

On the Structure of Oxyblepharismine and its Formation from Blepharismine

Dietrich Spitzner^{1*}, Gerhard Höfle^{2*}, Iris Klein¹, Silke Pohlen²,
Dieter Ammermann³, Lothar Jaenicke⁴

¹Institut für Chemie, Universität Hohenheim, Garbenstr. 30, D-70599 Stuttgart,

²Gesellschaft für Biotechnologische Forschung mbH, Abt. Naturstoffchemie, Mascheroder Weg 1, D-38124 Braunschweig,

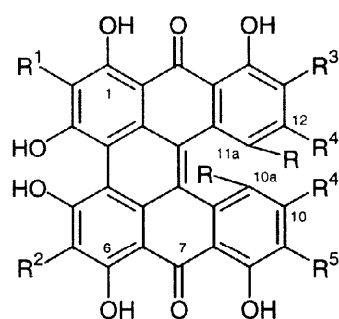
³Zoologisches Institut, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen,

⁴Institut für Biochemie, Universität Köln, Zùlpicher Str. 47, D-50674 Köln.

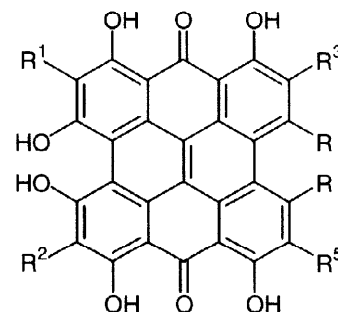
Received 5 March 1998; accepted 29 March 1998

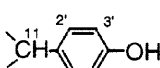
Abstract: The blepharismine (1) from *Blepharisma japonicum* give the corresponding oxyblepharismine (2) on irradiation *in vitro* and *in vivo*. The chemical structures of these compounds are elucidated and a mechanism is given for this unusual transformation. © 1998 Elsevier Science Ltd. All rights reserved.

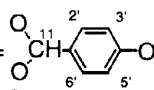
The pale purple colored ciliate *Blepharisma japonicum* has been studied extensively by Giese.¹ This organism shows negative phototaxis which was attributed to a photoreceptor. Exposure to bright light kills the organism due to the photodynamic activity of the pigments.² Giese has already demonstrated the existence of blepharismine (“red pigment”) and its photoproduct oxyblepharismine (“blue pigment”). The “red pigment” absorbs in both ethanol and in the living cell at $\lambda_{\max} = 576$ nm. The absorption maximum of the irradiation product (“blue pigment”), however, is shifted to $\lambda_{\max} = 592$ nm (ethanol). HPLC analysis of the coloring matter gave five blepharismine³ having a UV/Vis spectrum and structural features similar to compound 6c which was later isolated from *Stentor coeruleus*.⁴ According to HR-MS the major pigment of the blepharismine family possesses an elemental composition of $C_{41}H_{30}O_{11}$.³ The chemical structure of the major component of the blepharismine family was elucidated by Song et al. very recently.⁵ The structures of the other homologues were elucidated independently by Naoki et al..⁶ The blepharismine possess basically a



	1, 2, 5, 6	R ¹	R ²	R ³	R ⁴	R ⁵
A	a	Et	Et	H	OH	H
B	b	Et	<i>i</i> Pr	H	OH	H
C	c	<i>i</i> Pr	<i>i</i> Pr	H	OH	H
D	d	Et	<i>i</i> Pr	Me	OH	H
E	e	<i>i</i> Pr	<i>i</i> Pr	Me	OH	H



blepharismine A-E (1) R,R = 
 protohypericin (3) R, R¹, R², R³, R⁵ = H; R⁴ = Me
 protostentorin A-E (5) R = H

oxyblepharismine A-E (2) R,R = 
 hypericin (4) R = Me; R¹, R², R³, R⁵ = H
 stentorin A-E (6) R = OH

basically a protohypericin (2)⁷ like hydroxyquinone system attached in a unique fashion to a *p*-hydroxybenzylidene moiety as deduced from the spectra.

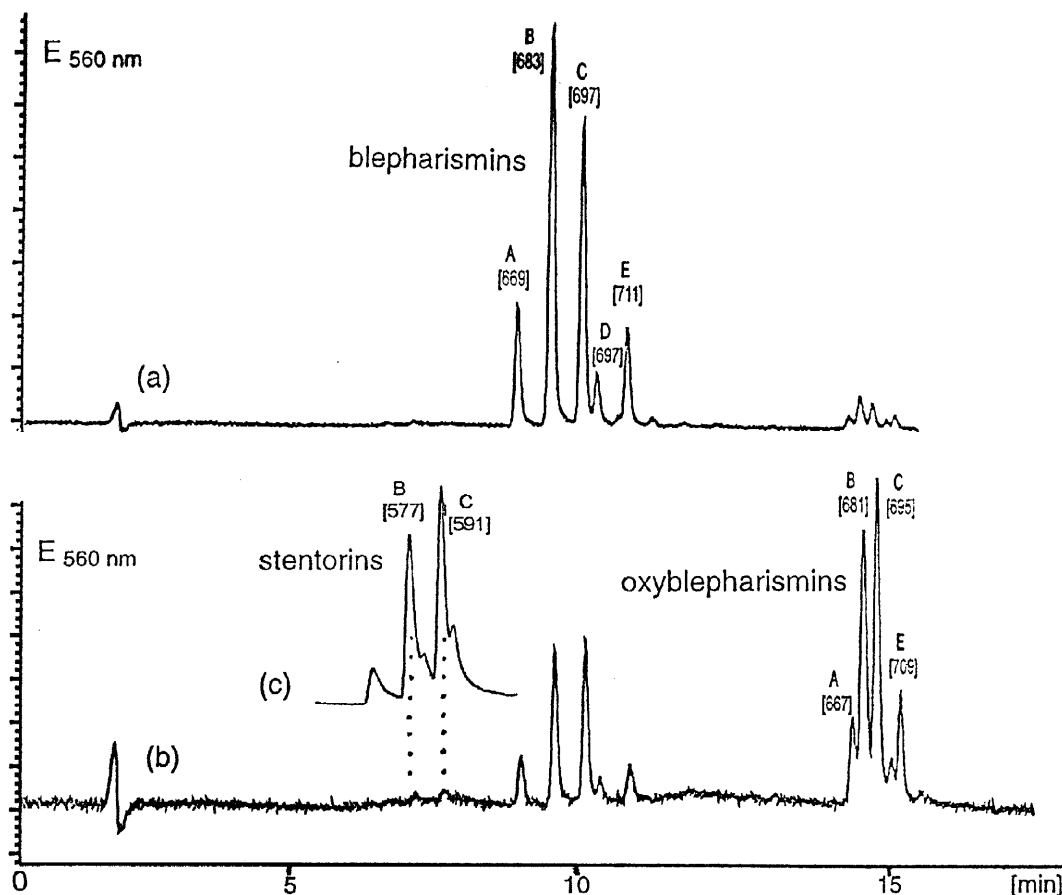


Fig. 1: *Ex-vivo* RP-HPLC/ESI-MS⁸ of *B. japonicum* grown in the dark (a) and in day-light (b) and of stentorin A-E (c).

Ex-vivo RP-HPLC-ESI-MS⁹ analysis of *B. japonicum* cultures¹⁰ confirmed the presence of at least three major and two minor components named blepharismins A-E (**1a-e**) according to the order of elution. Cultures grown in day-light contain in addition significant amounts of later eluting oxyblepharismins A-E and traces of the more polar stentorins A-E (**6a-e**) (Fig. 1). According to the molecular mass determination blepharismins C and D are isomers with (M-H)⁻ 697 mu, B and A lower homologues (683 and 669 mu, resp.), and E (711 mu) is a higher homologue. All members of the oxyblepharismine family contain consistently two hydrogen atoms less indicating an extra double bond or a ring. Blepharismine C (**1c**) was converted into oxyblepharismine C (**2c**) by irradiation in ethanol saturated with argon. The UV/Vis spectrum shows a number of isosbestic points (Fig. 2) which excludes accumulation of significant amounts of intermediates. The bathochromic shift of the long wavelength absorption pattern by going from blepharismins (**1**) to oxyblepharismins (**2**) reveals a striking similarity to protohypericin (**3**) and hypericin (**4**) and leads straightforwardly to structure **2** for the oxyblepharismins. This assignment is supported by the fact that the blepharismins (**1**) dissolve in alkaline solution with a blue color like **3**, the oxyblepharismins, however, dissolve under these conditions with a green color like **4**. In accordance, contrary to the

blepharismins, the oxyblepharismins are easily cleaved with 2% sulfuric acid in methanol to the stentorins **6** and *p*-hydroxybenzaldehyde. In order to prove the proposed structure of the oxyblepharismins, the quinones **1a-e** were isolated by preparative HPLC.¹¹ The substitution patterns of **1** determined by ¹H NMR are as described by Naoki et al.⁶ However, contrary to the published structure^{5,6} we propose that the *p*-hydroxyphenyl residue is placed above the quinoid system (Fig. 3). In this *endo* position it is shielded by the extended π -system of the quinone moiety which perfectly explains the upfield shifts of 2'-H and 3'-H by 1.13 and 0.77 ppm, resp., compared to e.g. *p*-cresol. On the other hand the *exo* 11-H is placed in the deshielding bay area of two phenyl rings and comes into close proximity of two hydroxyl groups at C-10 and C-12 resulting in the extreme low-field shift of δ 7.1.

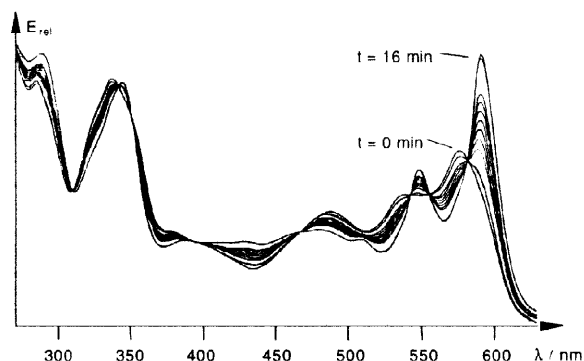
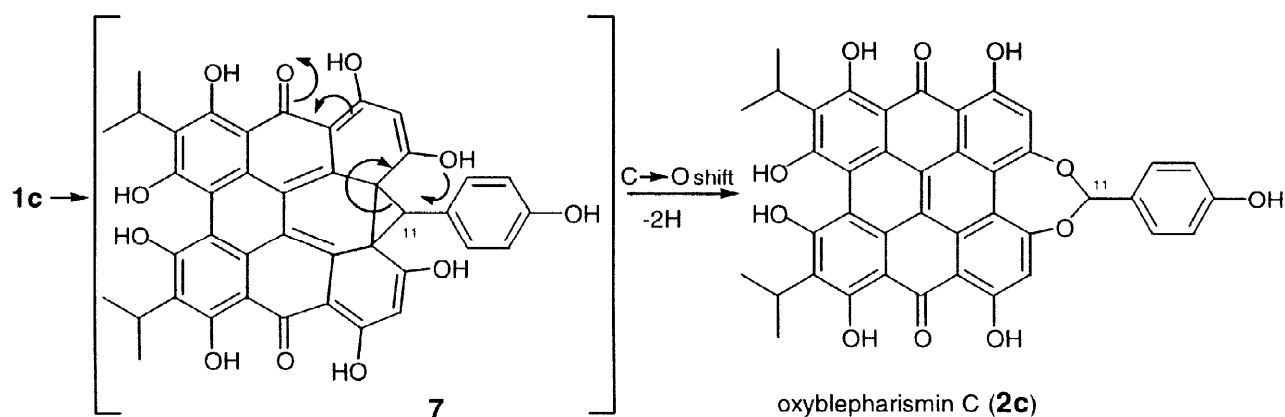


Fig 2: Irradiation of **1c** in ethanol with $\lambda = 400\text{--}600$ nm (Schott KL 1500, 150 W) under Ar.



Fig 3: Calculated (AM1) structure of *endo*-blepharismine C (**1c**)

1c was irradiated on a preparative scale to afford **2c**. Its 2D NMR spectral data are in full accord with the proposed structure. Of particular significance are the chemical shifts of C-11 at δ 100.8 and 11-H at δ 5.50 and the AA'BB' pattern of the *p*-hydroxyphenyl group at δ 6.90 and 7.30, resp.. In addition acidic hydrolysis of **2c** yields *p*-hydroxybenzaldehyde and stentorin C (**6c**) identical with an authentic sample.¹² As expected, the blepharismins (**1**) are not cleaved under these conditions. We propose as the mechanism of this rearrangement that the blepharismins exist as a rapidly interconverting mixture of the cycloheptatriene and norcaradiene structures **1** and **7**, resp. by a thermally allowed disrotatory process. Although the equilibrium



is greatly in favor of **1**, as can be estimated from the chemical shift of δ 126 for C-10a/C-11a, the norcaradiene tautomer undergoes photoinduced rearrangement: the C-11–C-10a and C-11–C-11a bonds migrate to the adjacent oxygen atoms. An irreversible dehydrogenation follows to restore the quinoid system. The small amounts of the stentorins (**6**) found in *B. japonicum* grown in the light [Fig. 1, trace (c)] may be formed either from the hypothetical protostentorins (**5**) by photocyclization or from **2** by hydrolysis. NMR spectroscopy at low-temperature, photochemistry under rigorous anaerobic conditions, and studies on other *Blepharisma* and *Stentor* spp. are in progress.

ACKNOWLEDGEMENTS: We thank the *Deutsche Forschungsgemeinschaft* (project: Sp 198/12-1) and the *Fonds der Chemischen Industrie e.V.* for financial support.

REFERENCES AND NOTES

1. Giese, A. C. "*Blepharisma The Biology of a Light-Sensitive Protozoan*", Stanford University Press: Stanford, Cal., 1973. Miyake, A., in: *Biochemistry of Differentiation and Morphogenesis* (L. Jaenicke, ed.), Springer Verlag: Berlin, 1982, p. 211-230.
2. Giese, A.; Grainger, R. M. *Photochemistry and Photobiology*, **1970**, *12*, 489-503.
3. Masters theses: Röhm, B. Univ. Köln, **1987**. Hupe, M. Univ. Köln, **1988**. Gieray U. Univ. Hohenheim, **1989**. Werner, H. Univ. Hohenheim, **1997**.
4. Tao, N.; Orlando, M.; Hyon, J.-S.; Gross, M.; Song, P.-S. *J. Am. Chem. Soc.* **1993**, *115*, 2526-2528.
5. Checcucci, G.; Shoemaker, R. S.; Bini, E.; Cerny, R.; Tao, N.; Hyon, J.-S.; Gioffre, D.; Ghetti, F.; Lenci, F.; Song, P.-S. *J. Am. Chem. Soc.* **1997**, *119*, 5762-5763.
6. Maeda, M.; Naoki, H.; Matsuoka, T.; Kato, Y.; Kotsuki, H.; Utsumi, K.; Tanaka, T. *Tetrahedron Lett.* **1997**, *38*, 7411-7414.
7. Brockmann, H. *Fortschr. Chem. Org. Naturst.*, **1957**, *14*, 141-185.
8. HPLC: Hewlett-Packard 1050; ESI-MS: Perkin Elmer API 100. Conditions: Nucleosil RP-18, 5 μ m; 125 x 2 mm column; acetonitrile/ 10 mM aq. ammonium acetate buffer pH 6.8 gradient, 28 : 72 to 95 : 5 in 15 min, flow 0.3 ml/min; detection: 580 nm and negative ions [M - H]⁻; ca. 50 specimens per HPLC run.
9. Höfle, G.; Pohlan, S.; Uhlig, G.; Kappe, K.; Schumacher, D. *Angew. Chem.* **1994**, *106*, 1561-1563; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1495.
10. Strain R 1072 of *Blepharisma japonicum* (klon) was grown in petri dishes (15 cm in diameter) at room temp. in neutral Pringsheim solution (0.85 mM Ca(NO₃)₂ 4H₂O, 0.35 mM KCl, 0.08 mM MgSO₄ 7H₂O, and 0.11 mM Na₂HPO₄ 2H₂O) in the dark and fed with *Chlorogonium elongatum*, which was grown under sterile conditions in a light-dark cycle. *Blepharisma japonicum* was harvested after 4 weeks. We thank Professor A. Miyake, Dept. of Cell Biol., Univ. Camerino, Italy, for providing this organism.
11. 25 ml wet cell mass (pellets from 750 petri dishes) was extracted with acetone. The extract was concentrated and partitioned between ethyl acetate and water. The organic layer was evaporated and the residue treated with petroleum ether to give a dark red solid (55 mg) which was separated by HPLC (Eurosil Bioselect 100, C18, 10 μ m, column: 16 x 250 mm, acetonitrile-water (with 0.1 % TFA), 60 : 40 as eluent) to give five fractions of the blepharismins: A (6 mg), B (16 mg), C (13 mg), D (2 mg), E (4 mg).
12. Cameron, D. W.; Riches, A. G. *Tetrahedron Lett.* **1995**, *36*, 2331-2334. Iio, H.; Zenfuku, K.; Tokoroyama, T. *Tetrahedron Lett.* **1995**, *36*, 5921-5924. We are indebted to Professor Iio, Osaka City University, Osaka, Japan, for a sample of synthetic stentorin C.