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ARTICLE TYPE

Characterization of [8-ethyl]-chlorophyll c_3 from *Emiliana huxleyi*Susana Álvarez,^a Manuel Zapata,^b José L. Garrido,^{*b} and Belén Vaz^{*a}

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We report herein the isolation and complete characterization of a member of the chlorophyll c family, designated as [8-ethyl]-chlorophyll c_3 ([8-ethyl]-chl c_3). Structural elucidation of this pigment rested on the analysis of mono- and bidimensional NMR, UV-VIS spectroscopy and ESI-MS data, and the configuration at the 13² position on chiral HPLC analysis.

Emiliana huxleyi (Haptophyta, Prymnesiophyceae) is the most abundant coccolithophore (haptophytes covered with calcified scales) in the ocean.² It is widely distributed in both coastal and oceanic environments and forms so extensive blooms that can be observed from satellites.³ Coccolithophores are responsible for the major production of calcium carbonate in the oceans which, from a climatological and ecological point of view, constitutes a critical factor in the regulation of the oceans pH. This is a major issue given the increasing acidity of seawater as a consequence of the rising atmospheric CO₂ partial pressure. In addition, calcium carbonate plays an important role in the biochemical cycling of carbon,⁴ where the precipitation of CaCO₃ in seawater is directly related to an increase in the concentration of CO_{2(aq)}, resulting in a higher partial pressure of carbon dioxide on the surface of the ocean.⁵ The balance between the calcification process and the photosynthesis in coccolithophores determines whether these unicellular marine algae constitute either a net source or a sink of CO₂ to the atmosphere.⁶ The significance of *Emiliana huxleyi* not only lies in its implication in the carbon cycle, but also in the sulphur cycle, of considerable importance given the ability of sulphur compounds to create an aerosol shield capable of reflecting ultraviolet radiation and potentially offset the global warming caused by the greenhouse effect.⁷ Since blooms formation of *E. huxleyi* is closely related to conditions of high light intensities in surface waters,⁸ the photosynthetic apparatus of these organisms has attracted the attention of the scientific community in efforts to understand and predict the environmental impact of these blooms. Much effort has been directed towards the identification of the pigment composition of *E. huxleyi*. Several new carotenoids and chlorophylls have been detected in this species.⁹⁻¹³ Chlorophyll c_3 , a compound of wide significance in marine ecology¹⁴⁻¹⁶ was first detected in *E. huxleyi* by Jeffrey and Wright¹⁷ as a very polar chlorophyll, and its tentative structure was subsequently reported.¹⁸ Chromatographic and spectroscopic evidence¹⁹ suggested that the isolated chl c_3 was in fact a mixture of two compounds, tentatively assigned as the chl c_3 and its 8-devinyl-8-

Figure 1. HPLC profile of the acetone extract of *E. huxleyi* using a C8 column and pyridine-containing mobile phases.¹ Detection at 440 nm.

ethyl- analogue of the same basic structure²⁰ based on mass spectrometry studies. In order to fully determine the structure of related chls c_3 , in this work we report an efficient isolation of both compounds from *E. huxleyi* and their full characterization by UV-Vis, ESI-MS and NMR spectroscopy, using mono- and bidimensional NMR experiments.

Emiliana huxleyi (strain CCMP 370) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences). Culturing techniques, cell harvesting and isolation of pigments (Fig. 1) were as previously described.¹²

In their UV-Visible spectra, the two compounds with the shortest retention time (Fig. 1) showed II bands of greater intensity than the corresponding I bands, which is characteristic of chlorophyll c_3 pigments (Fig. S1). In addition, a slightly hypsochromic shift for bands III and II was detected for the slow-eluting pigment (postulated as the [8-ethyl]-chl c_3 derivative) in comparison to the fast-eluting pigment (chl c_3). These differences were found in previously described mono- and divinyl pairs of chlorophylls, such as chl c_2 and chl c_1 ,²¹ divinyl-chl a and chl a ²² and divinyl-chl b and chl b .²² Considering that both chlorophylls have a light harvesting function,²³ the absorption differences may be related to the ability to absorb specific wavelengths. Previous results²⁴ have shown that under low light conditions the divinyl chl c_3

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predominates over [8-ethyl]-chl c_3 , whereas the relative proportions of both pigments are reversed under high light. The interconversion between both pigments could provide a competitive advantage to *E. huxleyi* in the variable light environments of marine ecosystems, in a similar way that the predominance of the cyanobacteria *Prochlorococcus marinus* in the low light deep waters has been associated with the presence of 3,8 divinyl chlorophyll *a* in its light harvesting antenna.²⁵

Mass analysis using electrospray ionization in negative mode ESI-MS showed molecular ion peaks at m/z 652.3 (chl c_3) and m/z 654.1 ([8-ethyl]-chl c_3), identical to the expected values for chl c_3 and [8-ethyl]-chl c_3 . Likewise, the mass difference in two units observed is consistent with the mass difference between a vinyl group and an ethyl group. Although a clear fragmentation could not be detected, the mass values observed are compatible with the presence of magnesium as the central metal in both cases, as reported previously.²⁰

Due to the solubility requirements and the position of the residual signal of the deuterated solvent, THF- d_8 was selected as a suitable solvent for the NMR experiments. Solutions of chlorophylls c_3 were prepared in THF- d_8 , and degassed with freeze-thaw cycles under Ar atmosphere in order to avoid the formation of oxidized derivatives of the natural pigments.

As expected, both compounds (chl c_3 and [8-ethyl]-chl c_3) showed similar NMR spectra with the exception of the downfield region, which showed significant differences (Fig. 2), and pointed to the saturation of one vinyl group in [8-ethyl]-chl c_3 . In order to extract analogous information for both compounds, the two chlorophylls isolated were carefully studied by NMR spectroscopy. However, only the results referring to the new chlorophyll are described here. The spin systems of the spectrum were straightforwardly deduced from COSY and TOCSY experiments and, due to the simplicity of the structure in this respect, also by the determination of the $^3J_{H-H}$ coupling constants. The 2D ROESY experiment proved to be a useful tool for the assignment of not only the chlorophyll protons, but also the substituents on the chlorophyll structure.

The *meso*-CH signals appeared as typically at the lowest field, in the order of 5, 10 and 20, moving upfield. These assignments were consistent with those reported earlier by Fookes *et al* for chl c_3 in pyridine- d_5 ,¹⁸ with the exception of the significant downfield shift of one of the *meso*-CH signal to $\delta = 11.10$ ppm. Although similar values were reported as corresponding to a formyl group,²⁶ this functionality was ruled out in our chl- c_3 related pigment due to the HSQC-based correlation signal at $\delta = 103.6$ ppm, characteristic of a *meso*-carbon. This remarkable downfield shift suggested the presence of an electron-withdrawing substituent in the vicinity of that position, which was confirmed by the presence of a singlet for a methyl ester at $\delta = 4.3$ ppm strongly influenced by the porphyrin π -skeleton ring current. An additional CO₂Me signal was observed ($\delta = 3.8$ ppm) corresponding to the methyl ester at C13². The *trans*-geometry of the C17¹=C17² bond of the acrylic moiety was confirmed by the vicinal coupling ($^3J_{H-H} = 17.9$ Hz), which is consistent with data reported for chl c_1 and chl c_2 ($^3J_{H-H} = 15.8$ and 16.0 Hz, respectively).²⁷ In contrast to the two vinyl moieties present in the structure of chl- c_3 , a single vinyl substituent was detected in the [8-ethyl]-chl c_3 . Instead, signals for an ethyl substituent [$\delta = 4.46$

Figure 2. ¹H-NMR comparison of the down-field region of spectra corresponding to chl c_3 and [8-ethyl]-chl c_3 .

ppm (q, $J = 7.8$ Hz), $\delta = 1.88$ ppm (t, $J = 7.8$ Hz)] were observed.

The characteristic spin system of the acrylic acid group at the 17-position was taken as a suitable starting point to proceed around the macrocycle and assign the ¹H-NMR signals based on ROESY data. The correlation between 17²-H and 18-CH₃ was clearly observed, which allowed the assignment of all the methyl and vinyl groups around the porphyrin skeleton. Noteworthy, the connection between the 7-CO₂CH₃ substituent of the B ring and 5-H and 8¹-H of the two vicinal groups established unambiguously the arrangement of both moieties (carboxymethyl and vinyl) in the same ring of the chlorophyll and ruled out the structure with a reversed substitution of the vinyl substituent in the C-ring (at C12) and a methyl group at C8. This result is in agreement with a recent study of Mizoguchi *et al* on the structure of chl c_3 , where the authors have established the positions of the peripheral groups attached to the porphyrin ring based on NOE correlations.²⁸ The same group also reported the characteristic *cisoid* conformation of the acrylate moiety attached to C17, which was also determined in our [8-ethyl]-chl c_3 by ROESY correlations. Spatial interactions between 17¹-H and both vicinal 13²-H and 13²-CO₂CH₃, as well as the proximity of 17²-H and 18-CH₃ were detected, which confirmed the *cisoid* arrangement of the peripheral unsaturated carboxylic acid. This conformation, together with the *E*-configuration of the acrylic moiety, may explain the lack of reactivity of this family of chlorophylls *c* in the enzymatic hydrogenation of the D-ring to form the corresponding chlorin.²⁹⁻³⁰

The absolute configuration at the 13²-position can tentatively be assigned as *R* based on precedents for chl *c* containing algae as recently reported by Mizoguchi *et al*.²⁸ In the course of their study, it was demonstrated that in all cases the chlorophylls *c* isolated under neutral conditions presented exclusively the (13²-*R*)-configuration, including the chl c_3 isolated from *Emiliania huxleyi*. Following this methodology, we have subjected the [8-ethyl]-chl c_3 sample extracted in the presence of pyridine to chiral HPLC separation, using isocratic reverse-phase chromatography [CHIRALPAK IC column, 0.5 mL/min, 1:2:2 (v/v/v) methanol:acetonitrile:100 mM aqueous ammonium acetate]. Two peaks were separated which showed identical absorption, characteristic of [8-ethyl]-chl c_3 . Taking into account (i) the recent enantiomer assignment of Mizoguchi *et al*²⁸ for chl c_3 [peak #1: (13²*R*)-configured chl and peak #2: (13²*S*)-configured chl] based on the comparison of reported CD signals for the structure-related PChlide-*a*, and (ii) the reversed elution order observed for the monovinyl chl c_1 ,³¹ we can unambiguously

Figure 3. Postulated biosynthesis mechanism for [8-ethyl]-chl c_3 .

assign the (13^2S) and the (13^2R) configurations to the fast and slow eluting [8-ethyl]-chl c_3 peaks, respectively. With the aim of elucidating the natural selection for one of the enantiomers of [8-ethyl]-chl c_3 , several attempts were made to obtain a non-epimerized sample from *Emiliana huxleyi*, following the previously described protocol.^{28, 31} However, the results were inconclusive, since a rapid racemization was observed even in the absence of pyridine or ammonium acetate. Considering that chls c proceed biosynthetically from the closely related (3)-mono- and (3,8)-divinyl protochlorophyllide- a , which are known to exhibit the $13^2(R)$ configuration,³² we surmise that the natural [8-ethyl]-chl c_3 pigment is likewise the R enantiomer.

One possible mechanism for the biosynthesis of [8-ethyl]- c_3 would involve the chl c_2 derived from 8-vinyl PChlide- a by dehydrogenation of the 17-propionate to the acrylic moiety and subsequent methoxycarbonylation at the 7 position followed by a final hydrogenation by the suitable 8-vinyl reductase. Nagata and co-workers have found that oxidized substrates such as 3,8-divinyl-chlide b and -chl b (having a formyl group at position 7) are not reactive substrates for this enzyme.³³ According to this evidence, a more likely biosynthetic pathway may consist of the hydrogenation of the 8-vinyl group either at the PChlide a or at the chl c_1 state, prior to the latter oxidation.

In summary, the structure of a pigment closely eluting with chlorophyll c_3 has been extensively studied by NMR spectroscopy, which has provided conclusive evidence on the monovinyl character of the new member of the chlorophyll c family. Unambiguous conclusions of the presence of an ethyl group attached to C8 have been reported, supporting the tentative structure suggested earlier²⁰ based on FAB-MS analysis.

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† Electronic Supplementary Information (ESI) available: UV-VIS absorption, ESI and NMR spectra, chiral-phase HPLC profile and detailed experimental procedures. See DOI: 10.1039/b000000x/

‡ Abbreviations: chl, chlorophyll; PChlide, protochlorophyllide; chlide, chlorophyllide.

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