Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron 64 (2008) 4103–4107
Tetrahedron
www.elsevier.com/locate/tet

Starter units of the biosynthesis of blepharismins: self-defense pigments of *Blepharisma japonicum*

Koichi Yoshioka [a], Sohei Tominaga [a], Yoshiyuki Uruma [b], Yoshinosuke Usuki [a], Hideo Iio [a,*]

[a] *Department of Material Science, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan*
[b] *Department of Materials Science, Yonago National College of Technology, Tottori 683-8502, Japan*

Received 27 December 2007; received in revised form 2 February 2008; accepted 5 February 2008
Available online 7 February 2008

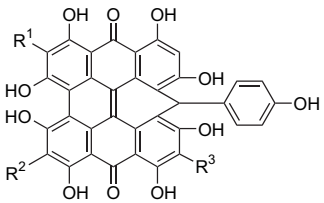
Abstract

The heterotrich ciliate *Blepharisma japonicum* produces red pigment blepharismins, which function as self-defense toxin against predators and as a photoreceptor for step-up photonegativity. The dibenzoperylenequinone moiety of blepharismins was shown to be biosynthesized via the polyketide pathway. In this paper, the starter units of the biosynthetic pathway of blepharismins were determined to be isovaleryl-CoA and butyryl-CoA by HPLC, LC/ESI-MS, and ^{1}H and ^{2}H NMR analyses of the pigments obtained from feeding experiment of L-leucine or sodium butyrate in excess and deuterium-labeled L-leucine.

Keywords: Blepharismin; Biosynthesis; Starter unit; Isovaleryl-CoA; L-Leucine-d_{10}

1. Introduction

The heterotrich ciliate *Blepharisma japonicum* contains red pigment blepharismins (BPs: **1a**–**1e**) in their extrusive organelles.[1] These pigments are known to have three functions: light perception,[2] chemical defense against predators,[3] and protection against UV radiation.[4] In addition to chemical defense against predators, BPs exhibit toxicity against other ciliates[5] and inhibit the growth of Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA).[6] The chemical structure of BP-C, the major component of BPs, was elucidated by Song et al.,[7] while the other homologs were elucidated independently by Naoki et al. (Fig. 1).[8] BPs are also known to be converted to oxyblepharismins by UV radiation.[9] BPs are structurally related to hypericin, a photodynamic toxin of *Hypericum*,[10] and stentorin extracted from the negatively phototactic ciliate *Stentor coeruleus*.[11] Stentorin and oxyblepharismin are also toxic to other ciliates, including predators.[12] Recently, maristentorin was isolated from the positively phototactic marine ciliate *Maristentor dinoferus*.[13] We previously reported that the dibenzoperylenequinone moiety of BPs was biosynthesized via the polyketide pathway by assignment of the ^{13}C NMR spectrum of BP-C based on analyses of 2D spectrum of ^{13}C-enriched samples obtained by feeding experiments using ^{13}C-labeled sodium acetate.[14] In feeding experiments of ^{13}C-labeled acetate, the incorporation of labeled carbon into C5, C6, and isopropyl group (C16, C17, C18) positions was one-tenth lower than that of other carbons of the dibenzoperylenequinone moiety of BPs; furthermore, the positions at which labeled acetates were incorporated did not agree with the expected normal position, indicating that these portions of the dibenzoperylenequinone moiety of BPs might be the starter unit of BP



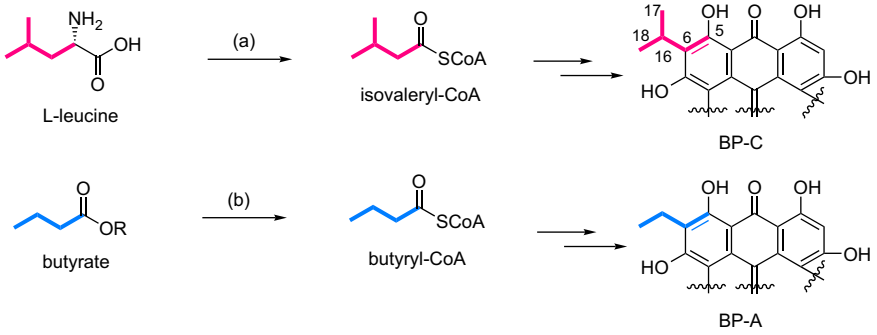
Blepharismin A-E (**1a-1e**)

BP-A (**1a**): R^1 = Et, R^2 = Et, R^3 = H
BP-B (**1b**): R^1 = Et, R^2 = *i*Pr, R^3 = H
BP-C (**1c**): R^1 = *i*Pr, R^2 = *i*Pr, R^3 = H
BP-D (**1d**): R^1 = Et, R^2 = *i*Pr, R^3 = Me
or R^1 = *i*Pr, R^2 = Et, R^3 = Me
BP-E (**1e**): R^1 = *i*Pr, R^2 = *i*Pr, R^3 = Me

Figure 1. Structures of blepharismins A–E.

* Corresponding author. Fax: +81 6 6605 2562.
E-mail address: iio@sci.osaka-cu.ac.jp (H. Iio).

0040-4020/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2008.02.004



Scheme 1. Starter units of the biosynthesis of blepharismins: (a) branched-chain amino acid transaminase and branched-chain keto acid dehydrogenase, (b) butyryl-CoA synthetase or butyryl-CoA transferase.

biosynthesis. It was also reported that the ethyl group attached at the C6 carbon atom of BP-A, BP-B, and/or BP-D was weakly enriched in pigments obtained by feeding ^{13}C-labeled acetates.[14] We previously proposed that isovaleryl-CoA and butyryl-CoA might be the starter units of BP biosynthesis; these compounds were derived from L-leucine or 4-methyl-2-oxopentanoic acid through incorporation of ^{13}C-labeled sodium acetate (Scheme 1). In this paper, we conclude that the starter units of biosynthesis of BPs are isovaleryl-CoA and butyryl-CoA based on HPLC and LC/ESI-MS analyses of samples obtained from feeding excess L-leucine or sodium butyrate and ^{1}H and ^{2}H NMR spectra of pigments obtained from feeding deuterium-labeled L-leucine.

2. Results and discussion

Polyketide synthase utilizes a wide assortment of starter units such as straight- and branched-chain fatty acids and amino acids.[15] In many cases, the nature of a starter unit confers important structural and biological features to the molecule.[16] It was reported that isovaleryl-CoA was used as a starter unit in the biosynthesis of myxothiazol, an inhibitor of the electron transfer system in the mitochondria of *Stigmatella aurantiaca*,[17] and an analogue of the lipase inhibitor lipstatin of *Streptomyces toxytricini*.[18] Isovaleryl-CoA is biosynthesized through a pyruvate pathway from acetyl-CoA and pyruvate via α-ketoisovalerate, and it can also be derived from the branched-chain amino acid L-leucine.[19] The first step in leucine degradation is transamination to 4-methyl-oxopentanoate, which is further processed by the Bkd (branched-chain keto dehydrogenase) complex into thioester isovaleryl-CoA.

BP-A has two ethyl groups in the molecule, while BP-C and BP-E each has two isopropyl groups. On the other hand, BP-B and BP-D each has an ethyl and an isopropyl group in the molecule. BP-D and BP-E have an additional methyl group in the carbon ring of dibenzoperylenequinone; however, the position of the methyl group in the carbon ring of BP-D has not been rigorously determined. We presumed that the ratio of BPs obtained from the culture medium of *B. japonicum* with excess precursors of the starter units differed from the ratio of BPs obtained from the culture medium of the protozoan cultivated under normal conditions. The starter units of the biosynthesis of BPs were estimated to be isovaleryl-CoA or butyryl-CoA based on the abovementioned result. Butyryl-CoA is also converted from butyrate by the butyryl-CoA synthetase (or butyryl-CoA transferase). In particular, we expected that feeding excess L-leucine would increase the ratio of BP-B, BP-C, BP-D, and BP-E, which have isopropyl group(s) in their molecules, through the use of L-leucine as the starter unit.

Feeding experiments of excess L-leucine and sodium butyrate were carried out according to the method described previously.[5] Pigments were isolated using a slightly modified method to prevent errors in determination of the ratio of each homolog during the purification procedure. The minimum concentrations for the lethal toxicities of L-leucine and sodium butyrate to *B. japonicum* cells were 125 and 31.3 mM, respectively. Considering the toxicities for *B. japonicum* cells, we prepared a culture of *B. japonicum* in a culture medium containing 12.8 mM of the putative precursors. After cultivation for three days in the dark, the cells collected from 1 L culture of *B. japonicum* were treated with a small amount of acetone, and ethyl acetate was added to the mixture to extract pigments. The cells were removed by centrifugation at 700*g* for 10 min, and the red-colored supernatant was washed with brine. Evaporation of the organic layer yielded crude pigments. These pigments were subjected to HPLC on an ODS column with 60% CH_3CN containing 0.05% TFA (Fig. 2). Based on the UV–vis absorption at 580 nm, the ratio of BP-A, BP-B, BP-C, BP-D, and BP-E obtained from normal cultivation was 2:21:68:3:20, while the ratios of BP-A, BP-B, BP-C, BP-D, and BP-E of the samples obtained from feeding excess L-leucine and sodium butyrate were 2:54:364:7:91 and 30:38:68:10:19, respectively (Table 1). In the case of feeding L-leucine, the ratios of BP-C and BP-E, which have two isopropyl groups in the molecule, increased by 5.4 and 4.6 times, respectively, while the ratios of BP-B and BP-D, which have one isopropyl group in the molecule, increased by 2.6 and 2.3 times, respectively (Table 1). In contrast, the feeding experiment of sodium butyrate showed that the ratio of BP-A, which has two ethyl groups in the molecule, increased by 15 times, while the ratios of BP-B and BP-D, which have one ethyl group in the molecule, increased by 1.8 and 3.3 times, respectively (Table 1). In the feeding experiments of 4 mM L-leucine or sodium butyrate, the results obtained were almost similar to those obtained after feeding 12.8 mM of additives. In the case of feeding 1 mM L-leucine, the ratio of BPs showed a slight change from the normal

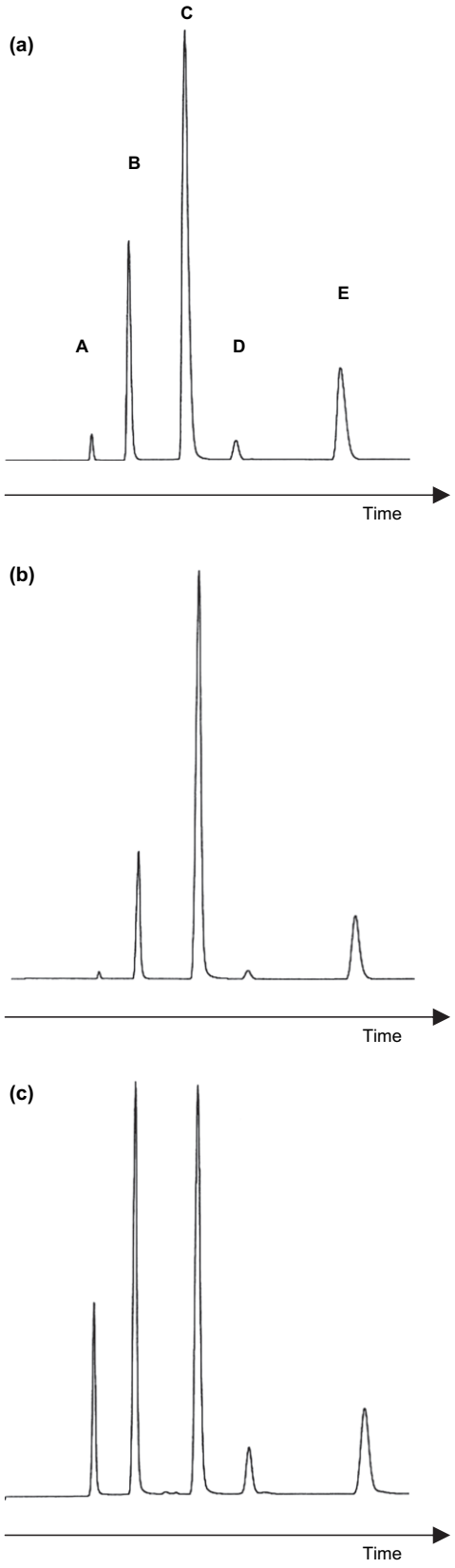


Figure 2. HPLC profiles of a mixture of blepharismins A–E: (a) normal cultivation (retention time: BP-A=5.2, BP-B=7.4, BP-C=10.8, BP-D=14.0, BP-E=20.6 min), (b) feeding of 4 mM L-leucine, (c) feeding of 4 mM sodium butyrate. UV–vis detection at 580 nm.

Table 1
The ratio of blepharismins A–E obtained by normal cultivation and feeding of excess L-leucine or sodium butyrate

Additive	Concentration (mM)	Ratio of BPs[a]				
		BP-A	BP-B	BP-C	BP-D	BP-E
—	—	2	21	68	3	20
L-Leucine	12.8	2 (1.0)	54 (2.6)	364 (5.4)	7 (2.3)	91 (4.6)
	4.0	2 (1.0)	37 (1.8)	174 (2.6)	5 (1.7)	42 (2.1)
	1.0	2 (1.0)	20 (1.0)	64 (0.9)	3 (1.0)	18 (0.9)
Butyrate	12.8	30 (15)	38 (1.8)	68 (1.0)	10 (3.3)	19 (1.0)
	4.0	19 (9.5)	51 (2.4)	68 (1.0)	9 (3.0)	24 (1.2)
	1.0	8 (4.0)	39 (1.9)	68 (1.0)	5 (1.7)	19 (1.0)

[a] The ratio of BPs based on UV–vis absorption at 580 nm. Numbers in parentheses denote an increase in the ratio compared to that in normal cultivation.

cultivation, whereas by feeding sodium butyrate, the ratio of BP-A, BP-B, and BP-D increased by 4.0, 1.9, and 1.7 times, respectively. These results indicate that the starter units of the isopropyl and ethyl groups of BPs were isovaleryl-CoA derived from L-leucine and butyryl-CoA, respectively. We also clarified the starter units of BP biosynthesis based on feeding experiments with deuterium-labeled L-leucine.

B. japonicum cells were harvested from a culture medium containing L-leucine-d_{10} (1 mM) by using the same cultivation method as described above. Incorporation of L-leucine-d_{10} into BPs was expected to be detected by LC/ESI-MS analyses, although a few changes in the ratio of BPs could be observed in the culture containing 1 mM L-leucine. LC/ESI-MS analyses revealed the incorporation of L-leucine-d_{10} into all BPs, except BP-A (Fig. 3). In the case of BP-B and BP-D, which have an isopropyl group, a molecular ion mass increase by 7 Da (BP-B_{d_7}, BP-B_{d_7}) from the original compound was newly detected in both pigments. On the other hand, in the mass spectra of BP-C and BP-E, which had two isopropyl groups, a molecular ion mass increase by 7 or 14 Da (BP-C_{d_7}, BP-$C_{d_{14}}$ and BP-E_{d_7}, BP-$E_{d_{14}}$) from the origin compound was newly observed in two molecular ions. Although the molecular ions of BP-C and BP-D are the same, the two isomers could be differentiated based on the number of incorporated L-leucine-d_{10} molecules.

The incorporation of L-leucine-d_{10} into the isopropyl groups of BPs was finally determined by ^{1}H and ^{2}H NMR spectra analyses. A relative decrease was observed in integration at δ 3.91 (2H, septet) for the methine proton and at δ 1.39 (6H, d) and 1.42 (6H, d) for the methyl groups of isopropyl in ^{1}H NMR of a mixture of BPs obtained from feeding of L-leucine-d_{10} (1 mM), while observation of the peaks at δ 3.80 and 1.32 in ^{2}H NMR in acetone (Fig. 4) unequivocally proved the incorporation of L-leucine-d_{10} into the isopropyl group of BPs.

3. Conclusions

The starter units of the biosynthesis of BPs were determined to be isovaleryl-CoA and/or butyryl-CoA derived from L-leucine and sodium butyrate, respectively, based on HPLC, LC/ESI-MS, and ^{1}H and ^{2}H NMR analyses of the pigments obtained from feeding experiments of their putative precursors

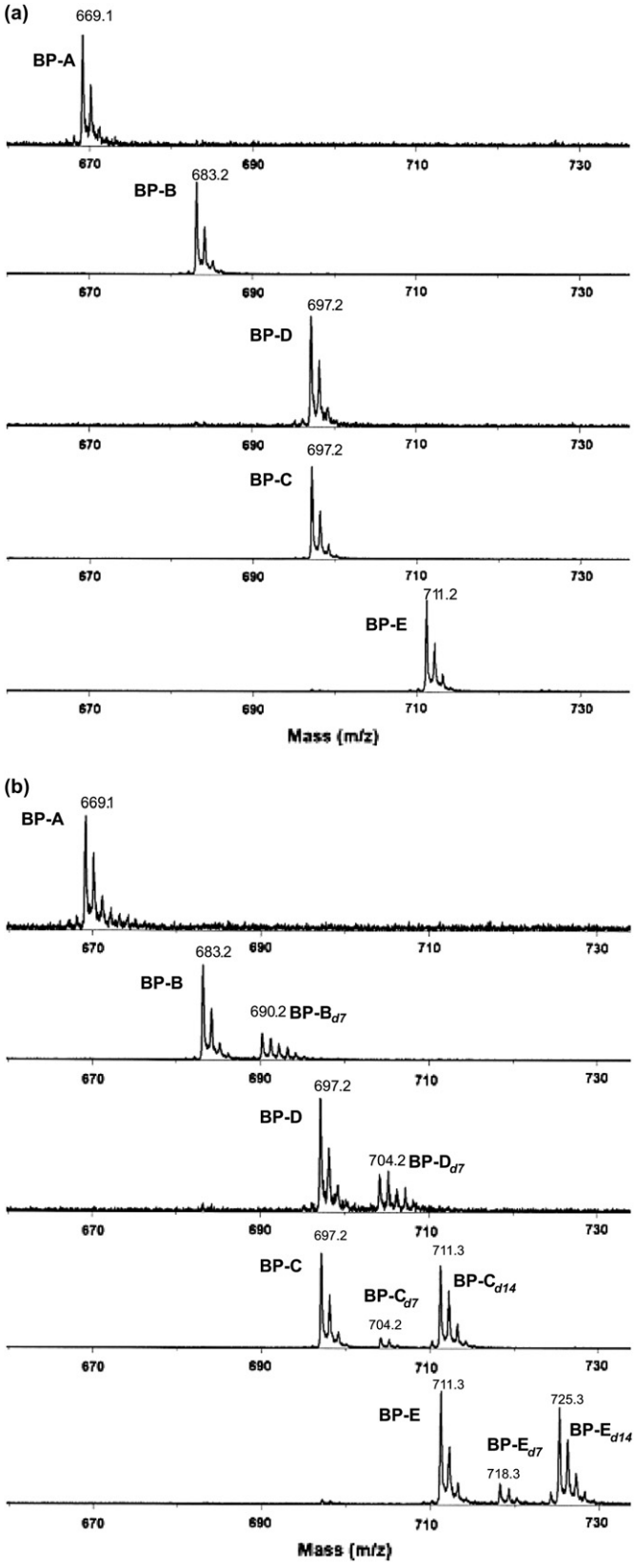


Figure 3. LC/ESI-MS spectra of blepharismins A–E: (a) BPs obtained by normal cultivation (retention time: BP-A=18.1, BP-B=19.6, BP-D=20.5, BP-C=21.1, BP-E=22.0 min), (b) BPs obtained by feeding L-leucine-d_{10}.

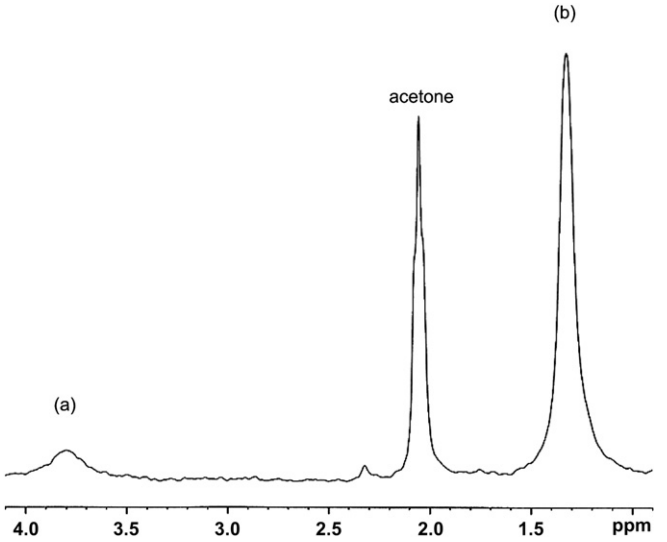


Figure 4. ^{2}H NMR spectrum of a mixture of blepharismins obtained by feeding L-leucine-d_{10} (1 mM): (a) methine deuterons of isopropyl groups, (b) methyl deuterons of isopropyl groups.

in excess and deuterium-labeled L-leucine. We are presently investigating the biosynthetic pathway of the *p*-hydroxybenzylidene unit of BPs and the precursor of the methyl group for BP-D and BP-E.

4. Experimental section

4.1. General

L-Leucine-d_{10} was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). HPLC analysis was carried out with a Shimadzu 10A series. LC and ESI-MS systems for LC/ESI-MS were Agilent 1100 series and Applied Biosystems Mariner™ spectrometers, respectively. ^{1}H and ^{2}H NMR spectra were obtained by Bruker Avance 600 in acetone-d_6 and acetone, respectively.

4.2. Cultivation of *B. japonicum* and extraction of BPs

Enterobacter aerogenes was inoculated into 500 mL of wheat grass powder (WGP; Pines international Co., Lawrence, KS) medium in a 2-L Erlenmeyer flask at 25 °C. After 2 days, 500 mL culture of *B. japonicum* was added and cultivated at 25 °C in the dark. The culture medium was centrifuged at 100*g* (3 min, two times), and the precipitated cells were washed with physiologically balanced solution of SMB (synthetic media for *Blepharisma*: 1.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.05 mM $MgSO_4$, and 2.0 mM sodium phosphate buffer, pH 6.8).[5] The collected cells were suspended in acetone, and the mixture was shaken. Ethyl acetate was added to the suspension and recentrifuged at 700*g* for 10 min. The red-colored supernatant was washed three times with brine, and the combined organic layer was dried over anhydrous Na_2SO_4. Concentration of the organic layer in vacuo yielded 2.8 mg of red-colored pigments. The pigments were subjected to HPLC and LC/ESI-MS.

4.3. Feeding experiments with excess L-leucine, sodium butyrate, and deuterium-labeled L-leucine

A typical feeding experiment was as follows. L-Leucine (1.68 g, 12.8 mmol) was added to the culture medium (500 mL) inoculated with *E. aerogenes*, followed by the addition of a culture of *B. japonicum* (500 mL). Cultivation and isolation of the pigments by using the abovementioned condition yielded 3.4 mg of a mixture of BPs. In feeding experiment of 1.41 g of sodium butyrate (12.8 mmol), 1.2 mg of a mixture of BPs was obtained. In feeding experiment of 19.7 mg of L-leucine-d_{10} (1.0 mM) in 150 mL culture medium, 1.0 mg of a mixture of BPs was obtained, which was used in LC/ESI-MS and ^{1}H NMR analyses. In feeding experiment of 65.6 mg of L-leucine-d_{10} (1.0 mM) in 500 mL culture medium, 3.3 mg of a mixture of BPs was obtained, which was used for ^{2}H NMR analysis.

4.4. HPLC analysis of blepharismins

The pigments extracted from *B. japonicum* were dissolved in CH_3CN and analyzed by HPLC on an ODS column (TSK gel ODS-120T, 4.6×150 mm; Tosoh, Japan). Elution conditions were as follows: isocratic mode with 60% CH_3CN in water containing 0.05% trifluoroacetic acid; flow rate with 1 mL/min at rt; UV–vis detection at 580 nm.

4.5. LC/ESI-MS analysis of blepharismins and deuterium-labeled BPs

The pigments were analyzed by time-of-flight mass spectrometer with ESI. The mass range was set from 100 to 1000 *m*/*z*. The column for LC was Develosil (ODS-HG-5, 2.0×150 mm; Nomura Chemical, Japan) connected to Guard cartridge (ODS-UG-5, 2.0×10 mm; Nomura Chemical). Elution conditions were as follows: gradient system from 28 to 95% (v/v) CH_3CN in 10 mM ammonium acetate buffer (pH 6.8) for 30 min; flow rate of 0.2 mL/min at 25 °C; UV–vis detection at 580 nm.

References and notes

1. Giese, A. C. *Blepharisma: The Biology of a Light-Sensitive Protozoan*; Stanford University Press: Stanford, Palo Alto, CA, 1973.
2. (a) Kraml, M.; Marwan, W. *Photochem. Photobiol.* **1983**, *37*, 313–319; (b) Matsuoka, T.; Matsuoka, S.; Yamaoka, Y.; Kuriu, T.; Watanabe, Y.; Takayanagi, M.; Kato, Y.; Taneda, K. *J. Protozool.* **1992**, *39*, 498–502.
3. Miyake, A.; Harumoto, T.; Slavi, B.; Rivola, V. *Eur. J. Protistol.* **1990**, *25*, 310–315.
4. Giese, A. C. *Science* **1965**, *149*, 540–541.
5. Miyake, A.; Harumoto, T.; Iio, H. *Eur. J. Protistol.* **2001**, *37*, 77–88.
6. Pant, B.; Kato, Y.; Kumagai, T.; Matsuoka, T.; Sugiyama, M. *FEMS Microbiol. Lett.* **1997**, *155*, 67–71.
7. 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.
8. Maeda, M.; Naoki, H.; Matsuoka, T.; Kato, Y.; Koutsuki, H.; Utsumi, K.; Tanaka, T. *Tetrahedron Lett.* **1997**, *38*, 7411–7414.
9. (a) Giese, A. C.; Grainger, R. M. *Photochem. Photobiol.* **1970**, *12*, 489–503; (b) Spitzner, D.; Hofle, G.; Klein, I.; Pohlan, S.; Ammermann, D.; Jaenicke, L. *Tetrahedron Lett.* **1998**, *39*, 4003–4006.
10. Brockmann, H.; Kluge, F.; Muxfeldt, H. *Chem. Ber.* **1951**, *84*, 865–887.
11. Tao, N.; Orlando, M.; Hyon, J. S.; Gross, M.; Song, P. S. *J. Am. Chem. Soc.* **1993**, *115*, 2526–2528.
12. (a) Harumoto, T.; Miyake, A.; Ishikawa, N.; Sugibayashi, R.; Zenfuku, K.; Iio, H. *Eur. J. Protistol.* **1998**, *34*, 458–470; (b) Terazima, M. N.; Iio, H.; Harumoto, T. *Photochem. Photobiol.* **1999**, *69*, 47–54.
13. Mukherjee, P.; Fulton, D. B.; Halder, M.; Han, X.; Armstrong, D. W.; Petrich, J. W.; Lobban, C. S. *J. Phys. Chem. B* **2006**, *110*, 6359–6364.
14. Uruma, Y.; Sakamoto, K.; Takumi, K.; Doe, M.; Usuki, Y.; Iio, H. *Tetrahedron* **2007**, *63*, 5548–5553.
15. Moore, B.; Hertweck, C. *Nat. Prod. Rep.* **2002**, *19*, 70–99.
16. Crawford, J. M.; Dancy, B. C. R.; Hill, E. A.; Udwary, D. W.; Townsend, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 16728–16733.
17. Mahmud, T.; Bode, H. B.; Silakowski, B.; Kroppenstedt, R. M.; Xu, M.; Nordhoff, S.; Hoefle, G.; Muller, R. *J. Biol. Chem.* **2002**, *277*, 32768–32774.
18. Eisenreich, W.; Kupfer, E.; Stohler, P.; Weber, W.; Bacher, A. *J. Med. Chem.* **2003**, *46*, 4209–4212 and references cited therein.
19. Xu, H.; Zhang, Y.; Guo, X.; Ren, S.; Staempfli, A. A.; Chiao, J.; Jiang, W.; Zhao, G. *J. Bacteriol.* **2004**, *186*, 5400–5409 and references cited therein.