NOTE

Hatomarubigin E, a biosynthetic intermediate of hatomarubigins C and a substrate of HrbU *O*-methyltransferase

Takashi Kawasaki¹, Yuki Yamada, Tomoka Maruta, Ayumi Maeda and Yoichi Hayakawa*The Journal of Antibiotics* (2010) 63, 725–727; doi:10.1038/ja.2010.118; published online 20 October 2010**Keywords:** biosynthesis; hatomarubigin; *hrb* gene cluster; *O*-methyltransferase

The angucycline antibiotics^{1,2} are characterized by containing the modified benz[*a*]anthraquinone chromophore and show antimicrobial, antitumor and/or enzyme-inhibitory activities. *Streptomyces* sp. 2238-SVT4 produced four angucyclines, hatomarubigins A, B, C and D (Figure 1a), which enhanced the cytotoxicity of colchicine in multidrug-resistant tumor cells.³ Recently, we identified the *hrb* gene cluster as consisting of hatomarubigin biosynthesis genes belonging to *Streptomyces* sp. 2238-SVT4.⁴ Several genes in the *hrb* cluster showed no homology to angucycline biosynthesis genes as previously reported. To clarify the function of *hrb* genes, biosynthetic intermediates of hatomarubigins were searched for in the culture extract. Color-guided fractionation resulted in the isolation of a new metabolite, hatomarubigin E (**1**). Herein, we report the isolation, structure elucidation and enzymatic conversion of **1**.

TLC and HPLC analyses detected a new metabolite in the 2-day culture of *Streptomyces* sp. 2238-SVT4 (Figure 2a). The fermentation broth (2.0 l) was centrifuged and the mycelium was extracted with acetone. The extract was concentrated to a small volume and then extracted with ethyl acetate. After evaporation, the residue was applied to a silica gel column and eluted with hexane-chloroform (1:1). The eluate was purified by reversed-phase HPLC (YMC-Pack D-ODS-7, YMC, Kyoto, Japan) with 80% methanol. A single-peak fraction was concentrated to dryness to give an orange powder of **1** (4.7 mg).

The molecular formula of **1** was established as C₁₉H₁₆O₅ by high-resolution FAB-MS. The ¹³C- and ¹H-NMR data of **1** resembled those of hatomarubigin C (**2**), except for a methoxy group in **2** (Table 1). All one-bond ¹H–¹³C connectivities were confirmed by the HMQC⁵ spectrum. The COSY and HMBC⁶ spectra of **1** revealed a proton spin network from a hydroxyl proton (δ 4.73) through 1-H, 2-H₂ and 3-H to 4-H₂ and 3-CH₃ (Figure 1b), and the presence of a

1,2,3,4-tetrasubstituted benzene ring. ¹H–¹³C long-range correlations joined the two partial structures as shown in Figure 1b. A quinone carbonyl (C-7) was attached to C-6a from a coupling between 6-H and C-7. HMCs from two phenolic hydroxyl protons (δ 12.95 and 12.92) and two overlapping aromatic protons (δ 7.29) to six aromatic carbons required them to be in a 2,3-disubstituted hydroquinone ring. Downfield chemical shifts for the two phenolic hydroxyl protons indicated a hydrogen-bonded arrangement of two quinone carbonyls constructing an anthraquinone framework (Figure 1b). These results established the structure of **1** as 8-*O*-demethylhatomarubigin C.

In the *hrb* cluster, *hrbU* shows homology to an *O*-methyltransferase gene involved in tetracenomycin C biosynthesis.^{7,8} To confirm the function of *hrbU*, the recombinant protein expressed in *Escherichia coli* was tested for its ability to convert **1**. HPLC analysis showed that **1** was converted to **2** by HrbU using *S*-(5'-adenosyl)-L-methionine as a cofactor (Figure 2b). This result indicated that **1** is a biosynthetic intermediate of **2** and a substrate of HrbU *O*-methyltransferase.

Hatomarubigins A–D possess a methoxy group at the 8-position. HrbU is a candidate for *O*-methyltransferase, as judged from its homology to TcmO *O*-methyltransferase in tetracenomycin biosynthesis.^{7,8} A new metabolite designated as hatomarubigin E is an appropriate tool for identification of the function of *hrbU*. Purified protein encoded by *hrbU* converted hatomarubigin E to hatomarubigin C in the presence of *S*-(5'-adenosyl)-L-methionine. This reaction corresponds to the 8-*O*-methylation step in hatomarubigin biosynthesis. HrbU contains a conserved motif for *S*-(5'-adenosyl)-L-methionine binding (DVGGARG).^{9–11} Our recent work has identified HrbY as a methylene-bridge-forming enzyme, which can convert hatomarubigin C to hatomarubigin D.⁴ The combined results establish the last two steps in hatomarubigin biosynthesis.

Department of Medicinal and Life Science, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba, Japan

¹Current address: Research Institute for Sustainable Humansphere, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan.

Correspondence: Professor Y Hayakawa, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan.

E-mail: hykw@rs.noda.tus.ac.jp

Received 30 June 2010; revised 25 September 2010; accepted 27 September 2010; published online 20 October 2010

Figure 1 (a) Structures of hatomarubigins A–E. (b) NMR analysis of hatomarubigin E. Bold lines show COSY networks and arrows indicate HMBCs.

EXPERIMENTAL PROCEDURE

Fermentation of hatomarubigin E

The seed medium consisted of 1.0% soluble starch, 1.0% molasses, 1.0% Polypepton and 1.0% meat extract (pH 7.2). Seed tubes containing 7.5 ml of the medium were inoculated with a stock culture of *Streptomyces* sp. 2238-SVT4 maintained on a Bennett's agar slant and were incubated on a reciprocal shaker at 27 °C for 3 days. The seed culture at 2% was transferred to 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of 2.5% soluble starch, 1.5% soybean meal, 0.2% dry yeast and 0.4% calcium carbonate (pH 6.2 before sterilization). The fermentation was carried out on a rotary shaker at 27 °C for 2–5 days. The fermentation broth was centrifuged and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was extracted with ethyl acetate. The extract was dissolved in methanol and the metabolites were examined by reversed-phase HPLC (YMC-Pak R-ODS-7, YMC, Kyoto, Japan) with 80%.

Physico-chemical properties of hatomarubigin E

Melting point 146–149 °C; high-resolution FAB-MS m/z 325.1075 ($M+H^+$, calcd. for $C_{19}H_{17}O_5$, 325.1076); UV λ_{max} (ϵ) 226 nm (26 700), 260 nm (26 900), 469 nm (8600) in methanol, 226 nm (27 100), 260 nm (26 800), 470 nm (8600) in 0.01 M HCl-methanol, 211 nm (39 700), 259 nm (30 000), 556 nm (10 400) in 0.01 M NaOH-methanol; IR (attenuated total reflection) ν_{max} 3529, 2953, 2926, 2873, 1617, 1570, 1452, 1251 and 1226 cm^{-1} ; $[\alpha]_D^{23} +663^\circ$ (c. 0.24, chloroform).

Spectroscopic measurements

Mass spectra were obtained on a JMS-SX102A spectrometer (JEOL, Akishima, Japan) in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. UV and IR spectra were measured on UV-1700 (Shimadzu, Kyoto, Japan) and FT/IR-410 (JASCO, Hachioji, Japan)

spectrometers, respectively. NMR spectra were obtained in $CDCl_3$ on a JEOL JNM-LA400 spectrometer with 1H -NMR at 400 MHz and with ^{13}C -NMR at 100 MHz. Chemical shifts are given in p.p.m. relative to $CHCl_3$ at 7.24 p.p.m. for 1H -NMR and $CDCl_3$ at 77.0 p.p.m. for ^{13}C -NMR.

Cloning and expression of *hrbU*

Streptomyces sp. 2238-SVT4 was used for the production of hatomarubigin E and the cloning of the *hrbU* gene. *E. coli* XL1-blue MRF' and the plasmid pGEM-11Z were used for the cloning experiment. *E. coli* M15 and pQE30 were used for expression of the *O*-methyltransferase gene. Restriction enzymes were obtained from Takara Bio Inc. (Otsu, Japan). T4 DNA ligase was obtained from Nippon Gene (Toyama, Japan). Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions using a Gene Pulser II electroporation system (Bio-Rad, Hercules, CA, USA). Other general procedures were performed as described by Sambrook *et al.*¹²

The *hrbU* gene (1.1 kb) was amplified from *Streptomyces* sp. 2238-SVT4 genomic DNA using primers with additional *Bam*HI and *Hind*III sites (5'-CGCGGATCCGCTGACCAATCGCCCGAGACGACGAG-3' and 5'-ACCAAGCTTTCAGGCGTCTTGTGGCCGATGAC-3'). *Bam*HI/*Hind*III-digested pQE30 and *hrbU* were ligated and introduced into *E. coli* M15, which was cultured in 500-ml Erlenmeyer flasks containing 100 ml of LB medium with kanamycin (25 $\mu g\ ml^{-1}$) and ampicillin (100 $\mu g\ ml^{-1}$). After isopropyl- β -D-thiogalactopyranoside (0.1 mM) was added, the culture was incubated at 18 °C for 20 h on a rotary shaker. The cells collected by centrifugation were washed and suspended in 100 ml of 50 mM Tris-HCl (pH 7.0)–5 mM dithiothreitol, followed by sonication on ice. The extract was centrifuged and the supernatant was applied to a nickel-nitrilotriacetic acid agarose column equilibrated with 50 mM Tris-HCl (pH 7.0), 10 mM imidazole and 5 mM dithiothreitol. The column was washed with 50 mM Tris-HCl (pH 7.0), 20 mM imidazole and 5 mM

Figure 2 HPLC analyses of hatomarubigins. (a) Mycelial acetone extract of 2-day cultured *Streptomyces* sp. 2238-SVT4. (b, c) Reaction mixture for hatomarubigin E conversion with (b) or without HrbU (c). 1: hatomarubigin E, 2: hatomarubigin C, 3: hatomarubigin B, 4: hatomarubigin A.

dithiothreitol, and eluted with 50 mM Tris-HCl (pH 7.0), 250 mM imidazole and 5 mM dithiothreitol. This fraction showed a single band at 37 kDa on SDS-polyacrylamide gel electrophoresis (15%). Protein concentration in the eluent was determined by Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Conversion of hatomarubigin E

The reaction mixture (500 μ l) consisted of 50 μ M hatomarubigin E, 50 μ g ml⁻¹ purified HrbU protein, 200 μ M S-(5'-adenosyl)-L-methionine chloride, 5 mM MgCl₂, 15% dimethyl sulfoxide, 5 mM dithiothreitol and 100 mM Tris-HCl (pH 7.0). After the mixture was stirred at 30 °C for 14 h, the solution was extracted with ethyl acetate and concentrated. The residue was dissolved in 150 μ l of dimethyl sulfoxide and analyzed by HPLC using a YMC-Pack R-ODS-7 column with 80% methanol at a flow rate of 1.0 ml/min. Visible absorption peaks for hatomarubigins C and E were detected at 465 nm.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Young Scientists (B) and a Grant-in-Aid for Scientific Research (C), The Ministry of Education, Science, Sports and Culture, Japan. We thank Dr T Daiiri, Toyama Prefectural University, for the generous gift of plasmids and *E. coli* strains. We thank F Hasegawa, Tokyo University of Science, for assistance with mass spectrometry.

Table 1 NMR data for hatomarubigins E and C in CDCl₃

Position	Hatomarubigin E			Hatomarubigin C ^a	
	δ_C	δ_H multiplicity (J in Hz)		δ_C	δ_H multiplicity (J in Hz)
1	66.8	5.58 m		66.8	5.54 ddd (9.0, 7.5, 4.0)
2	40.0	2.39 ddt (13.0, 7.5, 2.5), 1.62 ddd (13.0, 12.0, 9.0)		40.0	2.39 ddt (13.5, 7.5, 2.5), 1.64 ddd (13.5, 12.0, 9.0)
3	27.4	1.89 m		27.5	1.90 m
4	40.6	2.87 ddd (17.0, 3.5, 2.5) 2.67 dd (17.0, 10.5)		40.3	2.85 ddd (17.0, 4.0, 2.5), 2.67 dd (17.0, 11.0)
4a	147.0			145.4	
5	136.1	7.55 d (8.0)		136.3	7.53 d (9.5)
6	126.5	8.26 d (8.0)		126.9	8.19 d (9.5)
6a	134.2			136.1	
7	186.5			181.8	
7a	113.8 ^b			118.5	
8	158.3 ^c			153.8	
9	129.7 ^d	7.29 s		123.7	7.40 d (8.5)
10	129.5 ^d	7.29 s		126.6	7.32 d (8.5)
11	157.5 ^c			157.4	
11a	111.9 ^b			117.1	
12	190.9			192.8	
12a	132.0			130.9	
12b	143.8			142.5	
3-CH ₃	21.5	1.14 d (6.5)		21.5	1.15 d (7.0)
8-OCH ₃				57.0	4.01 s
1-OH		4.73 d (4.5)			4.67 d (4.0)
8-OH		12.92 ^e s			
11-OH		12.95 ^e s			12.74 s

^aHayakawa et al.³

^{b-e}Assignments are interchangeable.

- Krohn, K. & Rohr, J. Angucyclines: total syntheses, new structures, and biosynthetic studies of an emerging new class of antibiotics. *Top. Curr. Chem.* **188**, 127–195 (1997).
- Rohr, J. & Thiericke, R. Angucycline group antibiotics. *Nat. Prod. Rep.* **9**, 103–137 (1992).
- Hayakawa, Y., Ha, S.-C., Kim, Y. J., Furihata, K. & Seto, H. Studies on the isotetracenone antibiotics. IV. Hatomarubigins A, B, C and D, new isotetracenone antibiotics effective against multidrug-resistant tumor cells. *J. Antibiot.* **44**, 1179–1186 (1991).
- Kawasaki, T. et al. Cloning and characterization of a gene cluster for hatomarubigin biosynthesis in *Streptomyces* sp. strain 2238-SVT4. *Appl. Environ. Microbiol.* **76**, 4201–4206 (2010).
- Summers, M. F., Marzilli, L. G. & Bax, A. Complete ¹H and ¹³C assignments of coenzyme B₁₂ through the use of new two-dimensional NMR experiments. *J. Am. Chem. Soc.* **108**, 4285–4294 (1986).
- Bax, A. & Summers, M. F. ¹H and ¹³C assignments from sensitivity enhanced detection of heteronuclear multiple-band connectivity by multiple quantum NMR. *J. Am. Chem. Soc.* **108**, 2093–2094 (1986).
- Summers, R. G., Wendt-Pienkowski, E., Motamedi, H. & Hutchinson, C. R. The *tcmVI* region of the tetracenomycin C biosynthetic gene cluster of *Streptomyces glaucescens* encodes the tetracenomycin F1 monooxygenase, tetracenomycin F2 cyclase, and, most likely, a second cyclase. *J. Bacteriol.* **175**, 7571–7580 (1993).
- Summers, R. G., Wendt-Pienkowski, E., Motamedi, H. & Hutchinson, C. R. Nucleotide sequence of the *tcmII-tcmIV* region of the tetracenomycin C biosynthetic gene cluster of *Streptomyces glaucescens* and evidence that the *tcmN* gene encodes a multifunctional cyclase-dehydratase-O-methyl transferase. *J. Bacteriol.* **174**, 1810–1820 (1992).
- Haydock, S. F. et al. Cloning and sequence analysis of genes involved in erythromycin biosynthesis in *Saccharopolyspora erythraea*: sequence similarities between EryG and a family of S-adenosylmethionine-dependent methyltransferase. *Mol. Gen. Genet.* **230**, 120–128 (1991).
- Ingrasso, D., Fowler, A. V., Bleibaum, J. & Clarke, S. Sequence of the D-aspartyl/L-isoaspartyl protein methyltransferase from human erythrocytes. Common sequence motifs for protein, DNA, RNA and small molecule S-adenosylmethionine dependent methyltransferase. *J. Biol. Chem.* **264**, 20131–20139 (1989).
- Madduri, K., Torti, F., Colombo, A. L. & Hutchinson, C. R. Cloning and sequencing of a gene encoding carminomycin 4-O-methyltransferase from *Streptomyces peuceii* and its expression in *Escherichia coli*. *J. Bacteriol.* **175**, 3900–3904 (1993).
- Sambrook, J. & Russell, D. W. *Molecular Cloning: A Laboratory Manual* 1.1–1.162 (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2001).