

The Autofluorescent Substance in the Posterior Flagellum of Swimmers of the Brown Alga *Scytosiphon lomentaria*

Kimiaki Yamano^a, Hiroshi Saito^a, Yukie Ogasawara^a, Shoko Fujii^a, Hiroko Yamada^a, Haruhisa Shirahama^{a,*} and Hiroshi Kawai^b

^a Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060, Japan

^b Department of Biology, Faculty of Science, Kobe University, Kobe 657, Japan

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The flagellar autofluorescent substance of the brown alga *Scytosiphon lomentaria*, which is probably involved in the photoreception of the phototaxis of flagellate cells, was investigated. 4',5'-Cyclic FMN (**1**) was isolated from the extract of whole mature plants for the first time as a natural product. Since the concentration of 4',5'-cyclic FMN (**1**) was considerably low in vegetative plants, which do not contain fluorescent flagella, this substance is considered to correspond to the flagellar fluorescent substance.

Introduction

Brown algae are marine benthic algae and form various morphological types of flagellated reproductive cells (swimmers), according to the systematic groups and functions of the cells (e.g., zoospore, gamete, sperm). Many of them show phototaxis, a cellular response to detect the orientation of stimulation light and move toward or escape from the light. Those cells, which exhibit phototaxis, have been known to have stigma and flagellar swelling. Furthermore, Müller *et al.* (1987) and Kawai (1988) recently reported that these cells, which show phototaxis, have a green autofluorescence only in one of the two flagella, and the fluorescent substance is suggested to be involved in the photoreception of the phototaxis. This substance is widely distributed in all systematic groups of brown algae, except for some specialized cells which have lost phototaxis. Similar green autofluorescence is also noticed in some other chromophyte algae (e.g. Chrysophyceae, Synurophyceae, Prymnesiophyceae and Xanthophyceae), that are phylogenetically related with Phaeophyceae, and they are suggested to have a

similar photoreceptive mechanism (Kawai and Inouye, 1989). This autofluorescent substance is considered to be a kind of flavin based on its fluorescence spectra (Müller *et al.* 1987, Kawai, 1988), however, no chemical analysis has been done to date. The objective of this study was to isolate the flavin-like autofluorescent substance from the cosmopolitan brown alga, *Scytosiphon lomentaria*, and to identify the chemical composition.

Results

Because of the difficulty in detaching a large quantity of flagella from flagellated cells and extracting its flavins, we extracted a flavin-like autofluorescent substance from whole mature plants of *Scytosiphon lomentaria*, which contain abundant flagellated cells with fluorescent flagella. For comparison, the extraction was also made from young plants that do not contain flagellated cells. Extraction was also made from the mature thalli of *Laminaria japonica* (Laminariales) that lack flagellar autofluorescence and show no phototaxis. The results of the extractions are shown in Table I. Comparing the flavin compositions of the mature plants of *Scytosiphon* with that of the vegetative plants, mature plants contain a considerably higher concentration of Flavin 1, although the very little amount of FMN (and FAD) was contained. Flavin 2 is found only in mature plants but not in vegetative plants. In *Laminaria japonica* Flavin 1 could not be detected.

Reprint requests to Prof. Dr. H. Shirahama.
Telefax: 0198-51-0914.

* Present address: School of Science, Kwansei Gakuin University, Nishinomiya, Uegahara 662, Japan.

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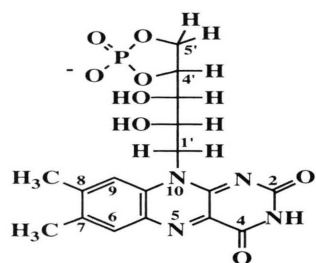
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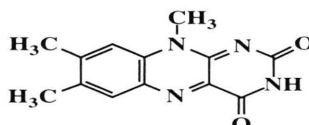
Table I. Composition of flavin compounds.

	<i>Scytosiphon lomentaria</i>		<i>Laminaria japonica</i>
	Mature alga ^a	Immature alga ^a	
Lumiflavin	not detected	not detected	major
FMN	<0.00001% ^b	0.0012% ^b	trace
FAD	0.00007%	0.00023%	trace
Flavin 1	0.00026%	not detected	not detected
Flavin 2 ^c	trace	not detected	not detected

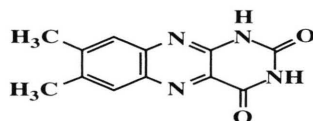
^a See experimental; ^b the rough weight percentage of the wet alga is shown; ^c structure is unknown. See text.



Flavin 1
Riboflavin-4',5'-cyclic phosphate
(4', 5'-cyclic FMN)



Lumiflavine



Lumichrome

Structure of Flavins 1 and 2

Flavin 1 exhibited an emission maximum at 525 nm when excited at 445 nm and UV maxima at 220, 265, 370 and 445 nm. In the ¹H NMR spectrum, signals at δ 2.29 and 2.41 (each 3H, *s*) are assigned to the two aromatic methyl groups and similarly signals at δ 7.67 and 7.68 (each 1H, *s*) to the two aromatic protons. These spectral data are characteristic of flavins (Table II), and the ¹³C NMR spectrum of Flavin 1 is very similar to that of FMN (Table III).

In the ¹H NMR spectrum (Table II), signals of six protons were observed in the region of δ 3.98–5.00. The ¹H-¹H COSY spectrum measurement revealed the presence of one more proton whose signal overlapped that of H₂O. These seven protons together with five signals in the ¹³C NMR [δ 48.4 (*t*), 67.3 (*t*), 70.3 (*d*), 73.4 (*d*) and 76.2 (*d*)] (Table

Table II. ¹H NMR data of Flavin 1 and riboflavin.

Positions	Flavin 1	Riboflavin
6	δ 7.67 (<i>s</i>) ^a	δ 7.99 (<i>s</i>) ^a
9	7.68 (<i>s</i>)	8.00 (<i>s</i>)
7a	2.41 (<i>s</i>)	2.60 (<i>s</i>)
8a	2.29 (<i>s</i>)	2.51 (<i>s</i>)
1'	5.00 (<i>m</i>)	5.18 (<i>brd</i>)
1'	4.72 ^b	4.72 ^b
2'	4.19 (<i>m</i>)	4.45 (<i>m</i>)
3'	4.28 (<i>m</i>)	3.99 (<i>dd</i>)
4'	4.58 (<i>m</i>)	3.95 (<i>m</i>)
5'	4.17 (<i>m</i>)	3.90 (<i>brd</i>)
5'	3.98 (<i>t</i> , <i>J</i> = 5.8)	3.75 (<i>dd</i>)

Chemical shifts in δ-values (ppm); ^a splitting patterns are designed as "*s*, *d*, *dd*, *t*, *m*, *brd*"; these symbols indicate "singlet, doublet, double doublet, triplet, multiplet, and broad", respectively; ^b the peak was overlapped with that of the solvent (water). It was detected by ¹H-¹H COSY spectrum.

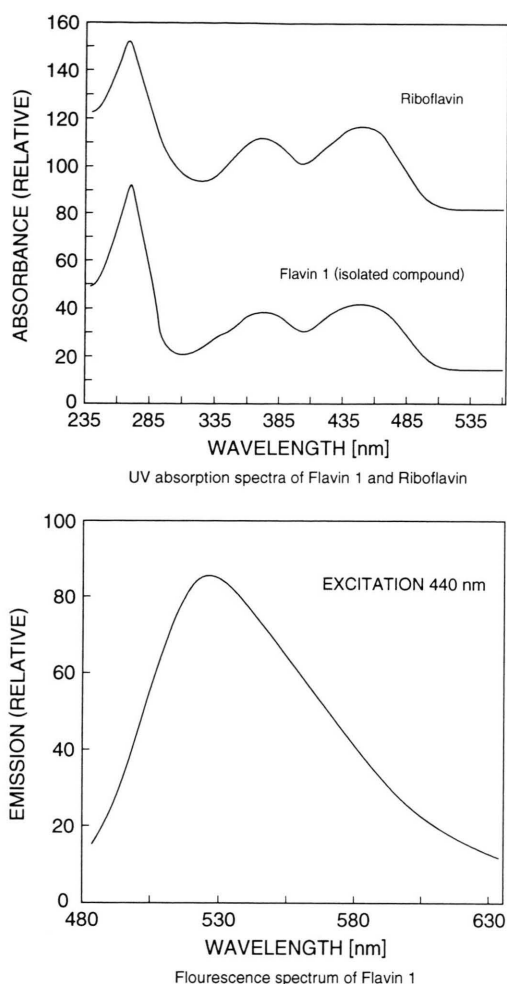


Fig. 1. Absorption and emission spectra of Flavin 1 accompanied with UV absorption spectrum of riboflavin.

III) clearly suggested the existence of a ribityl moiety. Additionally, the ^1H decoupled ^{31}P NMR spectrum exhibited the only singlet peak ($\delta +18.4$). Though these data (seven protons, five carbons and one phosphorus) immediately imply the structure of FMN, the retention time (R_t) in HPLC (5 min for FMN and 7 min for Flavin 1; LiChrosorb RP-18, 7% MeOH, 1 ml/min) and the chemical shift in the ^{31}P NMR spectrum of Flavin 1 were not consistent with those of FMN. The difference in the R_t value indicated that Flavin 1 is less polar than FMN. Since it is known that phosphorus-31 chemical shifts depend on the O-P-O bond angles in the phosphate esters (Gorenstein, 1975), the signal at $\delta +18.4$ implies that Flavin 1 has a five-

Table III. ^{13}C NMR data of Flavin 1 and FMN.

Carbon No.	Flavin 1	FMN
2	δ 159.8	δ 159.2
4	162.9	162.1
4a	135.1	135.2
5a	140.1	141.0
6	131.3	131.7
7	135.4	135.5
8	151.3	152.3
9	117.6	118.6
9a	132.5	133.0
10a	151.0	151.2
7a	19.5	20.2
8a	21.6	22.3
1'	48.4	49.1
2'	70.3	71.0
3'	73.4	74.2
4'	76.2	73.4
5'	67.3	66.8

Chemical shifts in δ -values (ppm).

membered cyclic phosphate ester moiety. The structure of Flavin 1 was, therefore, deduced to be 4',5'-cyclic FMN.

To confirm the structure, we prepared this compound from FAD by heat treatment (over 90°C) (Yagi and Matsuoka, 1960). The spectral data and chromatographic mobility of the synthetic product were identical to those of the natural one. This was the first isolation as a natural product, except for the separation by Yagi from a pig liver by boiled water extraction (Yagi, 1953). In this case riboflavin-4',5'-cyclic phosphate (4',5'-cyclic FMN) was probably obtained as the thermal decomposition product from FAD. Furthermore, Yagi *et al.* reported that the heat treatment of FAD (over 90°C) gave 4',5'-cyclic FMN (Yagi and Matsuoka, 1960). In our experiment, the handling of the natural product was performed in the absence of light at room temperature; there is no possibility that Flavin 1 is an artifact.

Flavin 2 was detected on the TLC of the mature plant extract. Though this compound was not isolated because of its very small quantity and being labile, it was clearly a flavin analog since it always gave lumichrome by decomposition (Yamano *et al.*, 1993).

Discussion

The difference in the concentrations of Flavin 1 as well as FMN between mature and vegetative

plants of *Scytosiphon* suggest that Flavins 1 and/or 2 correspond to the flagellar autofluorescent substance(s). The results that Flavins 1 and 2 were not detected in *Laminaria japonica*, which lacks flagellar autofluorescence, also gives indirect support to this explanation.

The photoreceptive mechanisms of algal phototaxis have been relatively well studied in the single-celled algae, *Chlamydomonas* (Chlorophyceae) and *Euglena* (Euglenophyceae). In *Chlamydomonas*, photoreceptive pigments are considered to be located on the surface layer of the stigma (Foster *et al.*, 1980, Kreimer and Melkonian, 1990). The photoreceptive pigment is suggested to be a II-*cis*-retinal, a common vision pigment in higher animals, based on experiments measuring the recovery of phototaxis in the *Chlamydomonas* mutant cells lacking stigmata by adding the analogs of retinal (Foster *et al.*, 1980).

In *Euglena*, photoreceptive pigments are considered to be located in the paraflagellar body, a lateral protuberance of one of the flagella. The paraflagellar body has been known to show green autofluorescence as in brown algal flagella, and both flavin and pterin are suggested to be contained in the paraflagellar body by fluorescence spectrometry studies (Benedetti and Checucci, 1975; Benedetti and Lenci, 1977; Galland *et al.*, 1990; Schmidt *et al.*, 1990).

On the other hand, brown algae are considered to have a different mechanism of phototaxis, because they are phylogenetically very distant and, moreover, they have different types of photoreceptive organs (e.g., flagella, stigma, action spectra and flagellar fluorescent substances).

The phototaxis of brown algae is one of the so-called blue light responses that use blue light as a stimulation signal (Kawai, 1992; Kawai *et al.*, 1990, 1991). Various kinds of blue light responses are known in many phylogenetic groups of organisms from bacteria to higher plants and animals, however, none of the receptive pigments have been isolated or identified to date. Therefore, if the flavins we isolated from brown algal plants are proved to be truly involved in the photoreception, this will become a breakthrough for the investigation of many other blue light responses.

The autofluorescence of brown algal flagella are rather unstable and easily deteriorated when excited. Our finding of the two flavins (Flavins 1 and

2), and the unstable flavin (Flavin 2) may be responsible for this flagellar fluorescence characteristic.

Experimental

General procedure

The ^1H NMR spectra (500 MHz) were recorded in D_2O ; chemical shifts were obtained as δ values in ppm relative to HDO (4.8 ppm) in D_2O . The ^{13}C NMR spectrum was measured in D_2O using dioxane as an internal standard. The ^{31}P NMR spectra were obtained in D_2O using 85% H_3PO_4 as an internal standard. Paper electrophoresis (PEP) was performed at pH 4.6 (pyridine-HOAc- H_2O , 3:3:994) and 600 V for 1.5 h. UV and fluorescence emission spectra were measured in H_2O .

Plant material

The mature and immature alga (*S. lomentaria*; 15kg and 1.2kg, wet weight respectively) were collected at Oshyoro Bay, Hokkaido, Japan. The mature *Laminaria japonica* (3kg, wet weight) was collected at Chikyuzaki, Muroran, Japan. Mature alga means the fertile plant bearing sporangia on the surface that contain flagellated cells with flagellar autofluorescence and immature alga does the young plant lacking sporangia and flagellated cells and therefore lacking flagellar autofluorescence.

Extraction

The fresh alga was soaked in methanol and allowed to stand for 2 or 3 days. For 15kg (wet) of mature, 1.2kg (wet) of immature *Scytosiphon lomentaria* and 3kg (wet) of mature *Laminaria japonica*, 25, 2, and 10 liters of methanol were used respectively. The methanol extracts were decanted and added to an equivalent volume of water. The methanol parts of extracts were removed *in vacuo* and the remained water solutions were partitioned with ethyl acetate. Ten, one and five liters of ethyl acetate were used for the extracts of the mature and the immature *S. lomentaria* and the mature *L. japonica*, respectively. The water layers were separated and the following isolation procedure was performed regarding above each extract.

Isolation of **1**

The water layer of the above extract was evaporated to give a copious residue which was dialyzed against water. Dialyzate was fractionated by column chromatography over HP-20. The 10–20% aq. methanol eluate was evaporated. The residue was rechromatographed on a ODS column. The 10–15% aq. methanol eluate was evaporated to yield a crude flavin derivative. This fraction was subjected to paper electrophoresis. The fluorescence area of +3–5 cm was cut out and the strips were extracted with water. After the solvent was removed *in vacuo*, the residue was purified by HPLC (LiChrosorb RP-18, 7% aq. methanol, Ex 445 nm, Em 525 nm) to give 4', 5'-cyclic FMN (**1**) (6 mg). **1**: a yellow powder; UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (log ϵ): 220 (4.46), 265 (4.48), 370 (3.97) and 445 nm (4.02); ^1H NMR (500 MHz): δ 2.29 (3H, *s*), 2.41 (3H, *s*), 3.98 (1H, *t*, *J*=5.8 Hz), 4.17 (1H, *m*), 4.19 (1H, *s*), 4.28 (1H, *m*), 4.58 (1H, *m*), 4.72 (1H, *m*), 5.00 (1H, *m*), 7.67 (1H, *s*) and 7.68 (1H, *s*); ^{13}C NMR (125 MHz): δ 19.5 (*q*), 21.6 (*q*), 48.4 (*t*), 67.3 (*t*), 70.3 (*d*), 73.4 (*d*), 76.2 (*d*), 117.6 (*d*), 131.3 (*d*),

132.5 (*s*), 135.1 (*s*), 135.4 (*s*), 140.1 (*s*), 151.0 (*s*), 151.3 (*s*), 159.8 (*s*) and 162.9 (*s*); ^{31}P NMR (161.9 MHz): δ +18.4 (*s*); FAB MS *m/z* (rel. int.): 462 [*M*+*Na*]⁺ (22) and 243 (49).

Conversion of FAD into **1**

FAD (83 mg) was stirred in water for 1 h at 110 °C. The solvent was removed *in vacuo*. The residue was chromatographed on a cellulose TLC plate (*R_f* 0.23, *n*-BuOH-HOAc-H₂O, 4:1:2) to yield **1** (29.4 mg). The NMR spectra, *R_f* value on TLC and *R_t* in HPLC of synthetic **1** were identical to those of natural **1**.

Detection of Flavin **2**

Ten percent aqueous methanol eluate of the HP-20 column chromatography described in the isolation of **1** was evaporated and the residue was chromatographed on cellulose tlc using *n*-BuOH-AcOH-H₂O (4:1:2) as a solvent. Flavin **2** appeared at *R_f* 0.19. This spot always accompanied with the spot of lumichrome on tlc (*R_f* 0.37).

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