ORGANIC LETTERS

2013
Vol. 15, No. 7
1588–1590

Structure of Chlorophyll *f*

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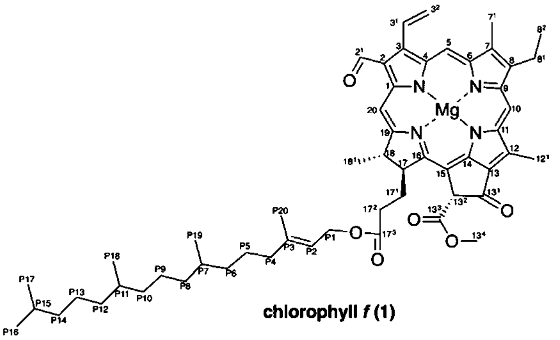
Received February 4, 2013

ABSTRACT



chlorophyll *f* (1)

Chlorophyll *f* (1) is the most red-shifted absorbing natural chlorophyll reported, and it is assigned the structure [2-formyl]-chlorophyll *a* ($C_{55}H_{70}O_6N_4Mg$). This structural assignment is confirmed based on the relative retention time on HPLC, mass spectroscopy, UV/vis absorption, and CD spectroscopy, and proton and carbon NMR of chlorophyll *f* purified from *Halomicronema hongdechloris*.

Chlorophylls are essential molecules for light-harvesting and energy transduction in oxygenic photosynthesis and are comprised of a phytoporphyrin or phytochlorin ring and in most cases a hydrocarbon tail, usually phytol.[1] The major functions performed by chlorophylls in photosynthetic reactions are absorbing light efficiently in the light-harvesting complexes; transferring the excitation energy with high quantum efficiency to the reaction centers; and performing with high yield the primary charge separation across the photosynthetic membranes and generating membrane potential that leads to ATP and strong reductants (NADPH).[2] There are five different forms of chlorophylls. Chlorophylls *a*, *b*, and *c* were identified in the 19th century,[1] and chlorophyl *d* was reported in 1943.[3] The fifth chlorophyll, chlorophyll *f*, was reported in 2010 with proposed structure **1** ([2-formyl]-chlorophyll *a* or more formally 2^1-oxo-chlorophyll *a* ($C_{55}H_{70}O_6N_4Mg$)).[4] However a full structural characterization has been lacking[5] because the available chlorophyll *f* was scarce. Recently, the cyanobacteria that synthesize chlorophyll *f*, *Halomicronema hongdechloris*, were isolated from stromatolites, Western Australia and cultured in the laboratory,[6] and a freshwater cyanobacterium (strain KC1) containing chlorophyll *f* has been isolated from Lake Biwa, Japan.[7] There are two types of chlorophylls in *H. hongdechloris*, chlorophyll *a* and chlorophyll *f*. The content of chlorophyll *f* can reach 20% of total chlorophylls when *H. hongdechloris* is grown under far-red light culture conditions.[8] Here the structure of chlorophyll *f* purified from *H. hongdechloris* is confirmed as [2-formyl]-chlorophyll *a* **1** ($C_{55}H_{70}O_6N_4Mg$) as detailed below.

H. hongdechloris was grown in K+ESM medium under continuous illumination (730 nm light) at 27 °C[6] with a

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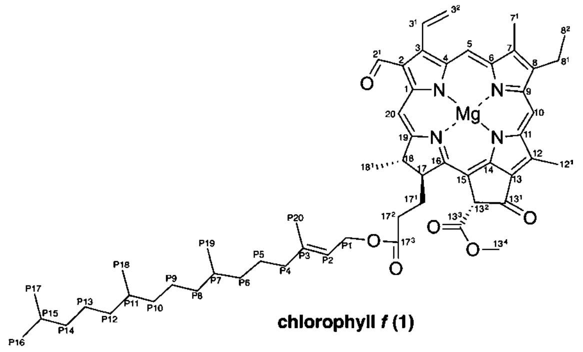
10.1021/ol400327j

doubling time of 4−5 days. Chlorophyll *f* (**1**) was purified from approximately 5 L of culture grown to late exponential phase over ~3 months. The cells were harvested by centrifugation, and the chlorophyll *f* was extracted into methanol followed by fractionation using a combination of liquid phase partition, normal phase chromatography on silica, and reversed phase HPLC, as described previously,[8] and as detailed in the Supporting Information, final purification by HPLC was conducted on an Agilent 1290 HPLC system with a reversed-phase C18 column (XRs Purasil 5, 250 mm × 10 mm, Agilent). The column was equilibrated, and pigments were separated using 100% 2-propanol at 2 mL/min over 20 min. Eluted pigments were detected with a photodiode array detector (1290, Agilent) at a wavelength range of 350−700 nm. Chlorophyll *f* (**1**) (~120 μg) eluted at 16.5 min.



chlorophyll *f* (1)

Absorbance spectral analyses and MS analysis of **1** were done as described previously, and data are shown in the Supporting Information.[4,6,8] The MALDI-MS spectrum has the molecular ion at 906 *m*/*z*, and the major fragment ion at 628 *m*/*z* (base peak) is due to the loss of a phytyl. The other major ion at 657 *m*/*z* is due to the terthiophene matrix. The circular dichroism spectrum in methanol had positive and negative extrema at 395 nm (Θ_M = 24 000) and 702 nm (Θ_M = −5000) respectively. This is consistent in terms of sign and position of the Soret and Qy absorbance peaks, except for the shifts which correspond to those of the absorbance spectra, with those of chlorophyll *a*.[9]

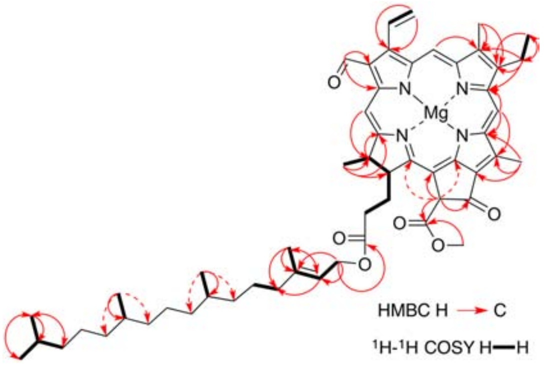


Figure 1. Key HMBC and COSY correlations of **1** (Dotted lines are one of two possibilities).

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of **1** in $CDCl_3$ with 0.5% d_5-Pyridine; Reference to $CHCl_3$ (δ in ppm, *J* in Hz)[a]

entry	δ_H	δ_C
1		149.5
2		130.3
2^1	11.165 (1H, s)	189.2
3		150.1
3^1	8.312 (1H, dd, 11.4, 17.5)	129.0
3^2	trans 6.266 (1H, d, 17.5) cis 6.227 (1H, d, 11.4)	126.1
4		
5	9.615 (2H, s) same as 10	104.8 or 107.0
6		150.1
7		135.7
7^1	3.29 (3H, s)	11.2
8		148.4
8^1	3.757 (2H, q, 7.6)	19.6
8^2	1.698 (3H, t, 7.6)	17.4
9		143.7
10	9.615 (2H, s) same as 5	104.8 or 107.0
11		150.4
12		138.5
12^1	3.671 (3H, s)	13.0
13		132.5
13^1		190
13^2	6.219 (1H, s)	65.4
13^3		170.2
13^4	3.847 (3H, s)	52.8
14		162.6[b]
15		104.6
16		162.6[b]
17	4.115 (1H, d, 7.2)	51.0
17^1 and $17^{1\prime}$	2.487 (1H, m) 2.25 (1H, m)	29.7
17^2 and $17^{2\prime}$	2.36 (1H, m) 1.913 (m)	30.5
17^3		172.9
18	4.46 (1H, q)	48.5
18^1	1.644 (3H, d, 7.3)	23.5
19		167.8
20	9.42 (1H, s)	96.8
P1	4.44 (2H, dd, 12.7, 7.0, 6.4)	61.4
P2	5.125 (1H, t, 7.0, 6.4)	117.7
P3		142.8
P4	1.88 (HSQC)	39.7
P5		
P6		37.2
P7	1.3	32.6
P8		37.2
P9		
P10		37.2
P11	1.3	32.6
P12		37.2
P13		
P14	1.1 (m)	39.2
P15	1.465 (H, n, 6.6)	28.0
P16 and P17	0.82 (6H, d, 6.6)	22.7
P18 and P19	0.783 or 0.765 (6H, d, 6.6)	19.6
P20	1.57 (3H, s)	16.1

[a] Data assigned by ^{1}H−^{1}H COSY, HSQC, HMQC, HMBC, and NOESY spectra. [b] Either 14 or 16.

The optical activity of chlorin-type chlorophylls is dominated by the configurations of C-17 and C-18.[10] The relative trans configuration of 17 and 18 in **1** is confirmed by the NOESY cross peak between 17H and $18H^1$ (Figure 2). Thus confirmation of this relative configuration together with the optical activity CD data establishes the same absolute configuration, 17S,18S, for chlorophyll *f* (**1**). Due to steric hindrance, the configuration at C-13^2 is, in turn, influenced by that of C-17[11] and, therefore, likely R as in chlorophyll *a*. In addition chlorophyll *a* and derivatives slowly isomerize to a mixture of epimers with the *a*′ or 13^2(S) epimer, representing 10−15% of the product.[12] The resonance of the H-13^2(S) epimer is shifted to higher field by up to 0.12 ppm compared to the R epimer.[12] When chlorophyll *f* is allowed to epimerize with a small amount of pyridine for 2 days, a resonance at 6.144 ppm appears as the only additional resonance in the ^{1}H spectrum. This resonance is 0.075 ppm higher field than the H-13^2 suggesting that this belongs to the H-13^2 (R) epimer, and thus confirming the 13^2(S) configuration of **1**.

Although chlorophyll *f* represents up to 20% of the total chlorophyll when *H. hongdechloris* is grown under 720 nm light conditions,[8] the slow growth rate coupled with the neccessity of growing *H. hongdechloris* under continuous 720 nm light to allow chlorophyll *f* production still limits the quantity of highly purified chlorophyll *f* for analysis. However, almost complete assignment of the proton and carbon resonances was possible from the 120 μg of **1** obtained (Table 1).

NMR analysis confirmed the structure of **1**. Beginning with the ^{1}H spectrum, all 10 CH_3 groups of **1**, 7^1, 8^2, 12^1, 13^4, 18^1, P16, P17, P18, P19, and P20, and the 3^1 and 3^2-vinyl group resonances are readily defined as they are at similar chemical shifts to the respective ones of chlorophyll *a* and chlorophyll *b*.[13−15] The chemical shifts from the 2^1 formyl proton and the meso position protons at 5, 10, and 20 are clearly visible, but as noted previously, the meso resonances are shifted to lower field[4] compared with chlorophyll *a* and *b*.[13,14]

In $CDCl_3$ the C5 and C10 resonances are indistinguishable, but these were resolved in CD_2Cl_2.[4] The coupling constants and COSY cross peaks clearly defined the 3^1−3^2, 8^1−8^2, 18^1−18−17, 17−17^1, 17−$17^{1\prime}$ 17^1−17^2, 17^1−$17^{2\prime}$, 17^1−$17^{1\prime}$, $17^{1\prime}$−17^2, $17^{1\prime}$−$17^{2\prime}$, 17^2−$17^{2\prime}$, P1−P2−P3−P20, P4−P5, and P14−P15−P16−P17 spin systems (Figure 1), but the P19−P7 and P18−P11 systems are indistinguishable. As the sample was limited, 120 μg, there was insufficient material for a ^{13}C spectrum. Thus the ^{13}C assignments and further structural confirmation were made using a combination of HSQC, HMQC, HMBC, and NOESY spectra as shown in Figures 1 and 2 and as detailed in the Supporting Information. The long- and short-range heteronuclear correlations using HSQC and HMBC have helped to define the ^{13}C resonances on chlorophyll derivatives previously.[16] The resonances at 17^1 and $17^{1\prime}$ and 17^2 and $17^{2\prime}$ observed in the ^{1}H spectrum are similar to those found for chlorophyll *a*[14,15] and are confirmed in the HSQC of **1**. The strong NOESY correlation between 17^1−17^2 and the absence of a correlation between $17^{1\prime}$−$17^{2\prime}$ suggests a partially eclipsed *anti* rotamer conformation around the 17^1−17^2 bond, and this compares favorably with the predominance of the *anti* rotamer, found on the basis of coupling constants, for chlorophyll *a*.[14,15] The final confirmation of the resonance assignments and overall structural assignment come from the NOESY spectra with clearly defined cross peaks linking; 2^1−20−18−18^1 and 18−17; 3^2−3^1−5−7^1; and 8^1−8^2 and (8^1 and 8^2)−10−12^1 as shown in Figure 2. The only assignments that could not be made or that are uncertain were the C4, C14, C16, and CH_2 groups in the phytyl chain. However, based on the assignment for 13^2(R)-methoxychlorophyll *a*[16] the three unassigned cross peaks at ~24.5 ppm in the HSQC belong to P5, P9, and P13.

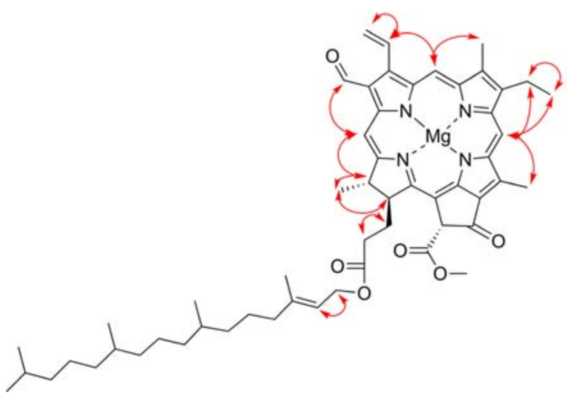


Figure 2. Key NOESY correlations of **1**.

Acknowledgment. M.C. holds an Australian Research Council Future Fellowship, and R.D.W. and M.C. thank the Australian Research Council for support. Y.L. is the recipient of a China Scholarship Council. The authors would like to thank Patrick Loughlin, School of Biological Sciences, Faculty of Sciences, University of Sydney, NSW 2006, Australia for MS data.

Supporting Information Available. All spectral data, CD, MS, UV−vis, ^{1}H NMR, HSQC, HMQC, HMBC, ^{1}H−^{1}H COSY, ^{1}H−^{1}H NOESY, and further details on the purification and experimental methodology are given. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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