Chemical Structure of Blepharismin, the Photosensor Pigment for *Blepharisma japonicum*

Giovanni Checcucci,[†] Richard S. Shoemaker,[‡] Elisabetta Bini,[‡] Ronald Cerny,[‡] Nengbing Tao,[‡] Jae-Seok Hyon,[‡] Domenico Gioffre,[†] Francesco Ghetti,[†] Francesco Lenci,[†] and Pill-Soon Song*[,‡,§]

CNR Institute of Biophysics, Pisa, Italy
Department of Chemistry, University of Nebraska
Lincoln, Nebraska 68588-0304
Kumho Life & Environmental Science Laboratory
Kwangju 506-712, Korea

Received March 5, 1997

Blepharisma japonicum, a motile unicellular ciliate,[1,2] is capable of both light intensity- and color (wavelength)-sensory perception. This primitive photosensing apparatus represents a unique "visual sensory system" based on blepharismin as the photodetector molecule.[3]

We report here the chemical structure of the photosensor molecule, the major component of blepharismins. [The blepharismin family includes at least five derivatives.] The proposed structure is illustrated in Figure 1. Mass spectrometry, FT-IR, and NMR methods have been employed to elucidate the chemical structure of blepharismin, a derivative of hypericin (a powerful photodynamic sensitizer in nature[1,2]). Blepharismin is also a member of the ciliate photosensory pigments that include stentorin, from *Stentor coeruleus*, whose structure has been recently elucidated.[4,5] Our preliminary data suggested that the blepharismin chromophore was similar, but not identical, to the stentorin chromophore. We show in this paper that blepharismin possesses a unique structure present neither in the parent molecule, hypericin, nor in the relative one, stentorin.

Experimental procedures for the isolation and purification of the major blepharismin species and the structural approaches used were similar to those described for stentorin.[4] Briefly, *B. japonicum* was grown in the dark as described previously.[6,7] [See the Supplementary Information for details.] Blepharismin pigments from the cell culture were extracted in acetone according to Ghetti et al.,[7] dried, and redissolved in methanol. The pigment extracts were purified by means of a Nucleosil C_{18} reversed phase column (Alltech; 250 × 10 mm, 5 μm) eluted with 72% methanol−18% ethyl acetate−10% water−0.04% trifluoroacetic acid. Five pigment fractions were resolved by HPLC. The third retention-time peak, which represented the major component, was further HPLC purified for its structure determination by rechromatography with 72% methanol−14% ethyl acetate−14% water−0.04% trifluoroacetic acid. The purified pigment collected from HPLC was red liquid when dissolved in an organic solvent or red powder when vacuum dried. The blepharismin sample was subjected to high-resolution mass spectrometry, proton and carbon-13 NMR (both 1- and 2-dimensional), and FT-IR.

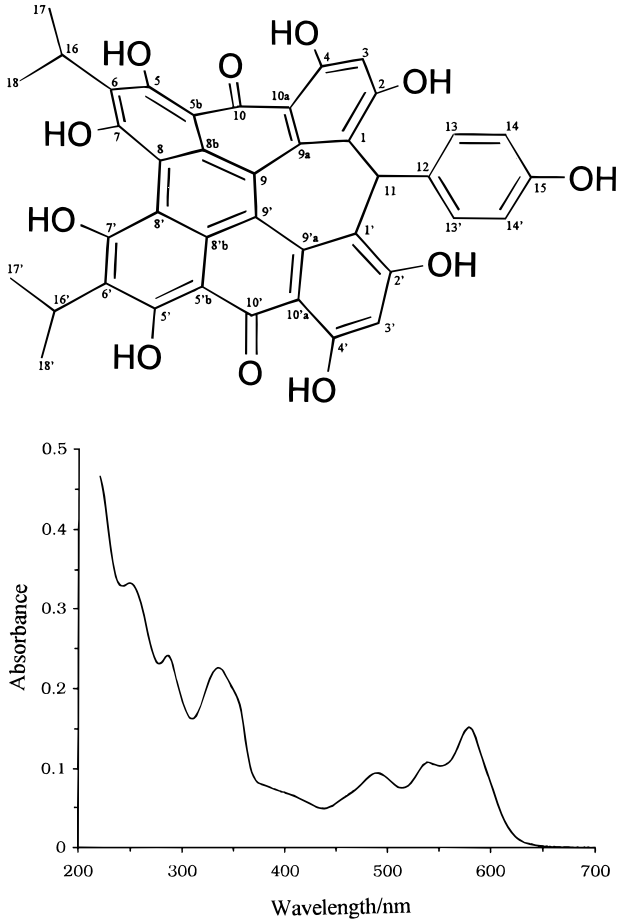

Figure 1. Chemical structure of blepharismin [2,4,5,7,2′,4′,5′,7′-octahydroxy-6,6′-diisopropyl-1,1′-(*p*-hydroxybenzylidene)naphthodianthrone]. The absorption spectrum of blepharismin (4 μM) in ethanol at room temperature is shown in the lower panel.

The MW of blepharismin was determined to be 698 for $C_{41}H_{30}O_{11}$ by mass spectrometry. The high-resolution FAB value for the negative ion [$(M - H)^-$] was 697.1693. The theoretical value for the blepharismin negative ion for $C_{41}H_{29}O_{11}$ is 697.1710; the relative error is −2.4 ppm. Enough highly purified sample has been collected to enable the acquisition of 2-dimensional H−H (COSY) and H−C (HMQC) spectra, including the proton-detected, H−C multiple-bond correlation experiment (HMBC). Two HMBC spectra were acquired, using different values for long-range H−C *J* coupling (6 and 3 Hz, respectively), so that both medium and weak long-range H−C couplings could be observed. From the 500 MHz proton NMR data, we identify two isopropyl groups (12H at 1.4 ppm; 2H at 3.9 ppm), a *para*-substituted phenol (4H at 6.1 ppm), and three peaks of hydroxyl protons (1H at 9.8 ppm, 1H at 13.8, and 1H at 14.8 ppm). Hydrogen exchange between the labile hydroxyl groups and deuterated solvent (acetone at 2.04 ppm) accounts for the decreased integrated intensities for these OH protons, or their absence from the spectrum altogether. The mechanism for this exchange is well known to be acid-mediated keto−enol tautomerism followed by exchange between the enol deuteron and the OH on the unknown compound. Deuterium (D_2O) exchange experiments have confirmed the assignment of the OH residues on this structure.

* To whom correspondence should be addressed. Phone: (402) 472-2749. FAX: (402) 472-2044. E-mail: pssong@unl.edu.
† CNR Institute of Biophysics.
‡ University of Nebraska.
§ Kumho Life & Environmental Science Laboratory.

(1) (a) Giese, A. C. *Blepharisma: The Biology of a Light-Sensitive Protozoan*; Stanford University Press: Stanford, CA, 1973; pp 366. (b) Giese, A. C. *Photochem. Photobiol. Rev.* **1981**, *6*, 139−180.
(2) (a) Song, P.-S. *Biochim. Biophys. Acta* **1981**, *639*, 1−29. (b) Song, P.-S. *Annu. Rev. Biophys. Bioeng*. **1983**, *12*, 35−68.
(3) (a) Scevoli, P.; Bisi, F.; Colombetti, G.; Lenci, F.; Passarelli, V. *J. Photochem. Photobiol., B* **1987,** *1*, 75−84. (b) Matsuoka, T. *J. Protozool.* **1983**, *30*, 409−414. (c) Matsuoka, T.; Matsuoka, S.; Mamaoka, Y.; Kuriu, T.; Watanabe, Y.;. Takayanagi, M.;. Kato, Y.; Taneda, K. *J. Protozool.* **1992**, *39*, 498−502.
(4) Tao, N.; Orlando, M.; Hyon, J.-S.; Gross, M.; Song, P.-S. *J. Am. Chem. Soc.* **1993**, *115*, 2526−2528.
(5) Cameron, D. W.; Riches, A. G. *Tetrahedron Lett.* **1995**, *36*, 2331−2334.
(6) Gioffre, D.; Ghetti, F.; Lenci, F.; Paradiso, C.; Dai, R.; Song, P.-S. *Photochem. Photobiol.* **1993**, *58*, 275−279.
(7) Ghetti, F.; Lenci, F.; Checcucci, G.; Heelis, P. F. *J. Photochem. Photobiol., B* **1992**, *13,* 315−321.

S0002-7863(97)00713-0 CCC: $14.00

Table 1. Carbon and Hydrogen Chemical Shifts (ppm) for Blepharismin[a]

Carbon	Carbon shift	Hydrogen shift
1	124.5	
2	159.5 or 163.2	
3	103.71	6.81 (2H, s)
4	159.5 or 163.2	
5	159.6*	
5b	119.6*	
6	121,3	
7	166.5*	
8	126.3*	
8b		
9		
9a	132.9*	
10	184.2*	
10a	110.4	
11	32.5	7.07 (1H, s)
12	136.5	
13	114.7	6.06 (2H, d)
14	127.6	6.11 (2H, d)
15	155.3	
16	25.3	3.89 (2H, m)
17	20.5 or 21.1	1.41 (6H, d)
18	20.5 or 21.1	1.44 (6H, d)
15-OH		9.72 (OH)
4-OH, 5-OH		14.11 (OH)
5-OH, 4-OH		14.8 (OH)

[a] Resonances labeled with an asterisk have been assigned according to chemical shift only. All other ^{13}C resonances have been assigned through direct correlation to hydrogens via HMBC or HMQC experiments.

There are two unknown ^1H NMR peaks at 6.8 ppm (2H) and 7.1 ppm (1H). The HMQC and HMBC spectra have enabled the identification of these two unknown proton NMR resonances through correlations with the carbon nuclei in the structure. The ^1H NMR peak at 6.8 ppm is connected to a carbon peak at 103.71 ppm, indicating an aromatic carbon. The ^1H peak at 7.1 ppm is connected to a carbon peak at 32.5 ppm, indicating a nonaromatic carbon [see later discussion on this assignment]. The HMBC ($J_{est} = 6$ Hz) spectrum correlates the CH proton at 7.1 ppm with two aromatic carbons at 124.5 and 135.5 (C1 and C12 in Figure 1). The 6.8 ppm ^1H NMR peak correlates to carbon peaks at 110.4 (C10a in Figure 1). The HMBC ($J_{est} = 3$ Hz) data also correlate the 6.8 ppm peak with the carbon resonances at 159.5 and 163.2 ppm, which are assigned to the aromatic C−OH carbons (C2 and C4 in Figure 1).

The ^1H and ^{13}C NMR data are presented in Table 1. All resonances except those marked with an asterisk were assigned through direct through-bond correlations in the 2D NMR experiments. The resonances labeled with an asterisk were assigned according to chemical shift only. The FT-IR spectrum shows a strong peak at 1597 cm^{-1}, clearly indicating that carbonyls are present. Mass spectra [FAB^+, MS/MS $(M + H)^+$, FAB^-, MS/MS $(M - H)^-$] clearly show the presence of two groups of MW 106 (CH-phenol) and 93 (phenol).

From the NMR data obtained, most importantly, the equivalence of the carbon and proton resonances at positions 3 and 3′, we conclude that the hydroxyl groups are symmetrically positioned, thus making the bridging carbon achiral. Circular dichroic analysis confirmed the achiral nature of the compound (data not shown), consistent with the symmetric structure shown in Figure 1. Surprisingly, the structure 2,4,5,7,2′,4′,5′,7′-octahydroxy-6,6′-diisopropyl-1,1′-(*p*-hydroxybenzylidene)naphthodianthrone derived from the present results deviates from the parent compound hypericin[8] and its related ciliate photoreceptor chromophore stentorin.[4] [A virtually identical structure was proposed by T. Matsuoka, private communication: Abstract No. S295, 12th Photobiol. Congress, Vienna, 1996.] The unique feature of the blepharismin structure is the tertiary carbon bridge to which a phenolic group is linked. Regarding the chemical shift of 7.1 ppm for the CH proton, the assignment of this proton (and attached carbon) relative to the aromatic rings reflects direct correlations in the 2D HMBC data, *vide supra*. For comparison, the CH of triphenylmethane appears at 5.5 ppm in the ^1H spectrum. When the bond strain combined with the extended, conjugated π-system in the proposed structure is considered, the 1.5 ppm difference is not unexpected. In addition, if one looks at the results of the modeling, the twist of the blepharismin rings places the CH in question directly on-edge to the aromatic rings (this will maximize the deshielding due to the ring current). Although the 7.1 ppm shift might at first glance seem to be large, it is not unreasonable. Also, note that the direct correlation between the CH proton in question and the adjacent ring systems has been unambiguously established.

The bridged structure introduces a distortion of the upper and lower halves of the naphthoanthraquinone groups, thus causing a blue shift of the absorbance maximum, relative to hypericin and stentorin (see below). A semiempirical calculation predicted that the bridged blepharismin ring system is 36 kcal/mol higher in energy than the hypericin ring system. This energy difference is consistent with the loss of aromaticity for one aromatic ring. Also, the structure assigned accounts for the facile photochemical transformation of blepharismin to "oxyblepharismin", with loss of two hydrogen atoms. We observed that the latter can actually be formed *in vivo* and *in vitro* even in the absence of oxygen, suggesting that the prefix "oxy" cannot refer to the photooxidation with oxygen as oxidant (unpublished results).

Interestingly, blepharismin that is isolated from the dark-adapted *Blepharisma* cells exhibits an absorbance maximum at 576 nm (Figure 1, lower panel; the red appearance is due to its fluorescence emission). This band red shifts to ca. 590 nm (blue blepharismin, weakly fluorescent) upon irradiation with red light, both *in vivo* and *in vitro*.[9] Both red and blue blepharismins appear to be functional. It remains to be studied whether or not the fluorescent-to-weakly fluorescent blepharismin phototransformation is part of its photocycle or both species independently serve as photodetector in the photosensory transduction in the cell.

Acknowledgment. This work was supported by the U.S. Army Research Office Grant No. DAAH04-94-G-0346. We thank Professor David Berkowitz for helpful discussion. This paper is dedicated to Professor Sang Chul Shim on the occasion of his 60th birthday. Kumho Life & Environmental Science Laboratory Publication No. 3.

Supporting Information Available: Description of blepharism isolation and mass, ^1H NMR, ^{13}C NMR, $^1H-^{13}C$ HMQC and HMBC, and FT-IR spectra (20 pages). See any current masthead page for ordering and Internet access instructions.

JA970713Q

(8) Etzlstorfer, C.; Falk, H.; Muller, N.; Schmitzberger, W.; Wagner, U. G. *Monatsh. Chem.* **1993**, *124*, 751−761.
(9) Checcucci, G.; Damato, G.; Ghetti, F.; Lenci, F. *Photochem. Photobiol.* **1993,** *57*, 686−689.