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

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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 blepharismine as the photodetector molecule.³

We report here the chemical structure of the photosensor molecule, the major component of blepharismine. [The blepharismine 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 blepharismine, a derivative of hypericin (a powerful photodynamic sensitizer in nature^{1,2}). Blepharismine 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 blepharismine chromophore was similar, but not identical, to the stentorin chromophore. We show in this paper that blepharismine 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 blepharismine 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.] Blepharismine 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 C₁₈ 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

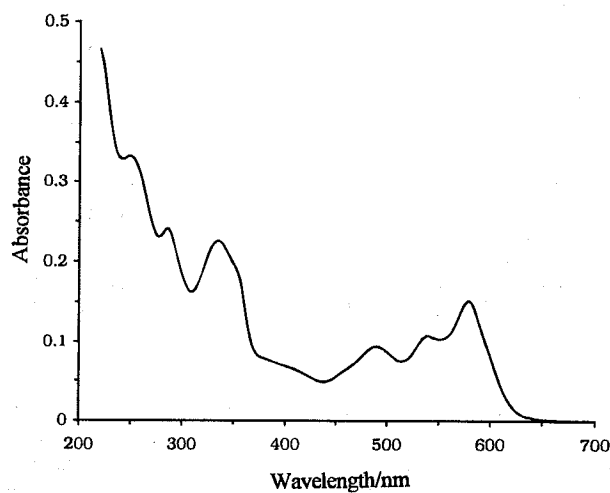
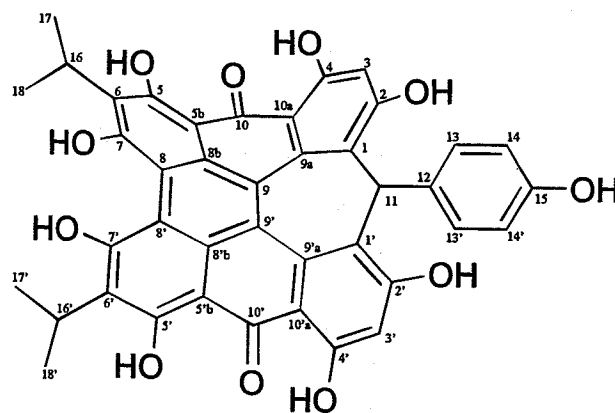


Figure 1. Chemical structure of blepharismine [2,4,5,7,2',4',5',7'-octahydroxy-6,6'-diisopropyl-1,1'-(p-hydroxybenzylidene)naphthodione]. The absorption spectrum of blepharismine (4 μM) in ethanol at room temperature is shown in the lower panel.

purified pigment collected from HPLC was red liquid when dissolved in an organic solvent or red powder when vacuum dried. The blepharismine sample was subjected to high-resolution mass spectrometry, proton and carbon-13 NMR (both 1- and 2-dimensional), and FT-IR.

The MW of blepharismine was determined to be 698 for C₄₁H₃₀O₁₁ by mass spectrometry. The high-resolution FAB value for the negative ion [(M – H)[–]] was 697.1693. The theoretical value for the blepharismine negative ion for C₄₁H₂₉O₁₁ 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 deuterium and the OH on the unknown compound. Deuterium (D₂O) exchange experiments have confirmed the assignment of the OH residues on this structure.

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Table 1. Carbon and Hydrogen Chemical Shifts (ppm) for Blepharismine^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 ¹³C 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 ($J_{\text{est}} = 6$ 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 ($J_{\text{est}} = 3$ 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 ¹³C 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 hypericin⁸ 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 blepharismine 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 blepharismine 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 blepharismine 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 blepharismine to "oxyblepharismine", 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, blepharismine 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 blepharismine, weakly fluorescent) upon irradiation with red light, both *in vivo* and *in vitro*.⁹ Both red and blue blepharismine appear to be functional. It remains to be studied whether or not the fluorescent-to-weakly fluorescent blepharismine 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 blepharismine isolation and mass, ¹H NMR, ¹³C NMR, ¹H-¹³C HMQC and HMBC, and FT-IR spectra (20 pages). See any current masthead page for ordering and Internet access instructions.

JA970713Q

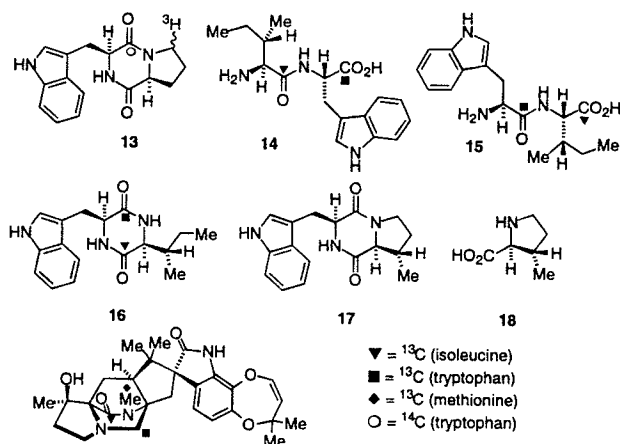
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Additions and Corrections

Studies on the Biosynthesis of Paraherquamide A. Origin of the β -Methylproline Ring [*J. Am. Chem. Soc.* **1996**, *118*, 7008–7009]. EMILY M. STOCKING, JUAN F. SANZ-CERVERA, ROBERT M. WILLIAMS,* AND CLIFFORD J. UNKEFER

The correct stereochemistry for compounds (16) *cyclo-L*-tryptophan-L-isoleucine, (17) *cyclo-L*-tryptophan-L- β -methylproline, and (18) L- β -methylproline in Figure 1 is shown below. The authors are indebted to Dr. Jeremy Everett for bringing this error to our attention.



JA9754158

Electron Transfer from C_{76} (C_{2v}) and C_{78} (D_2) to Radical Cations of Various Arenes: Evidence for the Marcus Inverted Region [*J. Am. Chem. Soc.* **1997**, *119*, 5744–5745]. DIRK M. GULD†* AND KLAUS-DIETER ASMUS

The correct notation for the C_{76} and C_{78} isomers throughout the paper should be C_{76} (D_2) and C_{78} (C_{2v}).

JA975412V

Facile Metathetical Exchange between Carbon Dioxide and the Divalent Group 14 Bisamides $M[N(SiMe_3)_2]_2$ ($M = Ge$ and Sn) [*J. Am. Chem. Soc.* **1996**, *118*, 10912–10913]. LAWRENCE R. SITA,* JASON R. BABCOCK, AND RIMO XI

After publication, we have become aware of prior work by Wannagat and co-workers in which they describe a related metathetical exchange process that occurs between carbon dioxide and $NaN(SiMe_3)_2$ to produce, along with 1,3-bis-(trimethylsilyl)carbodiimide (85% yield), a mixture of products arising from secondary reactions [Wannagat, U.; Kuckertz, H.; Krüger, C.; Pump, J. Z. *Anorg. Allg. Chem.* **1964**, *333*, 54–60]. We regret this omission, however, it does not affect the findings or conclusions of the present work in which the synthetic utility of this form of heterocumulene metathesis is demonstrated for subvalent Group 14 compounds.

JA975414F

Chemical Structure of Blepharismine, the Photosensor Pigment for *Blepharisma japonicum* [*J. Am. Chem. Soc.* **1997**, *119*, 5762–5763]. GIOVANNI CHECCUCCI, RICHARD K. SHOEMAKER, ELISABETTA BINI, RONALD CERNY, NENGBING TAO, JAE-SEOK HYON, DOMENICO GIOFFRE, FRANCESCO GHETTI, FRANCESCO LENCI, AND PILL-SOON SONG*

Page 5762: Richard Shoemaker's initial appeared incorrectly as S. in the journal.

Page 5762: The ring system of the structure shown in Figure 1 should be named benzodanthrone, instead of naphthodianthrone. The ring system linked by the bond 8-8' is dihedrally twisted, and the lack of a CD signal may be attributable to a racemic mixture of the two possible enantiomers. We thank Prof. Heinz Falk for pointing out the correct naming and asymmetry of the blepharismine structure.

JA975413N

Computer Software Reviews

Axum Version 5.0 for Windows. MathSoft, Inc.: 101 Main Street, Cambridge, Massachusetts, 02142; (617) 577-1017. \$199.95.

Axum 5.0 is a technical graphics and data analysis program. This version comes on eleven 3.5 in. 1.4 megabyte disks and is compatible with the DOS/Windows 3.x, Windows NT, and Windows '95 operating systems. MathSoft recommends a minimum 486-computer with 16 megabytes of memory and 14–20 megabytes of disk space depending on the installation procedure chosen. A math co-processor is also recommended but is not required. Hence, Axum is a reasonable choice for high-end academic research or industrial computers but will be more demanding of older, microlab academic computers.

Unlike many available software packages that also render technical graphics and perform data analysis, Axum is geared toward scientific applications rather than business applications. This is reflected throughout the program, including the ability to change the precision of the data with a single click on an icon and the inclusion of many mathematical functions, scientifically-important graph options, and a programming language to perform sophisticated data analysis. An important weakness of the program is the lack of an easy procedure within the program to insert complex characters into labels or titles (for example, Greek characters or mathematical symbols), although the

availability of tool bar buttons for fast addition of superscripts and subscripts to text is an improvement over previous versions of Axum. Additional features of the program include the ability to cut and paste comments, equations, and other figures (such as a chemical model) from any Windows-compatible word processor or graphics program into an Axum graph, in addition to the expected ability to cut and paste graphs generated with Axum into word processor programs.

In general, Axum is icon-driven and most commands are easily executed by using a tool bar that is similar to those used in most word processors and other graphics programs written for Windows and Macintosh platforms. As in older versions of Axum, the keyboard can be used to execute most commands by using combinations of the ALT, CTRL, and SHIFT keys. This is cumbersome, however, and the program works much more smoothly with the mouse.

Data can be imported in the usual ASCII format or can be imported directly from several popular spreadsheets like Excel, Quattro Pro, Paradox, and Lotus. Data can also be deleted or copied to new data sheets or new locations within the same data sheet by dragging or cutting and pasting individual data cells or entire data columns.

To make a graph, the user must click on the icon specifying a two-dimensional plot or a three-dimensional plot. This causes a menu of