The Journal of Antibiotics (2014) 67, 159–162

www.nature.com/ja

ORIGINAL ARTICLE

Functional analysis of hatomarubigin biosynthesis genes and production of a new hatomarubigin using a heterologous expression system

Masumi Izawa[1], Shoko Kimata[1], Ayumi Maeda[1], Takashi Kawasaki[1,2] and Yoichi Hayakawa[1]

The function of hatomarubigin biosynthesis genes was analyzed by heterologous expression of the *hrb* gene cluster. *Streptomyces lividans* carrying a gene cluster consisting of 25 genes (*hrbR1–hrbX*) with *hrbY* was found to produce all the known hatomarubigins including hatomarubigin D, which has a unique dimeric angucycline with a methylene linkage. Gene disruption was used in this heterologous expression system to analyze the function of *hrbF*, a gene with no homology to any known angucycline biosynthesis genes. A new metabolite was detected in the fermented broth of *S. lividans* expressing the *hrb* genes lacking *hrbF* and was designated hatomarubigin F. This compound was identified as 5-hydroxyhatomarubigin E by NMR spectroscopic analysis, suggesting that HrbF regulates the regiospecificity of oxygenation enzymes.
The Journal of Antibiotics (2014) **67,** 159–162; doi:10.1038/ja.2013.96; published online 16 October 2013

Keywords: angucycline; biosynthesis; hatomarubigin; *hrb* gene cluster

INTRODUCTION

Hatomarubigins A, B, C and D (Figure 1), multidrug-resistance reversal substances, were produced by *Streptomyces* sp. 2238-SVT4.[1] Hatomarubigin E and rubiginone B_2 were also found in the fermented broth as biosynthetic intermediates.[2] These compounds belong to the angucycline family,[3,4] which is characterized by containing a modified benz[*a*]anthraquinone skeleton. Among them, hatomarubigin D was a unique dimeric angucycline with a methylene linkage. In our previous study,[5] hatomarubigins except hatomarubigin D were produced by *Streptomyces lividans* carrying a gene cluster consisting of 25 genes (*hrbR1–hrbX*) to identify the *hrb* gene cluster (Figure 2) as hatomarubigin biosynthesis genes. Such a heterologous expression system is useful for functional analysis of unknown genes in the cluster. In this study, all the known hatomarubigins including hatomarubigin D were produced by heterologous expression of the 25 *hrb* genes with *hrbY*, and hatomarubigin F, a new member of the hatomarubigin family, was isolated from the fermented mycelium of *S. lividans* carrying the *hrb* cluster lacking *hrbF*.

RESULTS AND DISCUSSION

Expression of the *hrb* gene cluster including *hrbY* in *S. lividans*

Our previous study identified HrbY as an enzyme that can convert hatomarubigin C into hatomarubigin D in the presence of methylcobalamin.[5] To confirm the *in vivo* function of HrbY, we constructed pWHM3-HR-Y, a plasmid carrying the 25 *hrb* genes (*hrbR1–hrbX*) with *hrbY*. All the known hatomarubigins including hatomarubigin D were detected by HPLC analysis of the fermented broth of *S. lividans* carrying pWHM3-HR-Y, although *S. lividans* expressing *hrb* genes without *hrbY* did not produce hatomarubigin D (Figure 3). These results demonstrated that HrbY is functional *in vivo* and the hatomarubigin biosynthesis gene cluster exists in the region from *hrbR1* to *hrbR3*, a gene homologous to the angucycline biosynthesis regulator *jadR1* (61% identity).[5]

Expression of the *hrb* gene cluster lacking *hrbF* in *S. lividans*

The *hrb* gene cluster contains several genes with no homology to any known genes for angucycline biosynthesis (Figure 2). Among them, *hrbF* encodes a 234-amino-acid protein showing a weak similarity to antibiotic biosynthesis monooxygenase of *S. griseus* NBRC13350 SGR_5610 (34% identity) and ubiquinone/menaquinone biosynthesis methyltransferase of *Caulobacter crescentus* CB15 (34% identity). To investigate the function of *hrbF*, we constructed pWHM3-HR-YΔF, a plasmid that can express the *hrb* gene cluster lacking *hrbF*. The fermented mycelium of *S. lividans* carrying pWHM3-HR-YΔF contained all the hatomarubigins, thereby showing that *hrbF* is not essential for hatomarubigin biosynthesis. When the HPLC profiles were compared with those from *S. lividans* expressing the entire *hrb* gene cluster, a new metabolite was detected and the peak disappeared in the broth of the *hrbF*-reintroduced transformant (Figure 3). This substance was identified as a new member of the hatomarubigin family by spectroscopic analysis and designated hatomarubigin F (**1**).

[1]Department of Medicinal and Life Science, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda, Japan
[2]Current address: College of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Noji-higashi, Kusatsu, Shiga 525-8577, 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 December 2012; revised 27 June 2013; accepted 30 August 2013; published online 16 October 2013

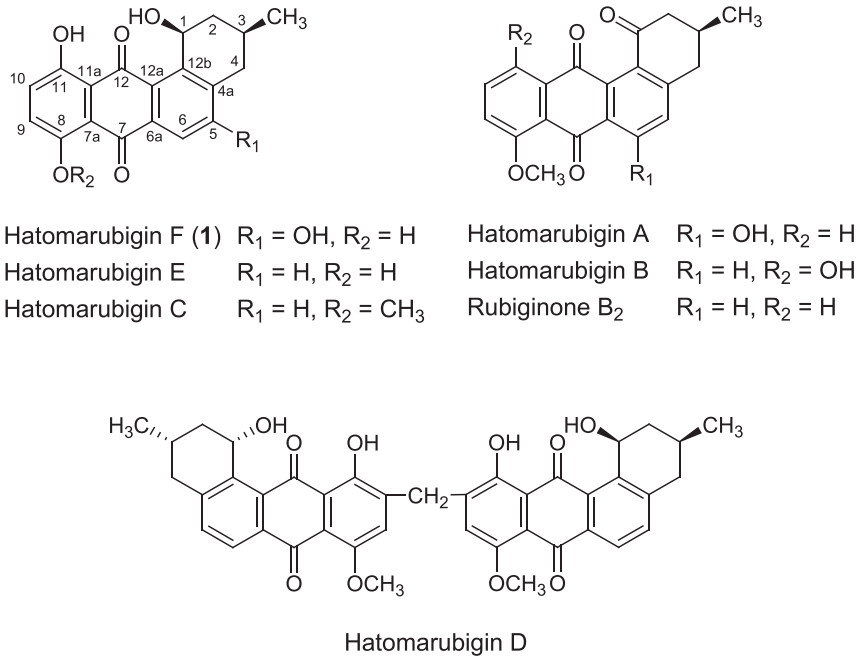

Figure 1 Structures of hatomarubigins A to F and rubiginone B_2.

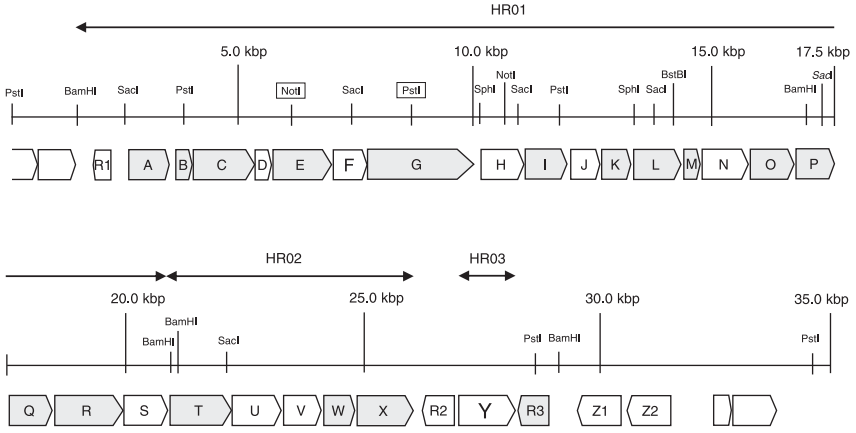

Figure 2 Hatomarubigin biosynthesis gene cluster from *Streptomyces* sp. 2238-SVT4. Arrows show DNA fragments used for heterologous expression. Gray boxes indicate genes homologous to known angucycline biosynthesis genes.

Structure determination of hatomarubigin F (1)

The molecular formula of **1** was established as $C_{19}H_{16}O_6$ by high-resolution EI-MS. The ^{13}C- and ^{1}H-NMR data summarized in Table 1 resembled those of hatomarubigin E.[2] All one-bond ^{1}H-^{13}C connectivities were confirmed by an HMQC[6] experiment. The COSY spectrum revealed a proton-spin network from a hydroxy proton (δ 4.96) through 1-H, 2-H_2 and 3-H to 4-H_2 and 3-CH_3 (Figure 4). In the HMBC[7] spectrum, a singlet aromatic proton at δ 7.58 displayed long-range correlations to four aromatic carbons (C-4a, C-5, C-6a and C-12a). Long-range couplings from both 1-H and 4-H (δ 2.16) to C-4a and C-12b (δ_C 146.8) constructed a tetrahydronaphthalene moiety. A quinone carbonyl (C-7) was attached to C-6a from a coupling between 6-H and C-7. HMBCs from *ortho* aromatic protons (δ 7.35 and 7.31) to four aromatic quaternary carbons including two oxygenated carbons (δ_C 155.9) required them to be in a 2,3-disubstituted hydroquinone ring (Figure 4). Downfield chemical shifts for the two phenolic hydroxy protons (δ 12.96 and 12.61) indicated a hydrogen-bonded arrangement of two quinone carbonyls to construct the 1,4-dihydroxyanthraquinone framework (Figure 4). The presence of a hydroxy group at C-5 was required by the molecular formula and the chemical shift of C-5 (δ_C 161.1), although the hydroxy proton was not observed. These results established the structure of hatomarubigin F (**1**) as 5-hydroxyhatomarubigin E.

Streptomyces sp. 2238-SVT4 produced rubiginone B_2, which can be converted into hatomarubigins A and B by oxygenation at C-6 and C-11, respectively. In the *hrb* cluster, the two tandem genes *hrbW* and *hrbX* encoding oxidoreductase and oxygenase are considered to be involved in 11-hydroxylation.[5] Putative oxygenase responsible for 6-hydroxylation is assignable to *hrbG*.[5] Disruption of *hrbF* caused the

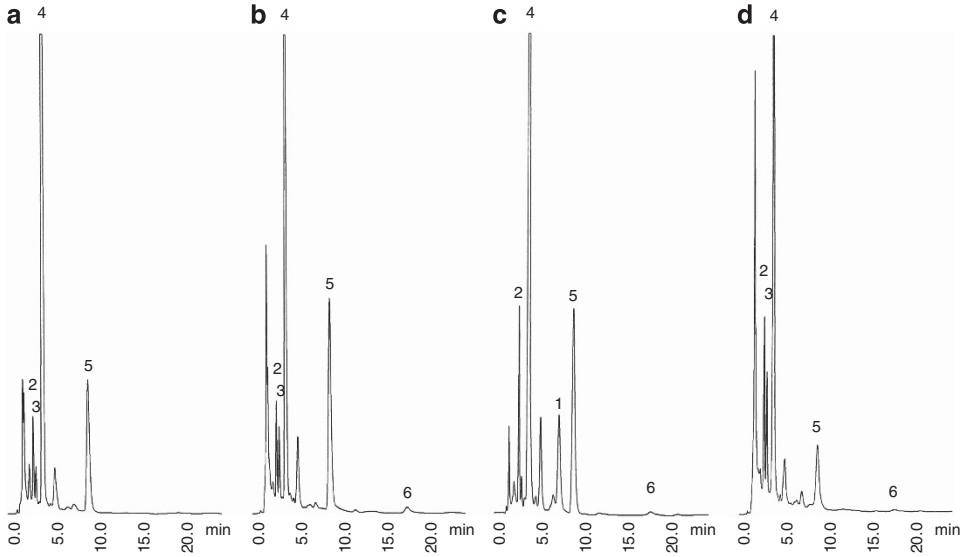

Figure 3 HPLC analysis of the mycelial extract of *S. lividans* carrying pWHM3-HR[5] (**a**); pWHM3-HR-Y (**b**); pWHM3-HR-YΔF (**c**) and pWHM3-HR-YΔF-F (**d**). **1**: hatomarubigin F, **2**: hatomarubigin B, **3**: hatomarubigin A, **4**: hatomarubigin C, **5**: hatomarubigin E, **6**: hatomarubigin D.

Table 1 NMR data for hatomarubigin F in DMSO-d_6 and hatomarubigin E in $CDCl_3$

	Hatomarubigin F		*Hatomarubigin E*[a]	
Position	δ_C	δ_H *multiplicity (J in Hz)*	δ_C	δ_H *multiplicity (J in Hz)*
1	65.9	5.58 t (8.0)	66.8	5.58 m
2	40.4	2.14 m, 1.35 td (12.5, 9.0)	40.0	2.39 ddt (13.0, 7.5, 2.5), 1.62 ddd (13.0, 12.0, 9.0)
3	26.0	1.75 m	27.4	1.89 m
4	33.0	2.81 ddd (12.5, 4.0, 2.5) 2.67 m	40.8	2.87 ddd (17.0, 3.5, 2.5), 2.67 dd (17.0, 10.5)
4a	133.7		147.0	
5	161.1		136.1	7.55 d (8.0)
6	110.3	7.58 s	126.5	8.26 d (8.0)
6a	134.8		134.2	
7	187.0		186.5	
7a	112.2[b]		113.8	
8	155.9		158.3	
9	127.5[c]	7.31[d] d (9.5)	129.7	7.29 s
10	129.6[c]	7.29[d] d (9.5)	129.5	7.29 s
11	155.9		157.5	
11a	113.7[b]		111.9	
12	187.7		190.9	
12a	123.0		132.0	
12b	146.8		143.8	
3-CH_3	21.8	1.08 d (6.5)	21.5	1.14 d (6.5)
1-OH		4.96 broad		4.73 d (4.5)
8-OH		12.61[e] s		12.92 s
11-OH		12.96[e] s		12.95 s

[a]Kawasaki *et al.*[2]
[b–e]Assignments are interchangeable.

oxygenation at an abnormal position C-5 and decreased production of hatomarubigin A (Figure 3), suggesting that HrbF controls regiospecificity of these oxygenating enzymes.

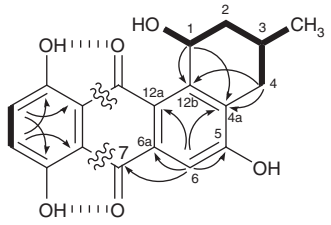

Figure 4 NMR analysis of hatomarubigin F (**1**). Bold lines show COSY networks and arrows indicate HMBCs.

EXPERIMENTAL SECTION

Bacterial strains and DNA manipulation

The media and growth conditions of *Streptomyces* sp. 2238-SVT4 and *S. lividans* TK23 were previously described.[1,5] *Escherichia coli* XL1-blue MRF' and JM110 were grown in LB medium supplemented with 100 μg ml^{-1} ampicillin. KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used for PCR amplification following the manufacturer's instructions. Other general procedures were performed as described by Sambrook *et al.*[8]

Construction of plasmids for expression of the *hrb* cluster

A 19.8-kbp fragment consisting of *hrbR1* to *hrbS* (HR01) was constructed as previously described.[5] A 5.2-kbp fragment consisting of *hrbT* to *hrbX* (HR02) was amplified from *Streptomyces* sp. 2238-SVT4 genomic DNA using primers with additional *Nsi*I and *Nhe*I sites (5′-TGCATGCATGTGCGAGAGCGC TGGGACGCACGC-3′ and 5′-ACCGCTAGCTCAGACGGACAGCAGCCCGC GTGC-3′). A 1.1-kbp *hrbY* fragment (HR03) was amplified from the genomic DNA using primers with additional *Nhe*I and *Hin*dIII sites (5′-ACCGC TAGCATGAGTGCAACAGTCGCGGTCGTC-3′ and 5′-ACCAAGCTTTCAG CCGGCGGTGGGACCGAACTG-3′). *Xba*I/*Nsi*I-digested HR01, *Nsi*I/*Nhe*I-digested HR02, *Nhe*I/*Hin*dIII-digested HR03 and *Xba*I/*Hin*dIII-digested pWHM3 were ligated to construct pWHM3-HR-Y, a plasmid carrying *hrbR1* to *hrbX* with *hrbY*. In this plasmid, *hrbA* to *hrbY* can be polycistronically transcribed.

Disruption of *hrbF* was carried out as described below. A 3.1-kbp *Not*I/*Pst*I fragment containing *hrbF* was cloned into pBluescript II SK (+) (Agilent technologies, Santa Clara, CA,USA). A 1.2-kbp fragment upstream of *hrbF* was amplified from the plasmid using M13 primer RV and a primer with an

additional *Bln*I site (5′-AACCTAGGCGTCTTTCACTCCCGGTGTCGTCG) and digested with *Not*I and *Bln*I. A 1.2-kbp fragment downstream of *hrbF* was amplified using M13 primer M4 and a primer with an additional *Bln*I site (5′-AGCCTAGGGCGCGCAGGTCGGCCCCAGAAGGA-3′) and digested with *Pst*I and *Bln*I. These two fragments were ligated to *Not*I/*Pst*I digested pBluescript II SK (+). A *hrbF*-deleted 2.4-kbp fragment obtained by *Not*I/*Pst*I digestion of the plasmid was swapped with an original 3.1-kbp *Not*I/*Pst*I fragment of pWHM3-HR-Y to construct pWHM3-HR-YΔF. A 0.7-kbp *hrbF* fragment was amplified from the genomic DNA using primers with additional *Bln*I sites (5′-GACCTAGGATGCCTGTAGCCTCCGACGCCCCA-3′ and 5′-CGCCTAGGTCAGTCGGCTGTCTTCTCCAGCGC-3′) and reintroduced into an artificial *Bln*I site between *hrbE* and *hrbG* of pWHM3-HR-YΔF in the same orientation to construct pWHM3-HR-YΔF-F for complementary analysis of *hrbF*.

Heterologous expression of hatomarubigin biosynthesis genes

The expression plasmids passaged through *E. coli* JM110 were introduced into *S. lividans* TK23. The transformants were inoculated into a seed medium consisting of 10.3% sucrose, 3.0% glucose, 1.5% Bacto Soytone, 0.1% glycine, 3 mM $CaCl_2$, 5 mM $MgCl_2$ and 10 μg ml^{-1} thiostrepton (pH 7.2). After incubating on a reciprocal shaker at 27 °C, 2 ml of the seed culture was transferred into 500-ml baffled Erlenmeyer flasks containing 50 ml of a production medium consisting of 2.5% soluble starch, 1.5% soybean meal, 0.2% dry yeast, 0.4% $CaCO_3$ and 10 μg ml^{-1} thiostrepton (pH 6.2). The fermentation was carried out on a rotary shaker at 27 °C for 2–3 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 analyzed by reversed-phase HPLC (YMC-Pack R-ODS-7, 4.6 mm id × 250 mm; YMC, Kyoto, Japan) with 80% methanol. Absorption peaks for the hatomarubigins were detected at 470 nm.

Isolation of hatomarubigin F (1)

The mycelium was obtained by centrifugation of the 2-day fermented broth (5 liters) of *S. lividans* TK23 carrying pWHM3-HR-YΔF and extracted with acetone. The extract was concentrated and partitioned between ethyl acetate and water. The organic layer was evaporated and dissolved in chloroform. The precipitate by addition of 10 volumes of hexane was dissolved in methanol and filtered. The filtrate was subjected to HPLC (YMC-Pack D-ODS-7, 20 mm id × 250 mm; YMC) with 80% methanol–0.2% phosphoric acid. One of the major peaks (retention volume: 280 ml) was further purified by HPLC (YMC-Pack D-ODS-7) with 75% methanol containing 5 mM disodium hydrogen citrate. The main fraction (retention volume: 395 ml) was evaporated and the aqueous concentrate was extracted with ethyl acetate. The extract was washed with water and concentrated to dryness to give an orange powder of **1** (1.2 mg).

Physico-chemical properties of hatomarubigin F (1)

m.p. 276–278 °C; high-resolution EI-MS *m/z* 340.0948 (M^+, calcd for $C_{19}H_{16}O_6$, 340.0947); UV λ_{max} (ε) 224 nm (20 300), 287 nm (18 000), 475 nm (6800) in methanol, 226 nm (22 000), 287 nm (20 000), 474 nm (7000) in 0.01 M HCl-methanol, 240 nm (14 200), 310 nm (15 000), 534 nm (8300) in 0.01 M NaOH-methanol; IR (ATR) ν_{max} 3438, 3127, 1619, 1568, 1449, 794 cm^{-1}.

Spectroscopic measurements

Mass spectra were obtained on a JMS-SX102A spectrometer (JEOL Ltd., Tokyo, Japan) in the EI mode. UV and IR spectra were measured on Shimadzu UV-1700 and JASCO FT/IR-410 spectrometers, respectively. NMR spectra were obtained in DMSO-d_6 on a JEOL JNM-LA400 spectrometer with ^{1}H-NMR at 400 MHz and with ^{13}C-NMR at 100 MHz. Chemical shifts are given in p.p.m. relative to DMSO at 2.49 p.p.m. for ^{1}H-NMR and at 39.5 p.p.m. for ^{13}C-NMR.

ACKNOWLEDGEMENTS

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

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