

## Isolation and Structure of a Novel Biindole Pigment Substituted with an Ethyl Group from a Metagenomic Library Derived from the Marine Sponge *Halichondria okadai*

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We screened a colored clone from a metagenomic library derived from the marine sponge *Halichondria okadai*. We isolated a yellow pigment, halichrome A (**1**), which was structurally elucidated to be a biindole, exhibited cytotoxicity against B16 melanoma cells and was substituted with an ethyl group. To the best of our knowledge, this is the first report of the isolation of a novel compound from a metagenomic library derived from a marine sponge.

Many structurally unique compounds with significant biological activity have been isolated from various marine invertebrates.<sup>1</sup> In particular, sponges, members of the porifera, are rich sources of many natural products. Recent research suggests that marine sponges harbor various microbial symbionts and that many bioactive compounds in sponges are produced by these symbionts.<sup>2</sup> Although microorganisms have potential as sources of bioactive compounds, only a small proportion of bacteria have been isolated from the environment.<sup>3</sup> Hence to use symbiotic bacteria efficiently as sources of natural products, a metagenomic approach is appropriate. Recently, several natural products have been isolated using metagenomic libraries derived from soil.<sup>4</sup> These metagenomic libraries were used directly as sources of natural products by screening of clones that produce bioactive compounds by heterologous expression of metagenomic DNA. We previously constructed a metagenomic library from the marine sponge *Halichondria okadai*.<sup>5</sup> In this study, we screened for colored clones and found a novel compound halichrome A (**1**) (Chart 1).

A colored clone was screened for the production of pigment on Luria–Bertani (LB) agar plates and grown in a liquid-shaker culture. This culture was extracted with EtOAc and chloroform. The extract was concentrated and partitioned between EtOAc and H<sub>2</sub>O. The EtOAc extract was subjected to fractionation with column chromatography (silica gel, toluene–EtOAc; ODS silica

gel, EtOH–H<sub>2</sub>O) and reversed-phase HPLC (Develosil ODS-UG-5, MeOH–H<sub>2</sub>O; YMC-pack AG-323, acetonitrile–H<sub>2</sub>O) to give halichrome A (**1**). The cytotoxicity of halichrome A (**1**) against B16 mouse melanoma cells was determined using the MTT method.<sup>6</sup> This compound showed cytotoxicity with an IC<sub>50</sub> value of 30.9 µg mL<sup>-1</sup> after 4 days.

Halichrome A (**1**) was isolated as a yellow pigment soluble in both methanol and chloroform. The UV-vis absorption maxima at 402 nm with a slight inflection at shorter wavelengths indicate the presence of an indole chromophore.<sup>7</sup> The molecular formula of **1** was determined to be C<sub>18</sub>H<sub>16</sub>N<sub>2</sub>O by HR-ESIMS (*m/z* 299.1160, [M + Na]<sup>+</sup>, Δ + 0.4 mmu). The NMR data for **1** are summarized in Table 1. The <sup>1</sup>H NMR spectrum of **1** in chloroform-*d* showed the presence of two aromatic ring ABCD spin systems (δ 7.65, 6.85, 7.49, 6.92, and 7.50, 7.02, 7.15, 7.34), one doublet aromatic proton (δ 7.22), one methylene (δ 2.30), one methyl group (δ 0.91), and two indole NH signals (δ 5.02 and 8.11). In the <sup>13</sup>C NMR spectrum, 18 carbon signals were observed, including one carbonyl carbon (δ 201.2), one methyl carbon (δ 6.1), one methylene carbon (δ 28.5), one quaternary carbon (δ 67.8), and 14 olefinic carbons (δ 159.0, 135.5, 135.0, 123.3, 123.1, 120.6, 120.5, 119.2, 118.2, 118.1, 117.1, 113.3, 110.5, and 109.6). The 9 olefinic carbon signals

Table 1. <sup>13</sup>C NMR and <sup>1</sup>H NMR data for **1**<sup>a,b,c</sup>

Position	δC	δH (mult., <i>J</i> in Hz)	HMBC (H to C)
1		5.02 (s)	2, 3, 3a, 8
2	67.8		
3	201.2		
3a	119.2		
4	123.1	7.65 (d, 7.7)	3, 7a
5	117.1	6.85 (dd, 7.2, 7.7)	3a
6	135.5	7.49 (dd, 7.2, 8.5)	7a
7	110.5	6.92 (d, 8.5)	3a
7a	159.0		
8	28.5	2.30 (m)	2, 3, 3'
9	6.1	0.91 (t, 7.3)	8
1'		8.11 (brs)	3a'
2'	120.6	7.22 (d, 2.4)	3', 3a', 7a'
3'	113.3		
3a'	123.3		
4'	118.2	7.50 (d, 8.0)	3', 7a'
5'	118.1	7.02 (dd, 8.0, 7.3)	3a'
6'	120.5	7.15 (dd, 8.0, 7.3)	7a'
7'	109.6	7.34 (d, 8.0)	3a'
7a'	135.0		

<sup>a</sup>Solvent: CDCl<sub>3</sub>. <sup>b</sup><sup>1</sup>H NMR (500 MHz), <sup>13</sup>C NMR (125 MHz).

<sup>c</sup>Recorded at 298 K.

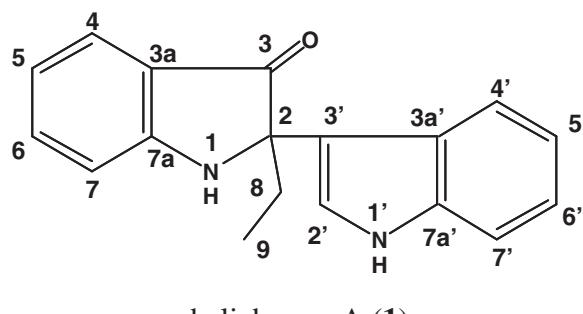
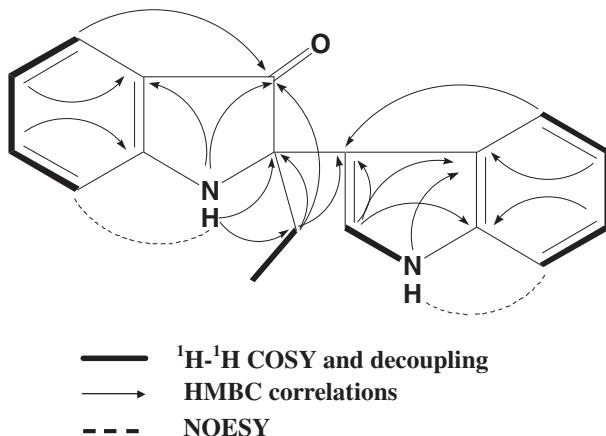


Chart 1.



**Figure 1.** Selected 2D NMR<sup>a,b</sup> correlations for halichrome A (1). <sup>a</sup>Solvent:  $\text{CDCl}_3$ . <sup>b</sup>Recorded at 298 K.

( $\delta$  135.5, 123.1, 120.6, 120.5, 118.2, 118.1, 117.1, 110.5, and 109.6) were assigned to the methines, based on the results of an HMQC experiment. The  $^1\text{H}$ – $^1\text{H}$  COSY spectrum and a decoupling experiment revealed the partial structures C4–C5–C6–C7, C4'–C5'–C6'–C7', and C8–C9, as shown in Figure 1. First, HMBC correlations at H5/C3a, H6/C7a suggested the connectivity of C3a–C4, C7a–C7, respectively. Then, the assignment of C3a and C7a were established by the similarity in the  $^{13}\text{C}$  NMR when compared to those of ketoindole moiety of Cephalinone B.<sup>8</sup> The correlations H1/C2, H1/C3, and H1/C3a in the HMBC spectrum suggested a ketoindole moiety. Furthermore, based on the HMBC correlations at H4/C3 and the NOESY correlations between H1 and H7, the ketoindole moiety should be located between N1 and C7a, as shown in Figure 1. Next, the HMBC correlations at H5'/C3a' and H6'/C7a' suggested the connectivity of C3a'–C4', and C7a'–C7', respectively. Then, the assignment of C3a' and C7a' were established by the similarity in the  $^{13}\text{C}$  NMR when compared to those of indole moiety of Arcyriarubin B 6-O-sulfate.<sup>9</sup> The decoupling of H1' and H2', and HMBC correlations at H2'/C3', H2'/C3a', H2'/C7a', and H1'/C3a' suggested an indole moiety. Furthermore, based on the HMBC correlations at H4'/C3' and the NOESY correlations between H1' and H7', the indole moiety should be located between N1' and C7a', as shown in Figure 1. Thus, these analyses showed two indole moieties and one ethyl group, and the connectivity between these partial structures was clarified by HMBC as follows. The HMBC correlations at H8/C2, H8/C3, and H1/C8 determined that the ethyl group was attached to a C2 quaternary carbon. And the HMBC correlations at H8/C3' suggest that a N1–C7a portion was connected to an N1'–C7a' portion at C2 and C3'. Consequently, the entire carbon chain was assembled and the gross structure of halichrome A (1) was determined to be as shown in Figure 1. The specific rotation of 1 is observed to be  $-0.9$  ( $c$  0.03, MeOH), and then determination of the absolute configuration at C2 is under investigation.

Many biosynthetic pathways of bacterial pigments have been investigated by a metagenomic approach. Recently, some clones that produce heterologously indigo, indirubin, and violacein were isolated from soil-derived metagenomic libraries and full or partial sequences of these gene clusters were

analyzed.<sup>4b,7b,10</sup> In this study we isolated a clone that produce halichrome A. Hence sequencing of the gene clusters is in progress and analysis of the genetic information of this clone would improve the study of the open reading frames, responsible for the halichrome A biosynthesis.

In summary, halichrome A (1) was isolated from a metagenomic library derived from the marine sponge *H. okadai*. The gross structure of halichrome A (1) was revealed to be biindole substituted with an ethyl group based on 2D NMR spectra. Halichrome A (1) exhibits weak cytotoxicity against B16 mouse melanoma cells with an  $\text{IC}_{50}$  value of  $30.9 \mu\text{g mL}^{-1}$ . To date, some natural products have been isolated by a metagenomic approach. Most of these natural products originate from soil, marine sediment, and no example from a marine sponge has been reported. To the best of our knowledge, this is the first report of heterologous expression of a novel natural product from a metagenomic library derived from a marine sponge. These results indicate the potential of metagenomic libraries derived from marine sponges as a genetic and chemical source.

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#### References and Notes

- 1 a) D. Uemura, *Proc. Jpn. Acad., Ser. B* **2010**, *86*, 190. b) D. Uemura, *Chem. Rec.* **2006**, *6*, 235.
- 2 C. A. Bewley, N. D. Holland, D. J. Faulkner, *Cell. Mol. Life Sci.* **1996**, *52*, 716.
- 3 a) R. I. Amann, W. Ludwig, K. H. Schleifer, *Microbiol. Rev.* **1995**, *59*, 143. b) V. Torsvik, J. Goksøyr, F. L. Daae, *Appl. Environ. Microbiol.* **1990**, *56*, 782.
- 4 a) D. E. Gillespie, S. F. Brady, A. D. Bettermann, N. P. Cianciotto, M. R. Liles, M. R. Rondon, J. Clardy, R. M. Goodman, J. Handelsman, *Appl. Environ. Microbiol.* **2002**, *68*, 4301. b) S. F. Brady, C. J. Chao, J. Handelsman, J. Clardy, *Org. Lett.* **2001**, *3*, 1981.
- 5 T. Abe, F. P. Sahin, K. Akiyama, T. Naito, M. Kishigami, K. Miyamoto, Y. Sakakibara, D. Uemura, *Biosci., Biotechnol., Biochem.* **2012**, *76*, 633.
- 6 T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55.
- 7 a) J. S. de Melo, A. P. Moura, M. J. Melo, *J. Phys. Chem. A* **2004**, *108*, 6975. b) H. K. Lim, E. J. Chung, J.-C. Kim, G. J. Choi, K. S. Jang, Y. R. Chung, K. Y. Cho, S.-W. Lee, *Appl. Environ. Microbiol.* **2005**, *71*, 7768. c) Physical properties of UV-vis absorption of 1 (in MeOH):  $\lambda/\text{nm}$  ( $\varepsilon/\text{M}^{-1} \text{cm}^{-1}$ ) 220 (29000), 258 (11000), and 402 nm (2000).
- 8 P.-L. Wu, Y.-L. Hsu, C.-W. Jao, *J. Nat. Prod.* **2006**, *69*, 1467.
- 9 K. Kamata, T. Suetsugu, Y. Yamamoto, M. Hayashi, K. Komiyama, M. Ishibashi, *J. Nat. Prod.* **2006**, *69*, 1252.
- 10 a) I. A. MacNeil, C. L. Tiong, C. Minor, P. R. August, T. H. Grossman, K. A. Loiacono, B. A. Lynch, T. Phillips, S. Narula, R. Sundaramoorthi, A. Tyler, T. Aldredge, H. Long, M. Gilman, D. Holt, M. S. Osburne, *J. Mol. Microbiol. Biotechnol.* **2001**, *3*, 301. b) C. Guan, J. Ju, B. R. Borlee, L. L. Williamson, B. Shen, K. F. Raffa, J. Handelsman, *Appl. Environ. Microbiol.* **2007**, *73*, 3669.