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Biosynthesis of Hibarimicins

II. Elucidation of Biosynthetic Pathway by Cosynthesis Using Blocked Mutants

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The biosynthetic pathway of hibarimicin (HBM) was proposed on the basis of the experimental results obtained by using blocked mutants of *Microbispora rosea* subsp. *hibaria* TP-A0121, the HBM producer. In its biosynthesis, the oxidative coupling of the aromatic undecaketide unit generates a symmetrical aglycon HMP-Y1 (hibarimicin-mutant product Y1), which is oxidatively modified to hibarimicinone, the HBM aglycon. The following glycosylation of hibarimicinone gives rise to the HBM complex. We identified that HMP-Y1 prepared by methanolysis of HMP-Y6, a glycosylated metabolite from a blocked mutant, was the key intermediate: transformation of ^{13}C-labeled HMP-Y1 to HBM B was confirmed by NMR measurements. Mutant strain produced another type of aglycon HMP-P1 in which the coupled polyketide units were intramolecularly bridged by the ether bond. This metabolite also arose by the spontaneous elimination of methanol molecule from hibarimicinone.

Hibarimicins (HBMs) were discovered from *Microbispora rosea* subsp. *hibaria* TP-A0121 as a complex of analogs in the screening for tyrosine kinase inhibitors[1,2]. The strain TP-A0121 produces more than ten HBM components detectable on TLC. Structural study of the major components revealed that HBM is the glycosylated aromatic polyketide derived from two undecaketides differing from each other by the number and type of deoxysugars attached on the amicetoses. HBM is one of the most complicated and largest molecules among the aromatic polyketide dimers of microbial metabolites. In the preceeding paper, we demonstrated that the aglycon of HBM arises by dimerization of two monomeric units of undecaketide through decarboxylation and skeltal rearrangement[3]. This study was attempted to elucidate the biosynthetic pathway of HBM regarding to the order of dimerization and glycosilation and the mode of rearrangement.

Materials and Methods

Bacterial Strains

Strains AN-0416, AN-0554, AN-0623, AN-0763, AN-0772, BN-Y185 and BN-Y218 used in this study are blocked mutants generated from *M. rosea* subsp. *hibaria* TP-A0121 by mutagenesis treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG).

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Media

Yeast starch (YS) agar medium containing yeast extract 0.2%, soluble starch 1.0% and agar 1.8% was used for the propagation and sporulation of strain TP-A0121 and its mutants. SPT agar medium consisting of soluble starch 4.0%, soybean meal (Ajinomoto Co.) 2.0%, $CaCO_3$ 0.3% and agar 1.8% was used for selection of blocked mutants. Seed medium (V-22) was prepared with soluble starch (Wako Chemical Co.) 1.0%, glucose 0.5%, NZ-case (Humco Scheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH was adjusted to 7.2 before autoclaving). Production medium (MP) was prepared with maltose 4.0%, Pharmamedia (Trader's Protein) 2.0% and $CaCO_3$ 0.3% (pH was adjusted to 7.0 before autoclaving).

Mutagenesis Treatment

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was used as a mutagen. Strain TP-A0121 was incubated on YS agar medium for 21 days at 30°C. After the full development, spores were scraped off and suspended in 10 ml of a solution containing Tween 80 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and NaCl 0.5%, sonicated and filtered through cotton wool. The spores were centrifuged at 5,000 rpm for 10 minutes and suspended in an aliquot of 10% DMSO solution. The spores (5×10^8) were inoculated into a 500-ml flask containing 50 ml of the V-22 medium and shaken at 120 rpm for 24 hours at 30°C. The germinated spores were harvested, suspended in 20 ml of 0.01 M Tris-HCl buffer (pH 8.0) and sonicated. This suspension was diluted 2-fold with the same buffer containing 6 mg/ml NTG and kept standing at 37°C for 30 minutes. This treatment gave the survival rate of *ca.* 0.1%. The treated spores were spread onto the SPT agar plates and incubated at 30°C for 14 days. Colonies were selected on the basis of the color difference of produced diffusible pigments. The isolated colonies were fermented and the metabolites were analyzed as described below.

Screening of Blocked Mutants in HBM Biosynthesis

The colonies obtained above were inoculated into small glass tubes containing 4 ml each of MP medium and cultivated on a reciprocal shaker (200 rpm) at 30°C for 10 days. The test culture was extracted with equal volume of ethyl acetate and 20 μl was spotted on a reversed phase silica gel thin layer plate (RP18 F_{254S} Art 15389, Merck), which was then developed with acetonitrile-distilled water (55 : 45)[1]. The developed spots were examined by the color and UV absorption and the strains producing new metabolites were selected as the candidates of biosynthetic blocked mutants.

Isolation of HBM-related Compounds from the Mutants

The metabolites produced by the blocked mutants and HBM of parent strain are used in cosynthesis experiment. Molecular formula, Rf value, color on silica gel TLC and retention time on HPLC of the compounds are summarized in Table 1. Isolation and structure determination of the new metabolites from mutants are discussed in the accompanying paper[4].

Methanolysis

Glycosylated metabolites were subjected to acid-catalyzed methanolysis in 1.5 N HCl-MeOH (100 μg/ml) to obtain the aglycons. The reaction was monitored by TLC. After incubation at 30°C for 3 hours, the solution was diluted with water and applied to a column of Diaion HP-20. The column was washed with water and eluted with acetone and the eluent was evaporated to dryness.

Preparation of ^{13}C-labeled HMP-Y1

Strain AN-0416 was inoculated in 500-ml K-1 flasks containing 100 ml of the MP medium and cultivated at 30°C on a rotary shaker. After 48 hours, one ml of sterilized 10 μg/ml sodium [1-^{13}C] acetate (99% ^{13}C, ISOTEC Inc.) solution was added into each flask and fermentation was continued for 8 days at 30°C on a rotary shaker. ^{13}C-labeled HMP-Y6 (hibarimicin-mutant product Y6) accumulated in the culture broth was obtained according to the isolation protocol described in the accompanying paper[4].

HMP-Y6 was degraded to the ^{13}C-labeled HMP-Y1 by methanolysis as described below. A solution of HMP-Y6 (5 mg) in 1.5 N HCl-MeOH (50 ml) was incubated at 30°C for 3 hours. After the completion of the reaction, the solution was diluted with excess water and applied onto a column of Diaion HP-20. The column was washed with water and eluted with acetone. The eluent was evaporated to dryness to give HMP-Y1 (3 mg) as a yellow powder: m.p. 183~185 °C; TLC Rf 0.54; UV λ_{max}^{MeOH} nm (log ε): 236 (4.43), 280 (4.64), 437 (4.17), $\lambda_{max}^{MeOH+HCl}$ 237 (4.47), 283 (4.67), 438 (4.12), $\lambda_{max}^{MeOH+NaOH}$ 238 (4.33), 277 (4.67), 444 (4.33); IR ν_{max} (KBr): 3450, 2940, 1620, 1455, 1405, 1320, 1260, 1110 cm^{-1}; FAB-MS *m/z* 927 $[M+H]^+$, 949 $[M+Na]^+$.

Cosynthesis in Agar Plate Culture

Cosynthesis test was performed with modifying the procedure of KAKINUMA *et al.*[5]. The compounds speculated to be the biosynthetic intermediate were dissolved in DMSO or methanol and added to SPT agar plate at the final

concentration of 100 μg/ml. Spores of a mutant grown on an agar slant (10~14 days old) were suspended in 5 ml of sterile physiological saline solution. The spore suspension of blocked mutants to be tested (0.1 ml each) was streaked on the above SPT agar plate and incubated at 30°C for 2 weeks. Production of diffusible red pigment around the mutant colony was considered the sign of HBM production. The agar layer containing the diffusible pigment was removed from the plate, frozen at −20°C overnight and defrosted at room temperature. The pigments were extracted from the agar plate with ethyl acetate and analyzed by TLC and HPLC.

Cosynthesis in Liquid Culture

Seed medium (V-22) was inoculated with a loopful of cells on a YS agar slant and incubated at 30°C on a reciprocal shaker for 4 days. 0.5 ml of the seed culture was inoculated into 10 ml of the fermentation medium and incubated at 30°C on a reciprocal shaker. After 5 days, DMSO solution of HMP-Y1 or HMP-Y6 was added to the medium and incubated at 30°C on a reciprocal shaker for 5 days. Culture broth was extracted with ethyl acetate and analyzed by TLC and HPLC.

Identification of Bioconverted Products

Metabolites produced in cosynthesis were extracted with ethyl acetate and evaporated to dryness and the extracts were analyzed by HPLC and TLC. HPLC analysis was performed in a HPLC system 1090 with a diode array detector (Hewlett Packard) using a YMC-ODS C18 column (Yamamura Chemical Lab., 100×4.6 mm, i.d.). Temperature was 40°C, flow rate 0.7 ml/minute, solvent 0.15% KH_2PO_4 buffer (pH 3.5) - acetonitrile (85 : 15) isocratic for 10 minutes, a linear gradient to 60% acetonitrile from 10 to 25 minutes and 60% acetonitrile isocratic for 10 minutes, detection at 254 and 430 nm. TLC plate (RP18 F_{254S} Art15389, Merck) was developed with acetonitrile - distilled water (55 : 45).

Results

Isolation of Blocked Mutants

Mutagenesis of strain TP-A0121 was carried out to obtain blocked mutants which produce novel metabolites relating to the biogenesis. At the first mutation, we obtained nine hundred sixty colonies on SPT agar producing colorless or diffusible pigments, the color of which was different from the characteristic red of HBM. The isolated mutants were fermented in the MP medium and no production of HBM was confirmed by TLC. Consequently, four stable mutants, AN-0416, AN-0554, AN-0623, AN-0763 and AN-0772, were selected as HBM non-producing strains. Among these mutants, strain AN-0416, the producer of HMP-Y6, was subjected to the second NTG mutation in order to generate mutants blocked in the earlier steps of biosynthesis. Two blocked mutants, BN-Y185 and BN-Y218, were selected from four hundred colonies.

Metabolites Produced by Blocked Mutants

TLC and HPLC were employed to analyze efficiently the metabolites produced by the blocked mutants. Because of the characteristic color of HBM visible on TLC plate, the structural variation in aglycon moiety was distinguished by color spot on TLC. In addition, UV spectrum obtained on HPLC with the diode array detector was compared with the typical UV spectral pattern of HBM. The number of attached deoxysugars on aglycon was speculated from the Rf value and retention time. Glycosylated metabolites were detected by comparing Rf values before and after the methanolysis.

Strain AN-0623 accumulated the glycosylated metabolite HMP-P4 (**6**). Its aglycon HMP-P1 (**5**) arose from hibarimicinone (**2**) through the formation of the ether bridge *via* addition-elimination reaction between the two aromatic groups with the release of a methanol molecule. Four deoxysugars of **6** were the same ones found in HBM. **5** was also the major product of another mutant AN-0623. The most attractive metabolite HMP-Y6 (**3**) was obtained from the culture broth of strain AN-0416. This symmetric compound was the dimer of the west half of HBM B (**1**). Since the transformation from **3** to **1** presumably requires two oxidation steps, we speculated that **3** or HMP-Y1 (**4**) is the intermediate of HBM biosynthesis. AN-0763 and two mutants BN-Y185 and BN-Y218 derived from the strain AN-0416 accumulated only shunt metabolites. Rf values and color of the mutant metabolites are summarized in Table 1.

Screening of Converter Strains

3 or **4** were speculated to be a biosynthetic intermediate. Screening of the strains which convert **3** or **4** to **1** was conducted using cosynthesis agar method. Four possible strains were isolated from 480 non-pigment-producing mutants, among which, the strain AN-0554 was found to be the most efficient converter. This strain transformed **4** to HBM including **1**, whereas **3** was not converted. Diffusible dark-green pigment around the colony of strain AN-0554

Table 1. Hibarimicin-related compounds used in this study.

Origins	Compounds	Molecular formula	TLC[a] Rf	color	HPLC[b] Rt (min)
Parent strain					
TP-A0121	HBM B (**1**)	$C_{85}H_{112}O_{37}$	0.21	red	17.9
	HBM C	$C_{83}H_{110}O_{36}$	0.25	red	12.7
	HBM D	$C_{85}H_{112}O_{38}$	0.30	red	12.1
	Hibarimicinone (**2**)	$C_{45}H_{48}O_{21}$	0.58	red	4.2
Mutant strains					
AN-0416	HMP-Y6 (**3**)	$C_{86}H_{118}O_{36}$	0.20	yellow	21.3
	HMP-Y1 (**4**)	$C_{46}H_{54}O_{20}$	0.54	yellow	4.5
AN-0623	HMP-P1 (**5**)	$C_{44}H_{44}O_{20}$	0.66	purple	4.3
AN-0722	HMP-P4 (**6**)	$C_{68}H_{84}O_{29}$	0.40	purple	5.4
BN-Y185	HMP-M1	$C_{22}H_{20}O_8$	0.30	pale yellow	18.8
BN-Y218	HMP-M2	$C_{21}H_{18}O_6$	0.31	yellow	18.1
BN-Y218	HMP-M3	$C_{22}H_{18}O_9$	0.73	colorless	4.9
AN-0763	HMP-M4	$C_{22}H_{18}O_9$	0.51	pale brown	5.0

[a] Plate: TLC RP-18_{F254S} (Merck, Art 15389). Solvent: CH_3CN-H_2O (55:45)

[b] Column: Cosmosil 5C18-AR (nacalai tesque, 250 mm × 4.6 mm, i.d.)
Solvent: CH_3CN-0.15%KH_2PO_4 (pH 3.5) (50:50). Temperature: 30°C.
Flow rate: 0.7 ml/min. Detection: 254 nm.

indicate the production of HBM on the agar medium containing **4** because the color of HBM is pH-dependent, showing red in acidic and green in basic conditions (Fig. 1).

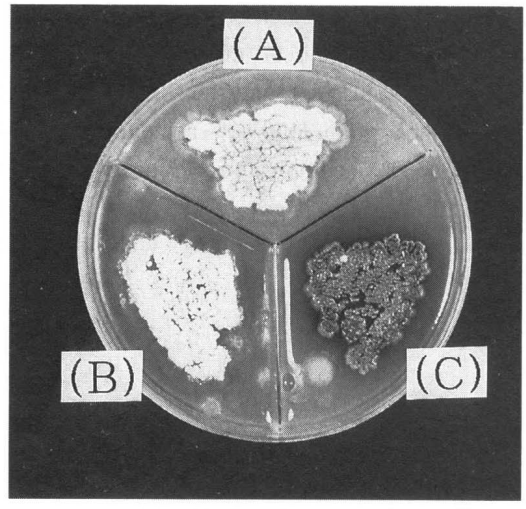
Bioconversion of HMP-Y1 and Hibarimicinone in Liquid Medium

Typical result of the bioconversion of **4** by the strain AN-0554 is shown in Table 2 and Fig. 2. After 48 hours, **4** was converted to **1** (9.8%) and HBM C and D (19.3%) along with trace amounts of minor components. **2** was also converted to **1** (11.4%), HBM C and D (33.6%) and minor components (30.6%). These results established that HBM was biosynthesized *via* **4** and **2**. Because of the lability in liquid culture, about fifty percent of **4** was transformed to an unknown compound in 96 hours or **2** spontaneously changed into **5** before the glycosylation took place.

Conversion of ^{13}C-Labeled HMP-Y1 to HBM B

To confirm the above result, isotope-labeled **4** was prepared and tested in the cosynthesis experiment. The blocked mutant AN-0416, the HMP-Y6 producer, was

Fig. 1. Cosynthesis of hibarimicin from HMP-Y1 by mutant strain AN-0554.

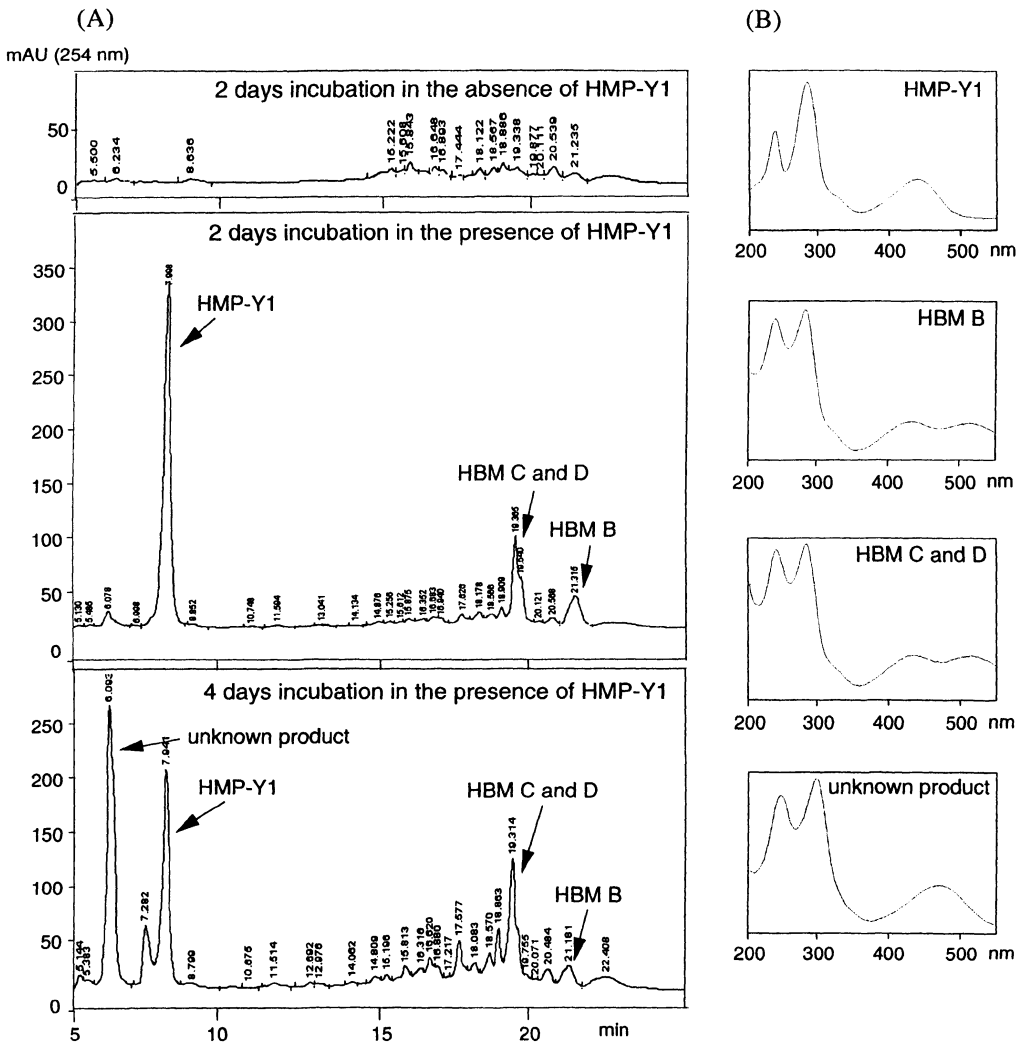


Strain AN-0554 grown at 30°C for 14 days on SPT agar in the absence (A) or presence of HMP-Y6 (B) or HMP-Y1 (C).

Table 2. Bioconversion of HMP-Y1 and hibarimicinone by growing culture of blocked mutant AN-0554.

Substrate	Incubation time (h)	Ratio of products (mol%)			Unreacted substrate (mol%)	Conversion yield (%)
		HBM B	HBM C/D	Other HBMs		
None	48	–	–	–	–	–
HMP-Y1	0	–	–	–	100	0
	48	9.8	19.3	14.0	56.9	100
	96	5.0	19.8	20.2	42.4	78.1
Hibarimicinone	0	–	–	–	100	0
	48	11.4	33.1	30.6	24.8	99.9

Fig. 2. Conversion of HMP-Y1 to hibarimicins by mutant strain AN-0554.



(A) HPLC chromatograms of EtOAc extract from the culture broth of mutant strain AN-0554 in the absence (control) or presence of HMP-Y1.
(B) UV-vis spectra of the peaks corresponding to HMP-Y1, HBM B, HBM C and D, and unknown product.

Table 3. Relative ^{13}C peak areas in aglycon part of hibarimicin B obtained from HMP-Y1 labeled with 1-^{13}C-acetate by cosynthesis.

Position	δ (ppm)	ratio	Position	δ (ppm)	ratio
1	152.04	2.2	1'	187.83	2.1
2	107.83	0.8	2'	125.32	0.8
3	153.26	2.1	3'	158.34	2.2
4	138.47	1.0	4'	184.77	1.2
5	135.41	2.2	5'	116.22	2.2
6	111.93	0.8	6'	150.87	0.9
7	139.33	2.0	7'	147.92	2.2
8	27.73	1.0	8'	67.83	1.1
9	44.43	2.3	9'	55.67	2.2
10	76.20	*	10'	*	*
11	70.66	2.3	11'	75.24	1.9
12	86.51	0.7	12'	85.32	0.9
13	79.38	2.3	13'	82.63	2.2
14	77.13	*	14'	85.66	0.9
15	203.38	2.1	15'	195.52	2.2
16	110.52	0.9	16'	124.78	1.1
17	164.31	2.2	17'	157.18	2.1
18	108.32	0.8	18'	112.93	0.9
19	37.05	0.8	19'	34.24	1.0
20	18.00	2.2	20'	16.57	2.3
21	15.13	1.0	21'	14.90	1.0
3-OMe	60.88	1.0**	3'-OMe	60.77	1.0**
4-OMe	61.12	1.0**			

* not determined because of peak overlapping.

** defined as 1.0.

cultured with feeding of sodium [1-^{13}C] acetate. After the chromatographic purification, the ^{13}C-labeled **3** was treated with methanolic hydrochloride to afford ^{13}C-labeled **4**. This labeled compound was converted to HBMs by strain AN-0554 under the condition of cosynthesis agar method. 10 mg of **1** was obtained from 500 mg of labeled **4**. ^{13}C NMR of **1** demonstrated that the intensities of the carbon signals derived from the carboxyl carbon of [1-^{13}C] acetate were 2.2 times larger than others in average (Table 3). This result confirmed **4** to be the intermediate of HBM biosynthesis.

Bioconversion of Other Mutant Metabolites

Metabolites from the blocked mutants were classified into two groups, monomeric and dimeric metabolites. In the cosynthesis experiments, these metabolites were supplied in the agar medium as a substrate for the converter strains. The monomeric metabolites, HMP-M1, M2 M3 and M4, were proved to be the shunt products which were not converted to HBM. **5** and **6** were glycosylated by blocked mutants AN-0554 and BN-Y185 which lost the capability of producing dimerized aglycon.

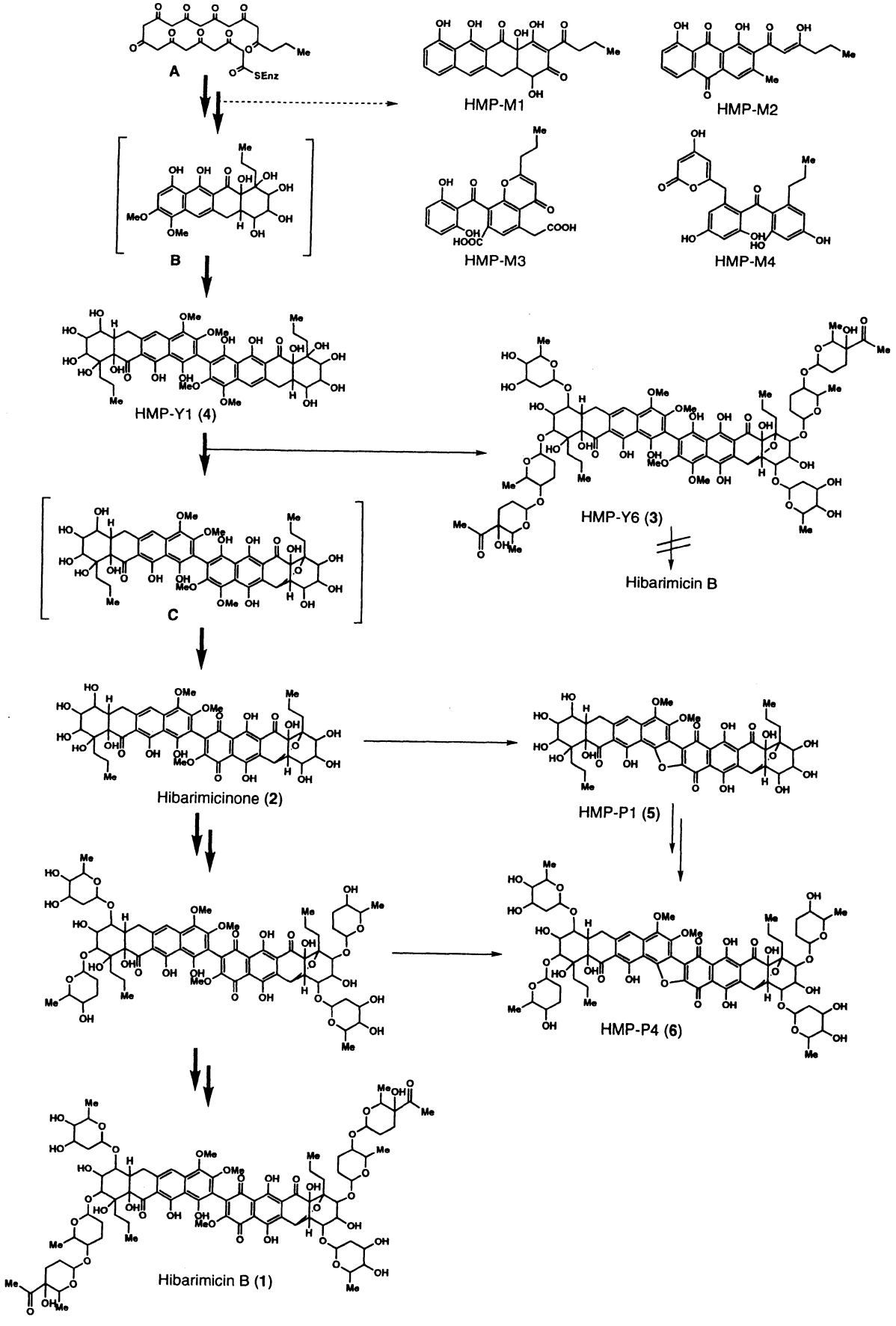
Discussion

On the basis of the experimental findings described above, biosynthetic pathway of hibarimicin is proposed as shown in Fig. 3. In the former half of the biosynthesis, the polyketide intermediate **A** arising from the eleven malonates is converted *via* cyclization and skeltal rearrangement to **B**, a presumed precursor for the oxidative coupling. Two monomer units are then coupled to yield the symmetric intermediate **4**, the glycoside of which, **3**, was isolated as the major product of mutant AN-0416. Mutagenesis of this strain resulted in the isolation of two mutants, BN-Y185 and BN-Y218, blocked in the steps involved with the biosynthesis of monomer unit. These mutants produced shunt metabolites, HMP-M1, M2 and M3. Their structures suggest that the cyclization from the polyketide chain takes place stepwise and generates a tetracyclic structure in a regular manner, followed by a carbon–carbon bond cleavage and formation in D-ring (The ring was conventionally denoted A, B, C and D from the left to the right). HMP-M4 was produced through the entirely different cyclization pattern from hibarimicins, suggesting the mutagenesis took place in the cyclase gene of the strain AN-0763.

In the next step, **4** is transformed into **2** by the oxidative cyclization of the ether ring between C-8′ and C-13′ and the following oxidation of A-ring to quinone. We identified a compound like **C** in the metabolites of strain AN-0416 showing the identical UV spectrum with that of **4** and the presence of the ether bridge at C-8′ though the full NMR assignment was not established due to the instability of the compound. The result that **3** was not converted to **1** by the converter strains definitely supports the idea that the formation of **2** is completed prior to the glycosylation in HBM biosynthesis. Accumulation of **3** was observed in the late stage of fermentation of the parent strain, indicating the feedback inhibition in the HBM production.

Addition of two amicetoses and two digitoxoses to **2** occurs immediately when it is generated because no accumulation of **2** was observed in fermentation broth. The glycosilation pattern on the amicetoses gives the structural

Fig. 3. Proposed biosynthetic pathway of hibarimicins.



variation in HBM components. Cosynthesis experiments revealed that **5** was glycosylated as well as **2** to yield higher glycosylated metabolites. These results indicate that HMP-P type metabolites can be produced *via* **5** and **2** and that the activity of glycosyltransferases is not effected by the slight structural modification in the aglycon moiety.

The aglycon of HBM spontaneously changes into the aglycon of HMP-P type products. This non-enzymic transformation occurs under physiological and non-physiological conditions with HBM components, suggesting that HMP-P type metabolite can be defined as an artifact rather than a natural product. The similar chemical transformation can be seen in the fungal metabolites: rubrosulphin arises from viomellein and viopurpurin from the hydroquinone-form of xanthomegnin presumably. It is not clear whether **6** is generated by the glycosilation of **5**, or by the cyclization of the corresponding HBM component, or by the both routes.

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