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Assignment of ^{13}C NMR spectrum for blepharismin C based on biosynthetic studies

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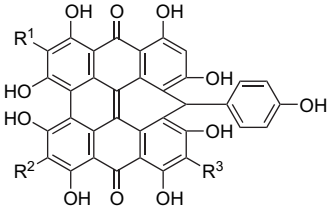
This paper is dedicated to Professor Dr. Yoshito Kishi on the occasion of his 70th birthday

Abstract—Blepharismins, toxic pigments of the ciliate *Blepharisma japonicum*, are polycyclic ring-condensed compounds. Assignment of ^{13}C NMR signals for blepharismin C, a major constituent of blepharismins, was achieved by analyses of the HMQC, HMBC, and INADEQUATE spectra of ^{13}C-enriched samples obtained by feeding experiments using sodium [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$]acetates.

1. Introduction

Blepharismins (BPs) are toxic pigments produced by the negatively phototactic ciliate *Blepharisma japonicum*. These pigments were first reported in 1905[1] and have been extensively studied by Giese.[2] The chemical structure of BP(s) was elucidated independently in 1997 by Song et al.[3] and Naoki et al.[4] by analyzing heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple-bond correlation (HMBC) NMR spectra of BP-C or tetramethoxy derivatives of BPs. BPs (**1**), which exist as a mixture of five congeners,[4] are structurally related to naturally occurring polycyclic phenanthroperylene quinones such as hypericin, a photodynamic toxin of *Hypericum*,[5] and stentorin isolated from *Stentor coeruleus*, another negatively phototactic ciliate.[6] Recently, the structure of maristentorin, a homologous pigment from the positively phototactic marine ciliate *Maristentor dinoferus*, was also reported.[7] BPs have been shown to be converted to stentorin via oxyblepharismines (OxyBPs) by UV radiation; their structures were determined by Spitzner et al.[8] Lensi et al. studied OxyBP–chromoprotein association[9] and the helical properties of OxyBP with or without the chromoprotein.[10] Although the function of BPs has not been fully elucidated, three functions have been clarified: light perception,[11] defense against predators,[12] and protection against UV irradiation.[2] BPs are highly ring-condensed compounds containing a high proportion of quaternary carbon. To date, the spectroscopic assignment of the ^{13}C NMR signals of BPs remains incomplete (Fig. 1).[3,4]



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
BP-E (**1e**): R^1 = *i*Pr, R^2 = *i*Pr, R^3 = Me

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

Figure 1. Structures of blepharismins A–E.

In this paper we report the full assignment of the ^{13}C NMR signals of BP-C (**1c**) using samples obtained by feeding ^{13}C-labeled sodium acetates to *B. japonicum* cells in culture media.

2. Results and discussion

A polyketide biosynthetic pathway comprising repetitive condensations of acetate derivatives was postulated for hypericin[5a] and stentorin.[6] In view of the structural similarities between BPs and hypericin and stentorin, we suspect that the phenanthroperylene quinone portions of BPs are also synthesized via the polyketide pathway. Armed with this working hypothesis, we studied the assignment of the ^{13}C NMR

Keywords: Blepharismin; Phenanthroperylene quinone; Polyketide; INADEQUATE spectrum.
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signals of BP enriched by feeding with ^{13}C-labeled acetates. In a typical feeding experiment, the cells of *B. japonicum* were cultivated for 3 d in the dark with the addition of 4 mM ^{13}C-labeled sodium acetate to the culture medium—a concentration of acetate one-tenth lower than that which effects *B. japonicum* cell growth. The cells of *B. japonicum* were collected, sonicated in acetone, and then the pigments extracted with ethyl acetate were purified by preparative TLC. The ratio of BPs A, B, C, D, and E was determined by HPLC (Mightysil RP-18, CH_3CN: 0.1% TFA/H_2O=3:2 v/v) to be 2:16:41:1:5. NMR spectra analyses were performed on a mixture of BPs, BP-C being the major congener. For the purpose of this study, we adopted the carbon numbering for BP-C described in Song et al.[3]

The ^{1}H and ^{13}C NMR spectra revealed the symmetrical nature of the molecule. In the ^{13}C NMR spectrum of the BPs containing incorporated sodium [1-^{13}C]acetate, highly enriched signals were observed at δ_C 182.97, 173.54, 163.14, 160.95, 135.63, and 125.96 ppm, and weakly enriched signals were observed at δ_C 119.30, 25.09, and 17.33 ppm. In the ^{13}C NMR spectrum of the BPs containing incorporated sodium [2-^{13}C]acetate, highly enriched signals were observed at δ_C 127.68, 125.68, 120.50, 107.88, 104.25, and 102.54 ppm, and weakly enriched signals were observed at δ_C 166.71, 21.19, 20.58, and 13.76 ppm (Fig. 2). Although no incorporation of sodium [1-^{13}C]- and [2-^{13}C]acetate by BPs was observed at the *p*-hydroxybenzylidene group, we confirmed from these incorporation results that, with the exception of *p*-hydroxybenzylidene moiety, BP is biosynthesized by the polyketide pathway (Table 1).

Singlet proton signals at δ_H 6.79 (2H, s) and 7.03 (1H, s) ppm in concentration of 1.2 mM—the chemical shift of these signals being concentration dependant—correlated, respectively, with the sp^2 carbon signal at δ_C 104.25 ppm and the sp^3 carbon signal at δ_C 33.2 ppm in the HMQC spectrum. This observation confirmed that these proton signals were

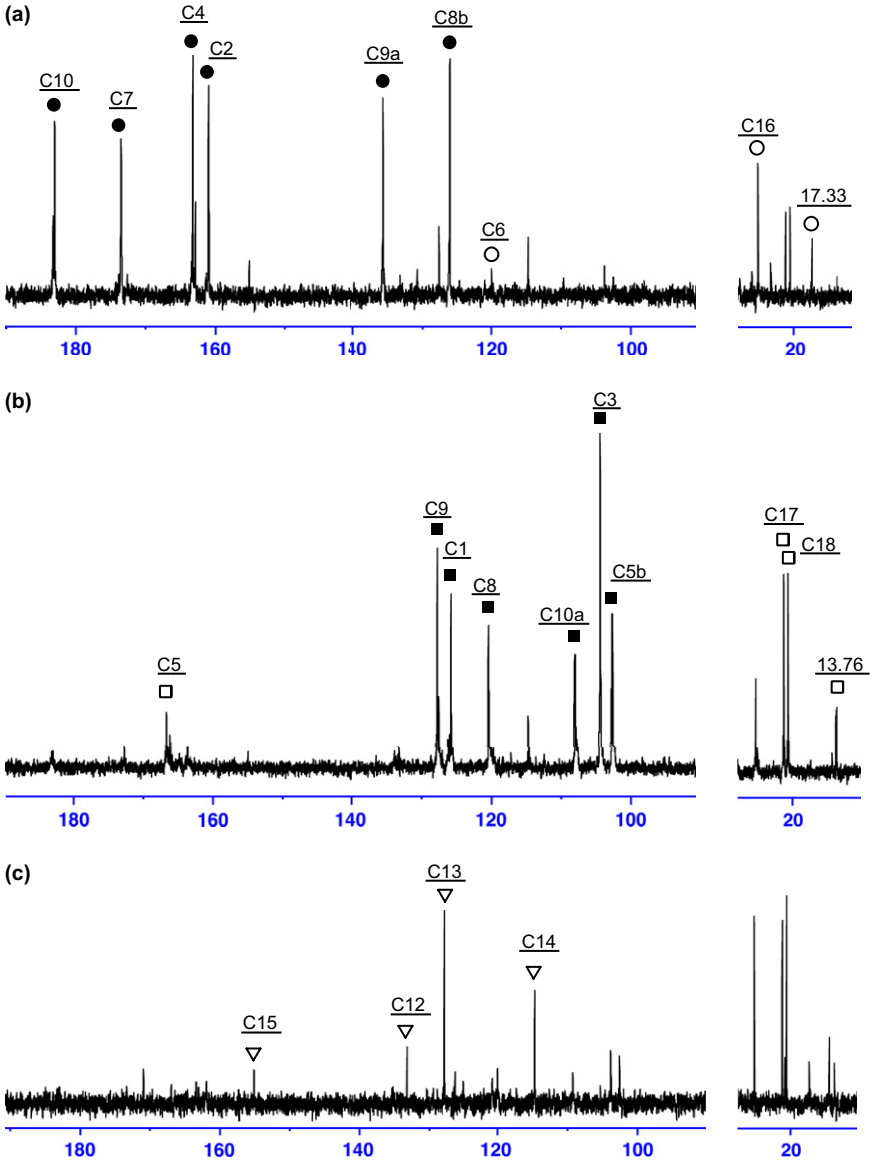

Figure 2. ^{13}C NMR spectra of (a) [1-^{13}C]acetate-enriched BP: ● high enrichment, ○ low enrichment; (b) [2-^{13}C]acetate-enriched BP: ■ high enrichment, □ low enrichment; (c) naturally abundant ^{13}C BP: ▽ signals of the *p*-hydroxybenzylidene group.

Table 1. NMR spectroscopic data (600 MHz, acetone-d_6) for blepharismin C (**1c**)

Position	Data from literature 3		Enriched by [1-^{13}C]acetate		Enriched by [2-^{13}C]acetate		δ_H	HMBC[b]	INADEQUATE[c]
	δ_H	δ_C	δ_C	%[a]	δ_C	%[a]			
1		124.5	—	—	125.68	12		H3, H11	C9a
2		159.5 or 163.2	160.95	19	—	—		H3, H11	C3
3	6.81 (2H, s)	103.71	—	—	104.25	14	6.79 (2H, s)	4-OH	C2
4		159.5 or 163.2	163.14	22	—	—		H3, 4-OH	C10a
5		159.6[d]	—	—	166.71	3.5		H16, 5-OH	C6 (w)
5b		119.6[d]	—	—	102.54	7.3		5-OH	C10
6		121.3	119.30	1.9	—	—		H16, 5-OH	C5 (w)
7		166.5[d]	173.54	18	—	—		H16	C8
8		126.3[d]	—	—	120.50	13		—	C7
8b		[e]	125.96	15	—	—		—	C8b
9		[e]	—	—	127.68	16		—	C9
9a		132.9[d]	135.63	23	—	—		H11	C1
10		184.2[d]	182.97	20	—	—		4-OH (w), 5-OH (w)	C5b
10a		110.4	—	—	107.88	8.3		H3, 4-OH	C4
11	7.07 (1H, s)	32.5	(33.04)[f]	—	(33.04)[f]	—	7.03 (1H, s)	—	—
12		136.5	(136.51)[f]	—	(136.51)[f]	—		H11	—
13	6.06 (2H, d)	114.7	(127.58)[f]	—	(127.58)[f]	—	6.13 (2H, d)	H11	—
14	6.11 (2H, d)	127.6	(114.60)[f]	—	(114.60)[f]	—	6.08 (2H, d)	—	—
15		155.3	(155.24)[f]	—	(155.24)[f]	—		—	—
16	3.89 (2H, m)	25.3	25.09	1.4	—	—	3.91 (2H, m)	H17, H18	C17 (w), C18 (w)
17	1.41 (6H, d)	20.5 or 21.1	—	—	21.19	2.5	1.39 (6H, d)	H16	C16 (w)
18	1.44 (6H, d)	20.5 or 21.1	—	—	20.58	2.2	1.42 (6H, d)	H16	C16 (w)
2-OH	[e]						19.19 (18.89)[g]		
4-OH	14.11 or 14.8						13.89		
5-OH	14.11 or 14.8						15.14		
7-OH	[e]						19.10 (18.74)[g]		
15-OH	9.72						9.72		

[a] Incorporations are commonly given as percentage enrichments.
[b] HMBC correlations are from proton(s) indicated and 'w' in a parenthesis denotes weak correlation.
[c] Correlation in the ^{13}C NMR spectrum enriched by feeding of sodium [1,2-$^{13}C_2$]acetate and 'w' in a parenthesis denotes weak correlation.
[d] It was claimed that the signals were assigned according to chemical shift only.[3]
[e] Data not available.
[f] The chemical shift in a parenthesis denotes the signal of *p*-hydroxybenzylidene group without ^{13}C-enrichment.
[g] The chemical shift in a parenthesis was measured in DMSO-d_6.

assigned to H3 and H11, respectively, and that the carbon signals were assigned to C3 and C11, as demonstrated by Song et al.[3] The lower magnetic field signal at δ_H 7.03 ppm for the sp^3 trityl proton (H11) is due to the *endo* configuration of the *p*-hydroxybenzylidene group exposing the proton to the deshielding position of the phenanthroperylene quinone chromophore, as pointed out by Spitzner et al.[8] The correlations of the signal at δ_H 6.79 (H3) with δ_C 160.95 and 163.14, and that at δ_H 7.03 (H11) with δ_C 160.95 and 135.63 in the HMBC spectrum enriched by feeding with sodium [1-^{13}C]-acetate confirmed that the signals at δ_C 163.14, 160.95, and 135.63 were assigned to carbons C4, C2, and C9a, respectively. Moreover, the correlations of the signal at δ_H 6.79 (H3) with δ_C 107.88 and 125.68, and that at δ_H 7.03 (H11) with δ_C 125.68 ppm in the HMBC spectrum enriched by feeding with sodium [2-^{13}C]acetate confirmed that the signals at δ_C 125.68 and 107.88 ppm were assigned to carbons C1 and C10a, respectively.

Hydroxyl protons were observed at δ_H 9.72, 13.89, 15.14, 19.10, and 19.19 ppm. In HMBC spectra, the hydroxyl proton signals at δ_H 13.89 and 15.14 ppm were correlated, respectively, to δ_C 163.14 (C4), 104.25 (C3), and 107.88 (C10a), and 166.71, 119.30, and 102.54 ppm. Moreover, in HMBC spectrum enriched by feeding with sodium [1-^{13}C]-acetate, weak 4-bond correlations $^4J_{CH}$ were observed between both of the hydroxyl protons and the lower magnetic field signal at the δ_C 182.97 ppm carbonyl carbon (C10).[13] These results demonstrated that the proton signals at δ_H 13.89 and 15.14 ppm could be assigned, respectively, to the peri-hydroxyl 4-OH and 5-OH that are hydrogen bonded to the carbonyl oxygen at C10. The signals of hydroxyl protons (δ_H 19.10 and 19.19 ppm) at a significantly lower magnetic field—the intensities of which are less than one proton due to intrinsically acidic nature of the hydroxyl protons at the bay region[5a]—could be assigned to 7-OH or 2-OH, and the remaining hydroxyl proton signal at δ_H 9.72 ppm was assigned to 15-OH.

The methine proton H16, which was observed at δ_H 3.91 ppm (2H, m), was correlated in the HMBC spectra with the sp^2 carbon signals at δ_C 119.30, 166.71, and 173.54 ppm, and the methyl carbon signals at δ_C 20.58 and 21.19 ppm. Because the signals at δ_C 119.30 and 166.71 ppm were each correlated to both of the proton signals at δ_H 3.91 (H16) and 15.14 (5-OH) ppm as mentioned above, these carbon signals could be assigned to C6 and C5, respectively. Consequently, the carbon signals at δ_C 173.54 and 102.54 ppm were assigned, respectively, to C7 and C5b. NOESY correlation between the signals at δ_H 15.14 (5-OH) and 1.39 (H17) ppm differentiated two methyl groups of an isopropyl group, resulting in the assignment of the sp^3 carbon signals at δ_C 21.19 and 20.58 ppm to C17 and C18, respectively. Weakly enriched sp^3 carbon signals at δ_C 17.33 and

13.76 ppm were assigned to an ethyl group attached at the C6 carbon atom of BP-A, B and/or D instead of the isopropyl group.

No correlation in the HMBC spectra was detected for the internal quaternary carbons C8, C8b, and C9; however, signal enrichment as a result of feeding with sodium [1-^{13}C]acetate at δ_C 125.96 ppm indicated that this signal could be assigned to C8b from a sequence of the polyketide biosynthetic pathway. At present, two signals at δ_C 120.50 and 127.68 ppm for C8 and C9 remain unassigned (Fig. 3).

As the mode of cyclization of the polyketide precursor, which could be converted to the phenanthroperylene quinone carbon framework in blepharismin, two patterns of ring formation—clockwise cyclization **A** and anti-clockwise cyclization **B**—can be considered (Fig. 4). In order to clarify C–C bond extension and the mode of ring formation, the feeding experiment using sodium [1,2-$^{13}C_2$]acetate was carried out. A blepharismin yield of 0.4 mg was obtained from this feeding experiment, and the 2D INADEQUATE ^{13}C NMR spectrum[14] of blepharismin enriched by double-labeled ^{13}C-acetate was measured (Fig. 5). In this spectrum, intense cross-peaks between δ_C 102.54 (C5b) and 182.97 (C10), 107.88 (C10a) and 163.14 (C4), 104.25 (C3) and 160.95 (C2), 125.68 (C1) and 135.63 (C9a), 127.68 and 125.96 (C8b), and 120.50 and 173.54 (C7) ppm, and weak cross-peaks between δ_C 119.30 (C6) and 166.71 (C5), 21.19 (C17) and 25.09 (C16), and 20.58 (C18) and 25.09 (C16) ppm were observed. These cross-peaks clearly indicate that cyclization of the polyketide to the anthrone carbon skeleton had occurred by clockwise cyclization **A**. Moreover, the cross-peaks between δ_C 127.68 and 125.96 (C8b), and 120.50 and 173.54 (C7) ppm unequivocally confirmed that the remaining signals at δ_C 127.68 and 120.50 ppm could be assigned to C9 and C8, respectively.

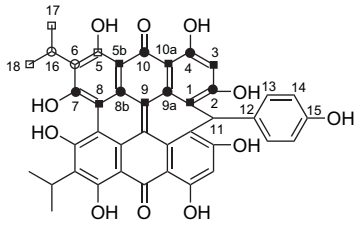

Figure 3. Carbon signals of BP-C enriched by sodium [1-^{13}C]acetate or [2-^{13}C]acetate. ● and ○: high and low incorporation of [1-^{13}C]acetate, respectively. ■ and □: high and low incorporation of [2-^{13}C]acetate, respectively.

The low incorporation of sodium [1-^{13}C]- and [2-^{13}C]acetates into the C5, C6, C16, C17, and C18 carbons, and the weak cross-peaks between C5 and C6, and C16 and C17 or C18 in the INADEQUATE spectrum obtained from the [1,2-$^{13}C_2$]acetate feeding experiment, relative to that between other anthrone carbons, might indicate that this part of the molecule is a starter unit derived from leucine or 4-methyl-2-oxopentanoate—the precursor for isovaleryl-CoA—for the biosynthesis of BP-C.[15] Studies on the biosynthesis of leucine and isovaleryl-CoA demonstrate that the carbons at C1 and C2 of acetate are incorporated, respectively, into C2 and C3, and C1 and C4 of isovaleryl-CoA;[16] this is consistent with the results obtained in our feeding experiment. We need, however, more experimental evidence in order to explain the weak incorporation of [1-^{13}C]acetate and [2-^{13}C]acetate into the C6 and C5 of blepharismin, respectively.

In conclusion, we assigned all the ^{13}C NMR signals of blepharismin C by analyses of the HMQC, HMBC, and INADEQUATE 2D NMR spectra of samples obtained by feeding experiment using sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates. In both sodium [1-^{13}C]- and [2-^{13}C]acetate feeding experiments, no carbon signal enrichment of the *p*-hydroxybenzylidene group in blepharismin was observed. The shikimate pathway might be the origin for the biosynthetic precursor of this group.[17] Studies designed to clarify this possibility are currently in progress.

3. Experimental section

3.1. Materials

Commercially available stable isotopes—sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates—were purchased from Cambridge Isotope Laboratories.

3.2. Cell culture, incubation with sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates, and isolation of blepharismins

B. japonicum (strain R1072) was grown in the dark at 25 °C in wheat grass powder (WGP, Pines) medium inoculated with *Enterobacter aerogenes* 2 d before use.[18]

A typical feeding experiment involved the cultivation of *B. japonicum* in 2 L of WGP culture media inoculated with *E. aerogenes* containing ^{13}C-labeled acetate (4 mM). The *B. japonicum* culture was harvested after a 3-d cultivation in the dark. The culture media were gently centrifuged

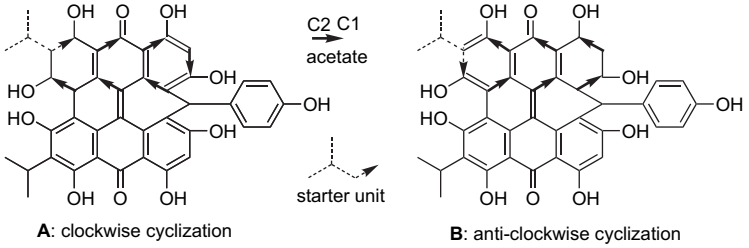

Figure 4. Possible cyclization modes in the biosynthesis of blepharismin C.

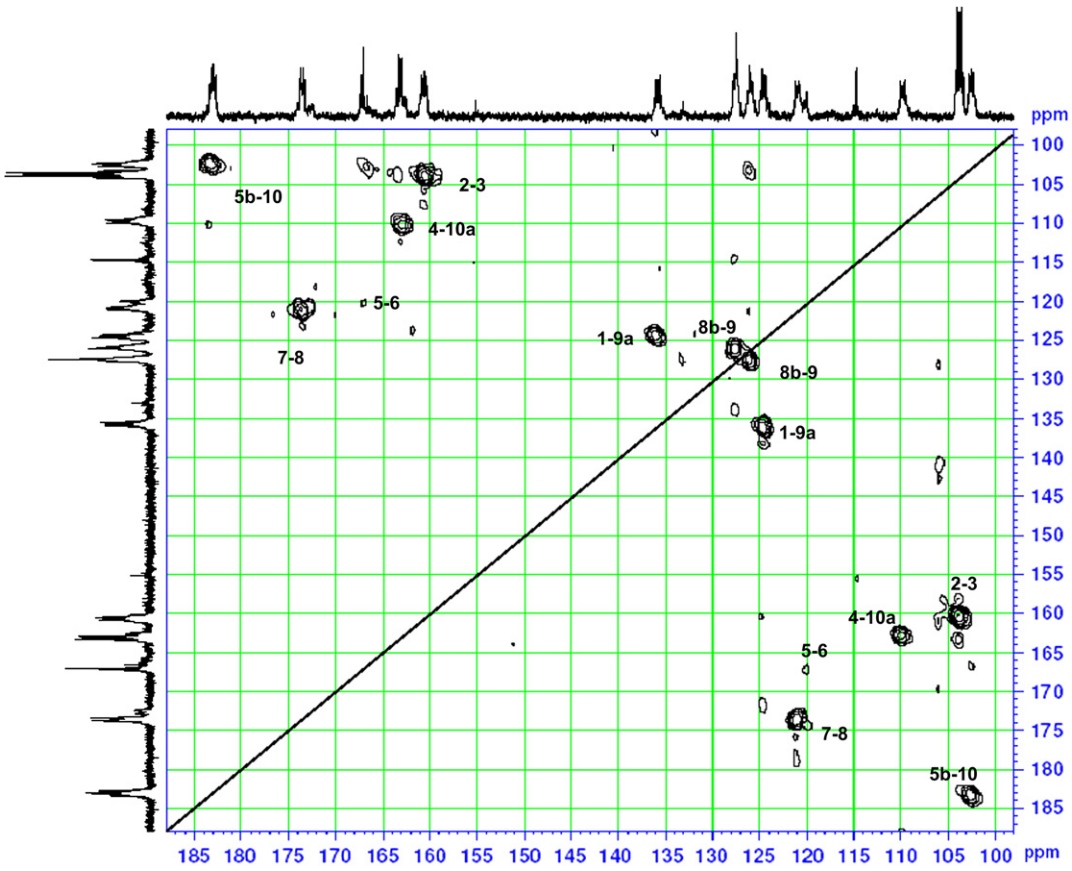

Figure 5. 2D INADEQUATE spectrum of blepharismin ^{13}C-enriched by feeding with sodium [1,2-$^{13}C_2$]acetate.

(100*g* for 3 min) and the cells were washed with physiological balanced solution SMB (a synthetic medium 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),[18] re-centrifuged, and treated with acetone for several minutes. After centrifugation at 2000*g* for 30 min, the supernatant was mixed with ethyl acetate and water, and the pigment was extracted using ethyl acetate. The organic layer was washed with water several times in order to remove acetone, and the ethyl acetate solution of the pigment was dried by adding anhydrous Na_2SO_4. After the solvent had been removed in vacuo, the residue was purified by preparative TLC ($60F_{254}$ plate, 0.5 mm thick; Merck) with a solvent system of acetone/ethyl acetate (3:8 v/v) under an Ar atmosphere in the dark to yield three fractions (red lower: BPR(l), R_f=0.20; red upper: BPR(u), R_f=0.25; and blue-purple upper: BPB(u), R_f=0.35) in the ratio of 8:2:1, and the major fraction BPR(l) was used for NMR analyses.

3.3. General procedure for ^{13}C NMR measurement

^{13}C NMR measurements in acetone-d_6 or dimethyl sulfoxide (DMSO)-d_6 were carried out with a Bruker AVANCE 600 spectrometer (150 MHz for ^{13}C) equipped with Bruker 2.0 mm NMR microtube for Bruker MATCH™ holder. Chemical shifts were referenced to the internal acetone at 29.8 ppm for carbon, and to tetramethylsilane at 0 ppm for proton. 2D HMQC and HMBC were measured with the usual pulse programs and acquisition parameters. For the 2D INADEQUATE measurements, delays were optimized for $^1J_{CC}$=62 Hz. Typically, 128 increments, each consisting of 512 scans separated by a 1, 2-repetition delay, were time-averaged over a 128-step phase cycle. Also, the protons were decoupled by using a Waltz-16 composite decoupling sequence for all ^{13}C measurements. The spectral width in F_1 and F_2 were 31,746 Hz, with a data block of 128×1 K, which after transformation yielded a 512×512 matrix. In both F_1 and F_2, a sine-squared function was used and the sine bell shift ($\pi/2$) window function was used. The total accumulation time was 3 d 15 h 58 min. All 2D spectra were displayed in the absolute mode.

The % enrichment of ^{13}C-labeled carbon to each signals was calculated by dividing the intensity in question by that of the corresponding signal in the natural abundant sample, normalized by the non-enriched peaks C11–C15 of the *p*-hydroxybenzylidene group as an internal standard.

Acknowledgements

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