Maristentorin, a Novel Pigment from the Positively Phototactic Marine Ciliate *Maristentor dinoferus*, Is Structurally Related to Hypericin and Stentorin

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The photoreceptor pigment of the heterotrich ciliate, *Maristentor dinoferus*, has been characterized. It is structurally similar to those of *Stentor coeruleus* and *Blepharisma japonicum* but differs significantly in that it bears no aromatic hydrogens. The structure of the pigment, maristentorin, is based upon the hypericin skeleton, and its spectra are nearly identical to those of hypericin but shifted toward the red. Within experimental error, its fluorescence lifetime is identical to that of hypericin, ∼5.5 ns in dimethylsulfoxide. It is remarkable that while the pigments are structurally similar in *S. coeruleus* and *M. dinoferus*, in the former there is an abrupt photophobic response, whereas in the latter there is a slow response toward light. The roles of the hypericin-like pigments in the heterotrich ciliates are discussed as potentially analogous in *Maristentor*.

Introduction

Several protozoa in a group of heterotrich ciliate families known collectively as stentorids have photoreceptor pigments that are not related to either rhodopsins (in animals) or to any of the photoreceptive pigments in photosynthetic eukaryotes.[1−4] Rather, they are structurally related to the hypericin group of naphthodianthrone quinone pigments. Hypericin is both toxic and medicinal, causing hypericism in animals and serving a variety of human needs including cancer and depression therapies. Hypericin occurs in the flowers of phylogenetically advanced species of *Hypericum*, including St. John's Wort (*H. perforatum*).[3,5] *Hypericum* spp. apparently do not use hypericin as a photoreceptor, but both hypericin and the stentorid pigments are toxic to potential predators or pathogens in their respective environments, in part through increasing photosensitivity.[3,6]

Structures have been determined for pigments from only two heterotrichs to date. *Stentor coeruleus* contains stentorin, which is very similar to hypericin,[7] while *Blepharisma japonicum* is colored by several blepharismins and oxyblepharismins, which have the ring structure modified by a "carbon bridge," [2,8−10] (Figure 1). The ciliate pigments occur in cortical granules just below the cell membrane. The pigments in *S. coeruleus* and *B. japonicum* are involved in the organisms' photophobic responses,[1,2,11,12] which can be abrupt. They are also involved in defense reactions, in which a cloud of pigment is released from a patch of cortical granules to deter raptorial ciliate predators.[13−15] Other heterotrichs are known to have spectrally similar pigments, but the structures have not yet been determined. Some other *Stentor* spp. have both pigment and symbiotic green algae (zoochlorellae) and are positively phototactic, but neither their photobiology nor their pigment structure has been studied.

A recently discovered marine ciliate, *Maristentor dinoferus*,[16,17] is prominently pigmented and also contains some 600 endosymbiotic golden algal cells (zooxanthellae) (Figure 2). It resembles *Stentor* but was placed in its own family within the stentorid group.[18] It is positively phototactic. Initial observations on the *Maristentor* pigment[16,17] showed that the pigment is fluorescent and has a UV−vis absorption spectrum similar to those of stentorin and hypericin. Here we report the structure of the chromophore and spectral properties of the pigment. Maristentorin is interesting both as a novel analogue of hypericin and as a new member of the multifunctional pigments from stentorids.

Materials and Methods

Materials. The solvents from Aldrich were used (freshly opened) without further purification. Silica gel was purchased from Sorbent Technologies, Atlanta, GA, having a porosity of 60 Å and a particle size of 32−63 μm (230−450 mesh).

Bulk Harvest of *Maristentor*. Seaweed (*Padina*) bearing dense populations of the ciliate was collected in gallon bags at a site in Apra Harbor, Guam, 3−5 m below low water.[16,17] During transport to the laboratory, many ciliate cells left the seaweed surface and were swimming; these were decanted into glass dishes. The seaweed and remaining ciliates were transferred to glass dishes without exposure to air. Dishes were kept at ambient temperatures (28−30 °C) and light (indirect daylight). Under these conditions the ciliates gathered on the lighted side of the dish just below the surface, and settled on the glass. When the water was poured out the other side of the dish, the ciliates remained stuck to the glass. Excess water and detritus were wiped from the bottom of the dish. The ciliate cells were then rinsed from the glass with 75% ethanol in water. Extracts, including cell debris, were shipped chilled to Ames, Iowa for analysis. The solution containing cells in 75% ethanol was centrifuged (at 7500 rpm for 20 min). The residue was resuspended in acetone to extract the pigment. This process was

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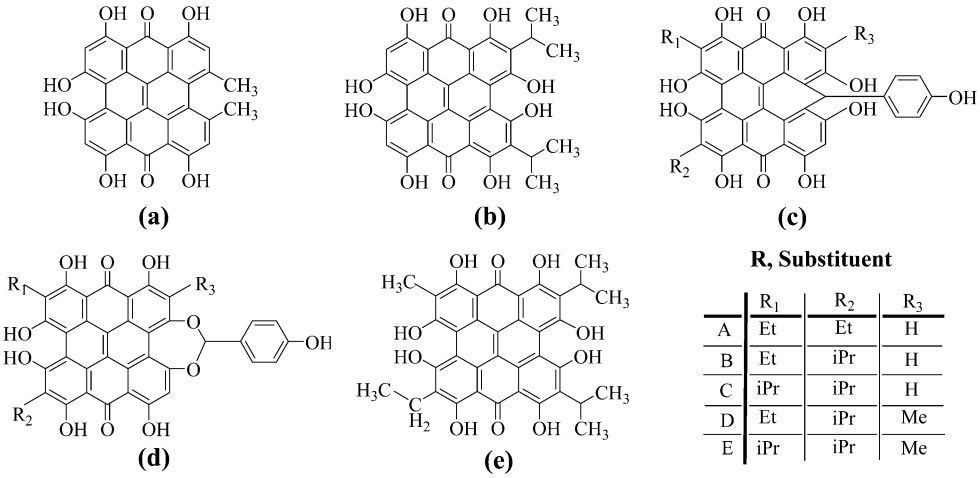

Figure 1. Structures of (a) hypericin, (b) stentorin, (c) blepharismins, (d) oxyblepharismins, and (e) a plausible form for maristentorin.

Figure 2. Portrait of *Maristentor*. The pigment is concentrated around the nucleus, just below the cap.

repeated five times. Solid compound recovered after acetone extraction was used to perform the silica gel column chromatography.

Chromatography. The compound obtained after acetone extraction was purified by silica gel column chromatography using a varying composition of mobile phase from 5:1 to 3:1 methylene chloride to methanol.

Steady-State and Time-Resolved Measurements. Steady-state excitation and emission spectra were obtained on a Spex Fluoromax with a 4-nm band pass and corrected for lamp spectral intensity and detector response. In all fluorescence measurements, a 1-cm-path-length quartz cuvette was used. The apparatus for time-correlated single photon counting is described elsewhere.[19] Fluorescence decay kinetics were recorded by exciting the sample at 407 nm and collecting emission at >500 nm with a cutoff filter. A maximum of 10 000 counts was collected at the peak channel.

Mass Spectroscopy. Electrospray mass spectra were obtained using the single quadrupole Shimadzu LCMS-2010 mass spectrometer (Shimadzu Corp. Kyoto, Japan). The mass range was from 50 to 1000 daltons. A 5-μL loop injection was performed for all experiments. The mass spectrometer was operated in the negative ion mode after proper tuning with known standards. The sample was dissolved in acetone.

NMR Spectroscopy. NMR spectra were collected on a Bruker Avance DRX 500 spectrometer equipped with a 5 mm ^{1}H/^{13}C/^{15}N gradient probe and operating at ^{1}H frequency of 499.867 MHz. Samples of maristentorin for NMR were dissolved in acetone-d_6. Chemical shifts were internally referenced to the methyl frequency of tetramethylsilane (TMS). All spectra were recorded at 298 K. One-dimensional ^{1}H (8 scans, sweep width 9000 Hz) and ^{1}H-decoupled ^{13}C spectra (1024 scans, sweep width 24 000 Hz) were acquired using standard experimental protocols. Two-dimensional double quantum filtered correlation spectroscopy (DQF-COSY),[20] ^{1}H−^{13}C heteronuclear single quantum coherence (HSQC) spectroscopy,[21] and ^{1}H−^{13}C heteronuclear multiple bond correlation spectroscopy[22] (HMBC) spectra were acquired. Pulse sequences utilizing PFG coherence selection were used for all three two-dimensional experiments. The DQF-COSY spectra consisted of 2048 × 256 complex points with a ^{1}H sweep width of 9000 Hz and 16 scans per free induction decay (FID). The HSQC and HMBC consisted of 1024 × 128 complex points with a ^{1}H sweep width of 4006 Hz and a ^{13}C sweep width of 18 855 Hz over identical spectral windows for each experiment. Exactly 32 and 160 scans per FID were used for the HSQC and HMBC spectra, respectively. A transfer delay of 60 ms corresponding to a long-range coupling constant of 8.3 Hz was used in the HMBC experiment. All NMR spectra were processed and analyzed using Broker Xwinnr 2.6 software.

Results

Two major pigments were separated chromatographically from the ethanol extract of ciliate cells. One has characteristic hypericin-like emission and excitation spectra (Figure 3), the other porphyrin-like spectra (Figure 4). The former, the maristentorin, has a single-exponential fluorescence lifetime in dimethylsulfoxide (DMSO) of 5.5 ± 0.2 ns (Figure 5), which is within experimental error identical to that of hypericin. The latter pigment is most likely a chlorophyll from the symbiotic dinoflagellate algae, the ~600 zooxanthellae in each ciliate.

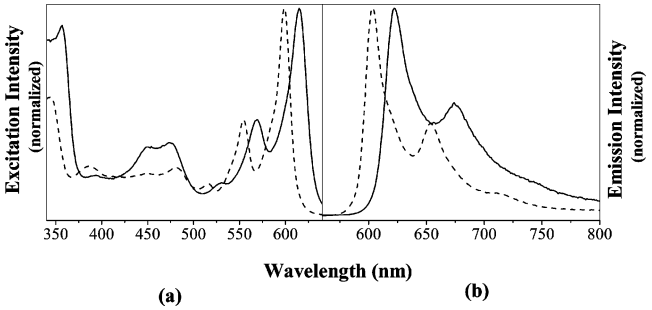

Figure 3. Normalized steady state (a) excitation and (b) emission spectra of maristentorin (solid line) and hypericin (dashed line) in DMSO. The excitation wavelength for the emission spectra is 550 nm. For the excitation spectra, the emission monochromator is set to 650 nm.

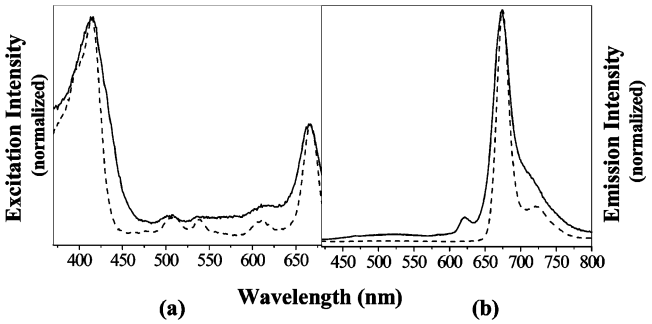

Figure 4. Normalized steady-state excitation and emission spectra of a porphyrin-like pigment obtained from *Maristentor* in DMSO (solid line). For purposes of comparison, the spectra of pyropheophorbide dissolved in DMSO (dashed line) are presented. The spectra are normalized to the reddest absorption maximum. For the emission spectra, the sample is excited at 410 nm, and for the excitation spectra, the emission monochromator is set to 700 nm.

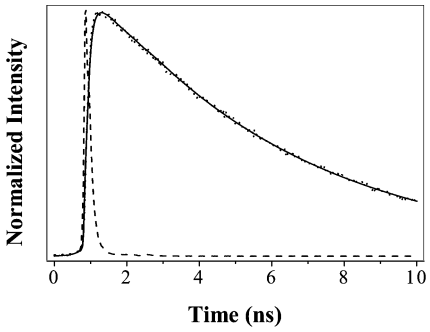

Figure 5. Fluorescence decay of maristentorin in DMSO. The sample is excited at 407 nm, and emission is collected at wavelengths greater than 500 nm with a cutoff filter. The decay is well described by a single exponential with $\tau_F = 5.5 \pm 0.2$ ns (based on the average of four measurements). For comparison, the fluorescence lifetime of hypericin in DMSO was found to be 5.6 ± 0.2 ns.[35]

When the maristentorin was subjected to analysis by electrospray mass spectrometry in the negative ion mode, it yielded a major peak at 619 *m*/*z* (Figure 6), corresponding to a molar mass of 620. This molecular mass differs from the structure proposed in Figure 1e by one methylene group. The presence of the methyl group was indicated by subsequent NMR measurements, and we reconcile the discrepancy by suggesting that a methylene group is lost during the ionization process.

NMR spectroscopy provided the key in determining the maristentorin structure. Figure 7 presents a one-dimensional ^{1}H NMR spectrum of maristentorin in acetone-d_6 acquired on a Bruker Avance DRX 500 NMR spectrometer. The lines at 15.6 and 15.4 ppm are due to the peri hydroxyl groups, as were

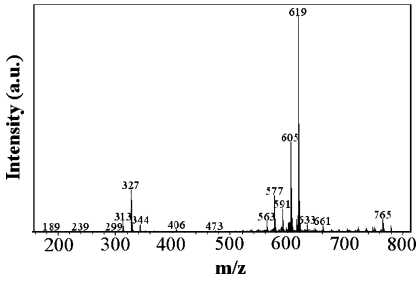

Figure 6. Electrospray mass spectra of maristentorin in the negative ion mode. The sample is dissolved in acetone. The dominant peak in the spectrum is at 619 *m*/*z*. This corresponds to a molecular mass that is one methylene group less than the suggested structure in Figure 1e. We believe that a methylene group is lost during the ionization process. The presence of the methyl group is borne out by the NMR spectra in Figure 10.

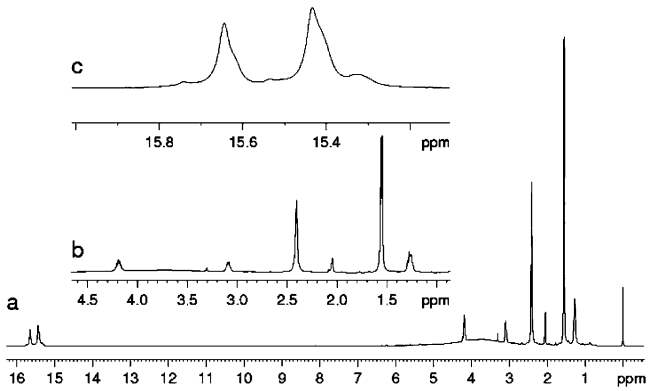

Figure 7. One-dimensional ^{1}H NMR spectrum of maristentorin in acetone-d_6 acquired on a Bruker Avance DRX 500 NMR spectrometer. Chemical shifts are internally referenced to the methyl frequency of TMS.

observed for all related pigments, namely, hypericin, stentorin, and blepharismin (Table 1). The spectrum also shows similar splitting for isopropyl groups as was found in stentorin[7] and blepharismin.[8] The major difference in the maristentorin spectrum is the appearance of a singlet at 2.4 ppm (comparable to hypericin),[23] a triplet at 1.3 ppm, and a quartet at 3.1 ppm. We assign these peaks to a methyl and an ethyl group, respectively. Most importantly, the absence of a signal at ~7 ppm clearly indicates the absence of any aromatic protons in maristentorin and is not consistent with the 619 *m*/*z* mentioned above. There are two lines due to aromatic hydrogens in hypericin (indicative of four aromatic protons), whereas only one is present in stentorin and blepharismin (indicative of two aromatic protons). The doublet due to aromatic hydrogens in blepharismin are from the extra phenyl ring, which is attached to the seven-membered ring. ^{1}H chemical shift values for all four pigments discussed here are tabulated in Table 1.

Figure 8 presents a one-dimensional ^{13}C NMR spectrum of maristentorin in acetone-d_6. The spectrum shows the presence of five alkyl carbons ($\delta = 10-30$ ppm), nine aromatic carbons ($\delta = 100-140$ ppm), and five carbonyl carbons ($\delta = 160-200$ ppm). The smaller and larger peaks correspond to one and two carbons, respectively, in the region $\delta = 100-140$ ppm. We have seen half the number of carbon peaks for aromatic and carbonyl carbons in maristentorin because of the structural symmetry. The total numbers of aromatic and carbonyl carbons in maristentorin are 18 and 10, respectively. The hydroxyl groups tend to have carbonyl characteristics, suggesting tautomeric forms.

TABLE 1: ^{1}H Chemical Shift Values (δ, ppm) for Hypericin,[23] Stentorin,[7] Blepharismin,[8] and Maristentorin in Different Solvents[a]

compound	hypericin/acetone-d_6	hypericin/DMSO-d_6	stentorin/DMSO-d_6	blepharismin/acetone-d_6	maristentorin/acetone-d_6
phenolic OH	14.82	14.74	15.3	14.8	15.6
	14.27	14.09	14.7	14.11	15.4
				9.72	
aromatic H	7.35 (s)	7.46 (s)	6.9 (s)	6.81 (s)	
	6.62 (s)	6.59 (s)		6.11 (d)	
				6.06 (d)	
nonaromatic H				7.07 (s)	
methylene					3.1 (q)
methyne			4.0 (septet)	3.89 (m)	4.2 (septet)
methyl	2.78 (s)	2.75 (s)	1.5 (d)	1.44 (d)	1.5 (d)
				1.41 (d)	1.3 (t)
					2.4 (s)

[a] The letters in the parentheses are the multiplicity of the signal.

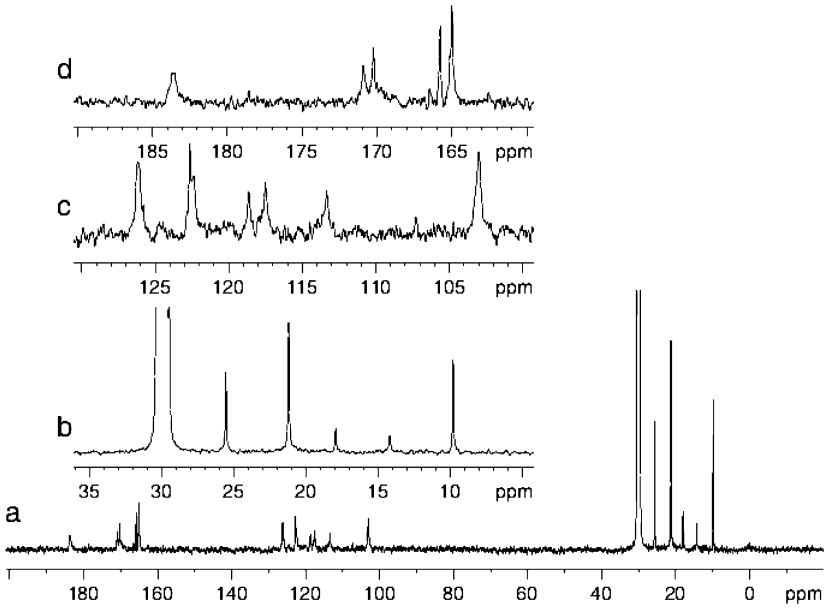

Figure 8. One-dimensional ^{13}C NMR spectrum of maristentorin in acetone-d_6. Chemical shifts are referenced to the solvent peak (at ~30 ppm).

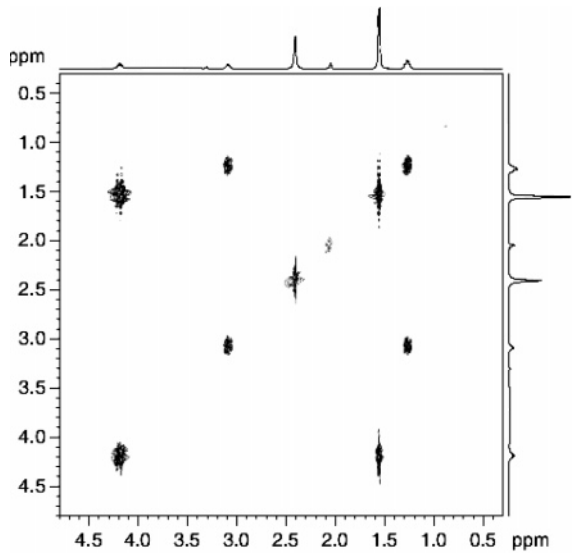

Figure 9. Two-dimensional COSY spectrum of maristentorin in acetone-d_6. The peak at 1.5 ppm is correlated with the peak at 4.2 ppm, whereas the peak at 1.3 ppm correlates with the peak at 3.1 ppm; these data confirm the presence of isopropyl and ethyl groups in maristentorin.

Figure 9 presents the two-dimensional COSY spectrum of maristentorin in acetone-d_6. The peak at 1.5 ppm is correlated with the peak at 4.2 ppm, whereas the peak at 1.3 ppm is in correlation with the peak at 3.1 ppm; these data confirm the presence of isopropyl and ethyl groups in maristentorin.

Finally, Figure 10 presents two-dimensional HSQC (panels a and c) and HMBC (panels b and d) spectra of maristentorin in acetone-d_6. The same contour levels are used to generate the spectra in panels a and c. Panel a shows the correlation of ^{1}H NMR peaks with ^{13}C alkyl peaks. In the HSQC experiment, there is no correlation between ^{1}H peaks with aromatic carbons, as shown in panel c. Panels b and d show the multiple bond correlation between ^{1}H and ^{13}C alkyl signals. All peaks from alkyl groups in the ^{1}H spectrum are correlated with aromatic carbons. The most compelling evidence for the existence of a methyl group in the structure is the strong correlation between the ^{1}H peak at 2.4 ppm and the ^{13}C peak at ~113.5 ppm.

Discussion

Structure. Upon the basis of the data presented above and upon analogy with stentorin[7,24,25] (Figure 1b), we propose the structure for maristentorin given in Figure 1e where the isopropyl groups are cis to the axis containing the carbonyl groups. We cannot, however, exclude the possibility of the trans isomer. The possibility of the isopropyl groups being on the same anthracene ring is virtually eliminated by consideration of biosynthetic pathways, since hypericin is produced in vivo through the polyketide pathway,[26] and this is presumably the pathway in the stentorids.

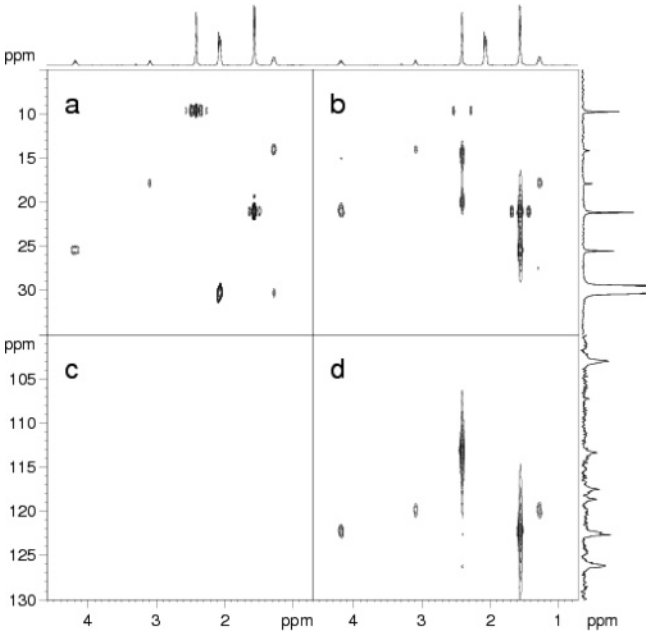

Figure 10. Two-dimensional (a and c) HSQC and (b and d) HMBC spectra of maristentorin in acetone-d_6. The same contour levels are used to generate the spectra in panels a and c.

Phylogenetic Considerations. There are two isopropyl groups in stentorin and the most common forms of blepharismin and oxyblepharismin and three alkyl groups in maristentorin. Blepharismin and oxyblepharismin have several derivatives with different substitutions; all have a *p*-hydroxybenzylidene "carbon bridge." The less common forms have ethyl and/or methyl in addition to or instead of isopropyl, but there are never more than three substituents [2] (Figure 1).

We now have several similar, presumably homologous, molecules from heterotrich ciliates (stentorin, blepharismin/oxyblepharismin and their derivatives, and maristentorin). The phylogeny of the heterotrichs is understood from only a few species,[18] and additional members of the stentorin group can be expected. Besides the stentorids mentioned so far, other stentorids such as some Folliculinidae have bluish pigments, some of which appear spectrally similar to stentorin. While *S. coeruleus* has stentorin, other species of *Stentor* have cortical pigments of other colors, and the structures of these have not yet been determined. Still other *Stentor* species have colorless cortical granules; these may contain unrelated compounds, such as the resorcinolic toxin climacostol in *Climacostomum virens*[27] that, like stentorin and blepharismin, acts as a predator deterrent. Pigments with hypericin/stentorin-like action spectra have been reported in two nonstentorid ciliates, the heterotrich *Fabrea salina* (Climacostomidae) and the hymenostome *Ophryoglena flava* (Ophryoglenidae) "although rhodopsin-like photoreceptors have also been suggested" for the photomovement of these ciliates.[2,28] It will be important in the context of phylogeny to establish whether these pigments in unrelated ciliates are hypericin analogues or not as well as whether other species of *Stentor* have stentorin-like pigments or not.

Possible Roles of Maristentorin. *Maristentor* has a large amount of pigment, and it must serve one or several roles. No role has yet been established, but the chemical similarity of maristentorin to stentorin and blepharismin suggests potentially similar biological functions, including light perception and defense against predators or UV irradiation. However, there are differences between *Maristentor* and *Stentor* that may provide illuminating comparisons. Most importantly, while both pigments may be involved in light detection, the results are quite different. Whereas *S. coeruleus* avoids light—in some strains very abruptly changing direction when encountering a light spot[2,29]—*Maristentor*, with its cargo of algal symbionts, moves toward light and does not change direction abruptly. Some *Stentor* (but not *S. coeruleus*) also have algal symbionts (zoochlorellae) and are positively phototactic. Some of these species (e.g., *S. amethystinus*, *S. fuliginosus*) have cortical pigments, but others (e.g., *S. polymorphus*) have colorless cortical granules.[30] *S. amethystinus* and *S. araucanus* would be particularly interesting for comparison with *Maristentor*, since their pigment colors are similar and they have zoochlorellae.

There are no known ciliate predators of *Maristentor* comparable to those that attack *Stentor* and *Blepharisma* in freshwaters (but the ciliate fauna of the reefs is barely known); however, fish predation of seaweeds on the reefs is significant. *Maristentor* occurs on various seaweeds and on rocks, and its clustering behavior should make it visible to foraging fish. If maristentorin is toxic or distasteful to fish, it could serve as a feeding deterrent, in which case its role in making the cells macroscopically visible could act as warning coloration.

The UV absorbption of blepharismin led Giese[31] to propose that it serves a protective role in *Blepharisma*, which is very light sensitive, and such a function could be useful to *Maristentor*, at least for shallow-water populations. *Maristentor* can redistribute its pigment; during the day specimens in bright light concentrate it around the macronucleus and away from the zooxanthellae.[16,17] *Maristentor*'s ability to move both pigment granules and algal symbionts within the host cell appears to be unique among the stentorids and provides the association more options than simply moving in response to environmental conditions.

Finally, an additional role for maristentorin is suggested by the work of Schlichter et al.[32] on deep-water corals, in which fluorescent pigment in the coral cells below the zooxanthellae enhanced photosynthesis of the algae. The fluorescence peak for maristentorin is close to the red absorbance of chlorophyll, and it is possible that it may serve as means of trapping and transferring light energy. None of these possible functions have yet been tested but remain for interesting future work on the biological side.

Conclusions

The photoreceptor pigment of the heterotrich ciliate, *Maristentor*, has been characterized. It is structurally similar to those of *Stentor* and *Blepharisma* but differs significantly in that it bears no aromatic hydrogens. The structure of the pigment, maristentorin, is based upon the hypericin skeleton, and its spectra are nearly identical to those of hypericin but shifted toward the red. Within experimental error, its fluorescence lifetime is identical to that of hypericin, ~5.5 ns in DMSO. It is remarkable that while the pigments are structurally similar in *Stentor* and *Maristentor*, in the former they give rise to an abrupt photophobic response and in the latter to a nonabrupt photophilic response. Clearly the immediate environment of the chromophore, for example, the protein to which it is attached, modulates its photoinduced properties. Such a modulation of physical properties was seen for hypericin itself in complex with the glutathione S-transferase isoforms A1-1 and P1-1.[33,34] Hypericin in complex with the former gives rise to significant oxidative damage, while in complex with the latter, almost none. It will be interesting to study the photophysical properties of the pigments in the heterotrich ciliates in complex with their respective proteins.

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