Pergamon

Tetrahedron Letters, Vol. 38, No. 42, pp. 7411-7414, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)01737-1

Blepharismin 1-5, Novel Photoreceptor from the Unicellular Organism *Blepharisma japonicum*[1]

Mitsuru Maeda,[a] Hideo Naoki,*[b] Tatsuomi Matsuoka,*[c] Yoji Kato,[c] Hiyoshizo Kotsuki,[d] Kozo Utsumi,[e] and Takaharu Tanaka[a]

[a]Institute for Biomedical Research and [b]Suntory Bioorganic Research Institute, Suntory Limited, Osaka 618, Japan
[c]Department of Biology and [d]Chemistry, Kochi University, Akebono-cho, Kochi 780, Japan
[e]Institute of Medical Science, Center for Adult Diseases, Kurashiki 710, Japan

Abstract: The structures of new photoreceptor molecules mediating the photobehavior of the unicellular organism, *Blepharisma japonicum*, were determined spectroscopically. These molecules had a 4-hydroxyphenyl-11H-dibenzo[a,o]cyclohepta[pqr]perylene moiety as a common chromophore structure.
© 1997 Elsevier Science Ltd.

It is generally believed that retinal, when bound to rhodopsin-like molecules, is the only photoreceptor responsible for photobehavior in many organisms. Recently, one of us has demonstrated that a pink-colored pigment called "blepharismin-2",[2] which occurs just beneath the plasma membrane of the ciliated protozoan, *Blepharisma*, is a primary photoreceptor pigment mediating the step-up photophobic response.[3] Blepharismin-2 has also been demonstrated to be bound to a 200 kD membrane protein.[4] In this paper we describe the isolation and structural elucidation of five blepharismins.

Blepharisma japonicum was cultured under dark conditions at 23 ℃ in a 0.1% cereal leaves infusion containing *Enterobacter aerogenes*, which was supplied by the Institute for Fermentation, Osaka, Japan. To extract the pigments, the packed cells (9.5 mL) were suspended in acetone (20 mL). The cells were centrifuged (8000 g, 10 min) and the pigment-containing supernatant was decanted, followed by complete solvent removal using a rotary evaporator in the dark. The pigments were dissolved in DMSO (10% aq) prior to application on a column (SEP-PAK tC_{18}, elution with acetonitrile/2-propanol = 1 : 1 v/v). The partially purified pigment was further subjected to an ODS column separation (Asahipak Hikarisil-C_{18}) with CH_3CN (70% aq) containing 0.05% TFA (final concentration) in the isocratic mobile phase to provide sequentially blepharismin (**BL**) 1, 2, 3, 4 and 5. For the purpose of their structural elucidation, each fraction was treated with diazomethane (30 min, 0 ℃) to afford **BL-1'** (1.7 mg), **BL-2'** (2.0 mg), **BL-3'** (2.1 mg), **BL-4'** (1.3 mg) and **BL-5'** (2.3 mg), respectively.[5] The ^1H NMR spectra of all of these pigments revealed the presence of four deuterium exchangeable protons, corresponding to hydrogen-bonded hydroxyl groups in the chemical shift region around 14 ppm. The molecular weight of the methylated blepharismins (**BL-1'-5'**) was increased by 56 mass units more than their native blepharismins (**BL-1-5**) as determined by high resolution FABMS measurement.[6] These results and their resemblance with those of hypericin[7] suggest that these blepharismins possess a naphthodianthrone skeleton with four peri-hydroxyl groups as a common structural component. Furthermore,

these compounds contain a *p*-hydroxybenzylidene unit (δ_H 5.95-6.0 (two sets of 2H, d, J = 8.7 Hz) and 6.90-7.0 (1H, s)). This was confirmed by a characteristic carbon signal at δ_C 31.3-33.2 in the HETCOR spectrum and the HMBC correlation of δ_C 31.3-33.2 (C-11) to δ_H 5.99-6.0 (two methines; H-2', 6'), which in turn showed a cross peak with the C-4' quaternary ipso carbon (δ_C 152.8) bearing a hydroxyl group.

The molecular formula of **BL-5**, $C_{42}H_{32}O_{11}$, was determined by high resolution FABMS (*m/z* 713.2030, $[M+H]^+$, Δ +0.7 mmu). In the ^1H NMR spectrum of **BL-5'**, four methoxyl signals (δ_H 3.28, 3.30, 3.95 and 4.13) and a singlet methyl (δ_H 2.45) were detected along with two isopropyl groups (δ_H 1.46, 1.50 (each 6H, d), and 3.8 (2H, m)) and an aromatic proton (δ_H 6.87 (1H, s)). In the HMBC spectrum of **BL-5'**, the C-1 quaternary carbon (δ_C 165.3) bearing a hydrogen-bonded hydroxyl group (δ_H 14.2) was correlated with a multiplet methine signal (δ_H 3.8, C-2-i-Pr). The C-6 quaternary carbon (δ_C 165.4) bearing a hydrogen-bonded hydroxyl group was also correlated with another isopropyl group (C-5-i-Pr). C-3 and C-4 quaternary carbon signals (δ_C 161.9 and 162.2) attached to methoxyl groups (δ_H 3.30 and 3.28) were correlated with two methyls (δ_H 1.46 (C-2-i-Pr) and 1.5 (C-5-i-Pr), respectively).[8] The C-10 quaternary carbon signal (δ_C 161.0) adjacent to a third methoxyl group (δ_H 3.95, s, C-10-OCH_3) showed a cross peak with an isolated methine (δ_H 6.90, H-11) and a methyl singlet (δ_H 2.45, C-9-CH_3). The C-12 quaternary carbon signal (δ_C 161.6) adjacent to a fourth methoxyl group (δ_H 4.13, C-12-OCH_3) showed a correlation with two methine singlets (δ_H 6.90, H-11 and 6.87, H-13). The C-14 quaternary carbon (δ_C 164.7) bearing a hydrogen-bonded hydroxyl group (δ_H 13.6, H-14) showed a correlation to a methine singlet (δ_H 6.87, H-13). The C-9 quaternary carbon (δ_C 121.2) attached to a methyl group (δ_H 2.45, C-9-CH_3) was correlated with a hydrogen-bonded hydroxyl group (δ_H 13.6, C-8-OH). Furthermore, NOE's were observed between the C-10-methoxyl group (δ_H 3.95) and the C-9-methyl group (δ_H 2.45) and also the H-11-methine singlet (δ_H 6.90). NOE's were also observed between the C-12-methoxyl group (δ_H 4.13) and the methine signal (δ_H 6.87, H-13). Accordingly, the C-11 carbon should be connected to C-10a and C-11a.

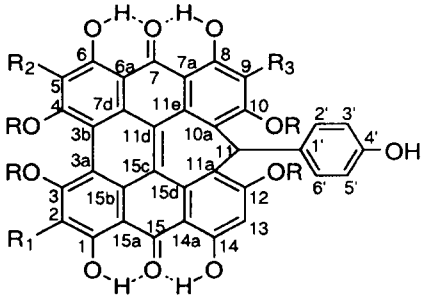

Fig. 1. Structures of blepharismin (**BL**) 1-5 (R = H) and their methylated derivatives 1'-5' (R = CH_3). **BL-1**: $R_1 = R_2$ = Et, R_3 = H; **BL-2**: R_1 = Et, R_2 = i-Pr, R_3 = H; **BL-3**: $R_1 = R_2$ = i-Pr, R_3 = H; **BL-4**: R_1 = Et, R_2 = i-Pr, R_3 = Me or R_1 = i-Pr, R_2 = Et, R_3 = Me; **BL-5**: $R_1 = R_2$ = i-Pr, R_3 = Me.

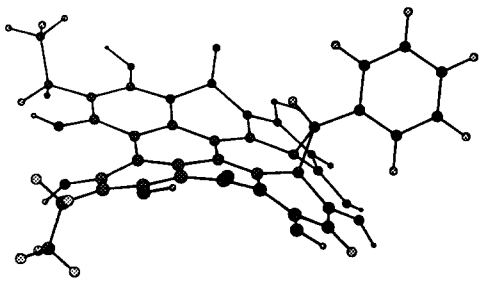

Fig. 2. The most favorable conformer of **BL-1** calculated with PM3.

These results lead to the structure for **BL-5** shown in Figure 1 with the name 1,3,4,6,8,10,12,14-octahydroxy-11-(4-hydroxyphenyl)-2,5-diisopropyl-9-methyl-11H-dibenzo[a,o]cyclohepta[pqr]perylene-7,15-dione. The other four blepharismins have alkyl group substituents at the C-2, -5 or -9 positions as shown by HMBC and NOE experiments: 2, 5-diethyl for **BL-1**; 2-ethyl-5-isopropyl for **BL-2**; 2,5-diisopropyl for **BL-3**;

Table 1. ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) Data for Methylated Blepharismins in $CDCl_3$.[a]

	BL-1'		BL-2'		BL-3'		BL-4'		BL-5'	
Position	δC	δH	δC	δH	δC	δH	δC	δH	δC	δH
1	164.4		164.2		165.1		165.3[b]		165.3	
2	125.3[b]		125.5		127.4[b]		125.8		128.0	
3	161.6		162.0		161.8		162.1		161.9[b]	
3a	127.2		126.3[b]		127.4[b]		126.1		127.5[c]	
3b	127.2		126.3[b]		127.4[b]		126.1		127.5[c]	
4	161.6		162.5		161.8		162.1		162.2[b]	
5	125.9[b]		127.3		127.4[b]		127.4		128.0	
6	164.4		165.0		165.1		165.3[b]		165.4	
6a	105.9		109.0[c]		106.3		106.3[c]		106.3[d]	
7	186.7		187.0		187.0		187.5		187.2[e]	
7a	108.6		108.9		109.0		111.0[c]		111.7	
7d	127.0		126.3[b]		127.2[b]		126.1		126.2[c]	
8	163.8		165.0		164.6		164.8[b]		164.7	
9	99.9	6.85 s	100.2	6.84 s[d]	100.2	6.85 s	121.2		121.2	
10	161.6		162.0		161.8		161.0		161.0	
10a	125.9[b]		125.7		125.5		129.5		129.5	
11	31.3	7.00 s	31.7	6.95 s	31.7	7.00 s	33.2	6.90 s	33.2	6.90 s
11a	125.9[b]		125.7		125.5		125.8		125.6	
11d	126.5		125.5[b]		126.1[b]		126.1		126.0[c]	
11e	134.7		135.0		134.9		133.0		135.2[f]	
12	161.6		162.0		161.8		161.6		161.6	
13	99.9	6.85 s	100.2	6.85 s[d]	100.2	6.85 s	100.2	6.88 s	100.2	6.87 s
14	163.8		164.2		164.6		164.4[b]		164.7	
14a	108.6		108.9		109.0		109.3[c]		109.3	
15	186.7		187.0		187.0		187.5		187.6[e]	
15a	105.9		106.2[c]		106.3		106.4[c]		106.4[d]	
15b	127.0		126.3[b]		127.2[b]		126.1		126.2[c]	
15c	126.5		125.5[b]		126.1[b]		126.1		126.0[c]	
15d	134.7		135.0		134.9		133.0		133.0[f]	
3-OMe	60.8	3.40 s	61.3	3.34 s	61.3	3.30 s	61.0	3.30 s	61.0	3.30 s
4-OMe	60.8	3.40 s	61.0	3.26 s	61.3	3.30 s	61.3	3.40 s	61.2	3.28 s
10-OMe	56.1	4.10 s	56.4	4.10 s	56.4	4.10 s	61.6	3.95 s	61.6	3.95 s
12-OMe	56.1	4.10 s	56.4	4.00 s	56.4	4.10 s	56.5	4.13 s	56.5	4.13 s
1'	133.1		133.4		133.5		133.8		133.8	
2', 6'	127.2	6.00 d[g]	127.3	6.00 d[g]	127.2[b]	6.00 d[g]	127.4	6.00 d[g]	127.4	5.99 d[g]
3', 5'	112.9	5.90 d[g]	113.2	5.95 d[g]	113.2	5.90 d[g]	113.3	5.95 d[g]	113.2	5.95 d[g]
4'	152.0		152.8		152.7		152.8		152.8	
1-OH		13.9 s		14.05 s		14.2 s		14.1 s		14.2 s
6-OH		13.9 s		14.05 s		14.2 s		14.0 s[d]		14.1 s
8-OH		13.5 s		13.6 s[e]		13.5 s		13.5 s[d]		13.6 s
14-OH		13.5 s		13.5 s[e]		13.5 s		13.7 s[d]		13.6 s

[a] ^{13}C and ^{1}H NMR data of R_1, R_2, and R_3 are shown in ref 5. [b,c,d,e,f] Signals are interchangeable within the same superscripts. [g] Coupling constant (J) = 8.7 Hz.

2-ethyl-5-isopropyl-9-methyl or 5-ethyl-2-isopropyl-9-methyl moieties for **BL-4**. ^{1}H and ^{13}C chemical shift assignments for these pigments are shown in Table 1. Interestingly, computational techniques using semi-empirical molecular mechanics calculations reveal the "propeller" conformation for **BL-1** as the most favorable conformer (Figure 2).[9]

In conclusion, we succeeded in determining the structures of five blepharismins that act as novel phtoreceptor molecules. In view of the great interest in their novel structures and their unusual biological and chemical properties,[10] the present work will constitute an important contribution to advancing knowledge of photodynamic action. Further studies are now in progress in these laboratories.

Acknowledgement: We thank Mr. T. Arita for help in purifying the blepharismin pigments.

References and Notes

1. This work was presented at the 12th International Congress on Photobiology. *Abstract of ICP.*, **1996**. p. 185 (S295). Review: Matsuoka, T. *Iden* **1997**, *51*, 73-77.
2. Giese, A. C. *Blepharisma. A Light Sensitive Protozoan*; Stanford Univ. Press: Stanford, CA., 1973.
3. a) Matsuoka, T.; Watanabe, Y.; Sagara, Y.; Takayanagi, M.; Kato, Y. *Photochem. Photobiol.*, **1995**, *62*, 190-193. b) Matsuoka, T.; Sato, M.; Maeda, M.; Naoki, H.; Tanaka, T. *Ibid.* **1997**, *65*, 915-921.
4. a) Matsuoka, T.; Murakami, Y.; Kato, Y. *Photochem. Photobiol.* **1993**, *57*, 1042-1047. b) Matsuoka, T.; Tsuda, T.; Ishida, M.; Kato, Y.; Takayanagi, M.; Fujino, T.; Mizuta, S. *Ibid.*, **1994**, *60*, 598-604.
5. **BL-1'**: FABMS (3-nitrobenzyl alcohol matrix) *m/z* 727 $[M + H]^+$; UV-VIS (MeOH) λ_{max} (log ε) 552 (4.17), 521 (4.12), 475 (4.18), 414 (3.98), 361 (4.04) nm: ^{1}H NMR (400 MHz) δ 1.3 (6H, t, *J* = 7.5 Hz, 2-, 5-$CH_2\underline{CH_3}$), 2.9 (4H, m, 2-, 5-$\underline{CH_2}CH_3$); ^{13}C NMR (100 MHz) δ 13.6 (2-$CH_2\underline{CH_3}$), 13.8 (5-$CH_2\underline{CH_3}$), 16.7*a* (2-$\underline{CH_2}CH_3$), 18.4*a* (5-$\underline{CH_2}CH_3$); *a* may be interchanged. **BL-2'**: FABMS *m/z* 741 $[M + H]^+$; UV-VIS (MeOH) λ_{max} (log ε) 554 (4.12), 523 (4.05), 476(4.10), 415 (3.91), 361 (3.97) nm: CD extrema/nm (Δε) 410 (2.57), 363 (-0.55), 333 (-0.61), 307 (0.80), 273 (-2.88), 243 (5.47); ^{1}H NMR (400 MHz) δ 1.3 (3H, t, *J* = 7.5 Hz, 2-$CH_2\underline{CH_3}$), 1.5 (6H, d, *J* = 7.1 Hz, 5-CH($\underline{Me}$)$_2$), 2.9 (2H, m, 2-$\underline{CH_2}CH_3$), 3.8 (1 H, m, 5-$\underline{CH}$(Me)$_2$); ^{13}C NMR (100 MHz) δ 14.0 (2-$CH_2\underline{CH_3}$), 17.1 (2-$\underline{CH_2}CH_3$), 20.5 & 20.8 (5-CH($\underline{Me}$)$_2$), 25.2 (5-$\underline{CH}$(Me)$_2$). **BL-3'**: FABMS *m/z* 755 $[M + H]^+$; UV-VIS (MeOH) λ_{max} (log ε) 555 (4.14), 523 (4.08), 476 (4.13), 416 (3.95), 361 (4.01) nm; ^{1}H NMR (400 MHz) δ 1.5 (6H, d, *J* = 7.1 Hz, 2-CH($\underline{Me}$)$_2$), 1.6 (6H, t, *J* = 7.1 Hz, 5-CH($\underline{Me}$)$_2$), 3.8 (2H, m, 2-, 5-$\underline{CH}$(Me)$_2$); ^{13}C NMR (100 MHz) δ 20.5 & 20.8 (2, 5-CH($\underline{Me}$)$_2$), 25.4 (2, 5-$\underline{CH}$(Me)$_2$). **BL-4'**: FABMS: m/z 755 $[M + H]^+$; UV-VIS (MeOH) λ_{max} (log ε) 555 (4.11), 522 (4.05), 481(4.12), 426 (3.87), 364 (3.92) nm: CD extrema/nm (Δε) 476 (4.46), 363 (-5.13), 308 (7.25), 284 (-5.13), 268 (-0.98), 257 (-3.18), 245 (0.07); ^{1}H NMR (400 MHz) δ 1.3 (3H, t, *J* = 7.5 Hz, 2-$CH_2\underline{CH_3}$), 1.5 (6H, d, *J* = 7.1 Hz, 5-CH($\underline{Me}$)$_2$), 2.45 (3H, s, 9-Me), 2.91 (2H, m, 2-$\underline{CH_2}CH_3$), 3.83 (1H, m, 5-$\underline{CH}$(Me)$_2$); ^{13}C NMR (100 MHz) δ 14.0 (2-$CH_2\underline{CH_3}$), 17.2 (2-$\underline{CH_2}CH_3$), 20.4 & 20.9 (5-CH($\underline{Me}$)$_2$), 25.2 (5-$\underline{CH}$(Me)$_2$), 41.0 (9-Me). **BL-5'**: FABMS m/z 769 $[M + H]^+$; UV-VIS (MeOH) λ_{max} (log ε) 557 (4.08), 524 (4.02), 483 (4.10), 425 (3.82), 361 (3.87) nm: CD extrema/nm (Δε) 386 (4.46), 363 (0.53), 314 (-0.90), 285 (0.95), 266 (-0.56), 248 (1.21); ^{1}H NMR (400 MHz) δ 1.46 (6H, d, *J* = 7.1 Hz, 2-CH($\underline{Me}$)$_2$), 1.5 (6H, d, *J* = 7.1 Hz, 5-CH($\underline{Me}$)$_2$), 2.45 (3H, s, 9-Me), 3.8 (2H, m, 2-5-$\underline{CH}$(Me)$_2$); ^{13}C NMR δ 20.4 (2-CH($\underline{Me}$)$_2$), 20.7 (5-CH($\underline{Me}$)$_2$), 25.3 (2-$\underline{CH}$(Me)$_2$), 25.5 (5-$\underline{CH}$(Me)$_2$), 41.0 (9-Me).
6. FABMS (glycerin or triethanolamine matrix): **BL-1**, *m/z* 669; **BL-2**, *m/z* 683; **BL-3**, *m/z* 697; **BL-4**, *m/z* 697; **BL-5**, *m/z* 711 $(M - H)^-$.
7. Falk, H. ; Schmitzberger, W. *Monatsh. Chem.* **1992**, *123*, 731-739.
8. Marshall, J. L. *Carbon-Carbon and Carbon-Proton NMR Couplings: Applications to Organic Stereochemistry and Conformational Analysis*: Verlag Chemie International, Inc. 1983; pp 42-51.
9. Kotsuki, H.; Matsuoka, T. unpublished results. Calculations were performed by MOPAC '93 (PM 3) implemented in ANCHOR II™, a molecular design system for SUN™ computers, developed by FUJITSU, Ltd. and Kureha Chemical Industry Co., Ltd., 1992. MOPAC 93: J. J. P. Stewart and Fujitsu, Ltd., Tokyo, Japan, 1993.
10. Kato, Y.; Watanabe, Y.; Sagara, Y.; Murakami, Y.; Sugiyama, M.; Matsuoka, T. *J. Photochem. Photobiol., B: Biology* **1996**, *34*, 29-33.

(*Received in Japan* 22 *July* 1997; *accepted* 22 *August* 1997)