oceanogr*. **2008**, *53*, 255.
(22) Porra, R. J.; Olster, U.; Scheer, H. In *Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography*; Roy, S., Llewellyn, C. A., Egeland, E. S., Johnsen, G., Eds.; Cambridge University Press: Cambridge, U.K., 2011; p 78.
(23) Mizoguchi, T.; Kimura, Y.; Yoshitomi, T.; Tamiaki, H. *BBA-Bioenergetics* **2011**, *1807*, 1467.

The authors declare no competing financial interest.