Interconversion among 16-Membered Macrolide Antibiotics Belonging to Leucomycin-Maridomycin Group*

Masaru SUZUKI, Tsutomu TAKAMAKI, Ken-ichiro MIYAGAWA, Hideo ONO, Eiji HIGASHIDE and Minoru UCHIDA

Microbiological Research Laboratories, Central Research Division,
Takeda Chemical Industries, Ltd., Japan

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To clarify the biosynthetic pathway of maridomycin, the biosynthetic relation among 16-membered macrolide antibiotics belonging to leucomycin-maridomycin group was examined with intact cells or growing cultures of *Streptomyces hygroscopicus* No. B-5050-HA and its mutants.

Carbomycin A, B and leucomycin A₃ were converted to maridomycin II in the pH range of 5.5~7.5 with an optimum between 6.0~6.5. But, maridomycin II was not converted to leucomycin A₃ or carbomycin B. These findings were confirmed by culture experiment. Leucomycin A₃, carbomycin A and B added to the culture at 48 hr were almost completely converted to maridomycin II.

On the basis of these facts, we propose a biosynthetic relation among leucomycin A₃, carbomycin A, carbomycin B and maridomycin II.

Recently, much attention has been paid to the biosynthesis and the interconversion of 16-membered macrolide antibiotics such as carbomycin A,^{1~3)} platenomycin,^{4~7)} leucomycin,^{8,9)} tylosin,^{10,11)} maridomycin,^{12~17)} etc. The 16-membered macrolide antibiotics can be classified into two major groups depending on the difference in substituents at the 4-, 12-, 14-, and 15-positions of the lacton ring, as follows: (1) the leucomycin-maridomycin group containing carbomycin A and B, platenomycin, SF-837,¹⁸⁾ etc., and, (2) the tylosin-cirramycin¹⁹⁾ group containing rosamicin,²⁰⁾ relomycin,¹¹⁾ etc.

The leucomycin-maridomycin group is further divided into four groups: (1) leucomycin group containing platenomycin, SF-837, etc., (2) carbomycin A group containing delta-mycin,²⁵⁾ (3) carbomycin B group containing YL-704W,²⁶⁾ etc., (4) maridomycin group containing YL-704C,²⁶⁾ etc. These four groups differ from each other in the structures at C-9, and C-12, C-13 positions of the aglycone, as illustrated in Fig. 1.

In the course of a fermentation study on

FIG. 1. Structures of Maridomycin II, Leucomycin A₃, Carbomycin A and Carbomycin B.

maridomycin, it was found that some mutants derived from a maridomycin producer, *Streptomyces hygroscopicus* No. B-5050-HA, produced a small amount of the other 16-membered macrolide antibiotics such as leucomycins, carbomycin A and B, as by-products along with maridomycin. In the fermentation by these mutants, the appearance of leuco-

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mycins, carbomycin A and B was observed mainly in the late phase of the fermentation. This observation suggested the two possibilities of microbiological interconversion among those antibiotics mentioned above, that is, (1) leucomycins, carbomycin A and B once produced were converted microbiologically to maridomycin in the early phase but not the late phase of the fermentation, or (2) maridomycin once produced was converted to leucomycins, carbomycin A and B in the late phase of the fermentation.

It would be great interest, therefore, to make clear the biosynthetic relation among 16-membered macrolide antibiotics belonging to leucomycin-maridomycin group because of their structural similarity. A review on the structural relation of 16-membered macrolide antibiotics has been published by Omura²¹⁾ *et al.*, but microbiological interconversion of these macrolides has hardly been dealt with.

In this paper, we report the microbiological interconversion among 16-membered macrolide antibiotics belonging to leucomycin-maridomycin group.

MATERIALS AND METHODS

Microorganisms. *Streptomyces hygroscopicus* No. B-5050-HA, a maridomycin producer, and its mutants were used.

Media and cultivation. A spore suspension (0.5 ml) of *S. hygroscopicus* No. B-5050-HA or its mutants was used to inoculate 200-ml shake flask containing 20 ml of seed medium consisting of 30 g of glucose, 30 g of corn steep liquor, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 3 g of CaCO_3 per liter (pH 6.5). The inoculum was incubated at 28°C for 48 hr on a rotary shaker. One milliliter of the culture broth thus obtained was transferred into a 200-ml shake flask containing 20 ml of the medium consisting of 80 g of glucose, 30 g of corn steep liquor, 10 g of meat extract, 0.5 g of NH_4Cl , 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 g of CaCO_3 per liter (pH 6.5). Incubation was carried out on the rotary shaker at 28°C for 40 hr. Cells were harvested from the culture broth by centrifugation and washed once with sterile water, and then used as intact cells. In the fermentation experiment, 1 ml of the seed culture described above was transferred into a 200-ml shake flask containing 16 ml of the medium consisting of 120 g of glucose, 20 g of Proflo, 5 g of soy bean meal, 5 g of

NH_4Cl , 1.2 g of K_2HPO_4 , 0.5 g of $\text{MnSO}_4 \cdot 4\text{-}6\text{H}_2\text{O}$, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g of DL-methionine, 5 g of DL-alanine and 12 g of CaCO_3 per liter (pH 6.5), and was incubated at 26°C for 144 hr on the rotary shaker.

Analytical methods. Maridomycin was measured by the colorimetric method reported by Uchida *et al.*²²⁾ Leucomycin, carbomycin A and B were assayed by UV absorption at 231 nm ($E_{1\%}^{1\text{cm}}$ 350), 238 nm ($E_{1\%}^{1\text{cm}}$ 185) and 278 nm ($E_{1\%}^{1\text{cm}}$ 276), respectively. Bioautography or high speed liquid chromatography was also employed, when it is necessary. Total potency of 16-membered macrolides was assayed by bioassay method using *Bacillus subtilis* PCI 219.

Chemicals. Maridomycin II was purified by partition or adsorption chromatography from the maridomycin complex. Leucomycin A₃ was extracted from commercial preparation by the method of Suzuki *et al.*²³⁾ Carbomycin A and B were prepared from maridomycin II by the method of Muroi²⁴⁾ *et al.*

RESULTS

Interconversion of the 16-membered macrolide antibiotics belonging to the leucomycin-maridomycin group

To determine whether leucomycin A₃, carbomycin A, carbomycin B and maridomycin II were convertible to each other, the intact cells of *Streptomyces hygroscopicus* No. B-5050-HA and its mutant M-1382, which lacked the enzyme for the reduction at C-9 position of aglycone, were incubated with or without macrolides described above. As shown in Table I, carbomycin A was completely converted to maridomycin II with the intact cells of maridomycin producer (strain HA). Carbomycin B was converted to leucomycin A₃ and maridomycin II. The maridomycin II formed from carbomycin B might be synthesized *via* leucomycin A₃, because leucomycin A₃ was easily converted to maridomycin II (carbomycin B → leucomycin A₃ → maridomycin II). The other route, that is, carbomycin B → carbomycin A → maridomycin II is also possible, because carbomycin B was converted to carbomycin A with the intact cells of strain M-1382. Leucomycin A₃ and maridomycin II were converted to carbomycin B and carbo-

TABLE I. INTERCONVERSION AMONG LEUCOMYCIN A₃, CARBOMYCIN A, CARBOMYCIN B AND MARIDOMYCIN II BY INTACT CELLS

The reaction mixture contained 2.50 μ moles of substrate and 100 mg of washed cells (as dry weight) in a total volume of 5.0 ml of 0.2 M phosphate (pH 6.5) or Tris-maleate buffer (pH 8.5), and was incubated at 30°C with shaking.

Cells used ^{a)}	Substrate ^{b)}	Reaction condition		Reaction products (μ moles/ml)			
		pH	Time (hr)	LM A ₃	CM A	CM B	MDM II
No. B-5050-HA	LM A ₃	6.5	4.0	—	ND	ND	0.37
	LM A ₃	8.5	4.0	—	ND	0.10	0.01
	CM A	6.5	1.0	ND	—	ND	0.50
	CM B	6.5	1.0	0.19	ND	—	0.30
	MDM II	6.5	4.0	ND	ND	ND	—
	MDM II	8.5	4.0	ND	0.11	ND	—
	None	6.5	1.0	ND	ND	ND	0.01
	None	6.5	4.0	ND	ND	ND	0.02
	None	8.5	4.0	ND	ND	ND	0.01
No. B-5050-M-1382	CM A	6.5	18.0	ND	—	ND	ND
	CM B	6.5	18.0	ND	0.38	—	ND
	None	6.5	18.0	ND	0.01	ND	ND

^{a)} The cells were harvested after cultivation for 40 hr.

^{b)} Each substrate was dissolved in the aqueous solution with succinic acid.

ND: Not detectable.

Abbreviations: LM A₃, leucomycin A₃; CM A, carbomycin A; CM B, carbomycin B; MDM II, maridomycin II.

mycin A, respectively. The optimum pH of the reduction at C-9 ($\text{O}=\text{C}\langle\rightarrow\text{OH}-\text{C}\langle\text{H}$) was 6.0~6.5, and that of the reverse reaction ($\text{HO}-\text{C}\langle\text{H}\rightarrow\text{O}=\text{C}\langle$) was 8.0~8.5. The conversion of leucomycin A₃ to maridomycin II, and carbomycin B to carbomycin A was observed with the intact cells of strain HA and those of strain M-1382, respectively. The optimum pH of the epoxidation at C-12 and 13 ($-\text{HC}=\text{CH}-\rightarrow-\text{HC}\langle\text{O}\rangle\text{CH}-$) was 6.0~6.5.

From the data obtained above, the interconversion among these 16-membered macrolides with the intact cells is summarized as follows;

Relation between conversion activities and the accumulation of leucomycin-maridomycin group macrolides

In the course of strain development, we have

succeeded in the isolation of a predominantly leucomycin-accumulating mutant (A23-6), a carbomycin A and B-accumulating mutant (M-1382), and a both leucomycin and maridomycin-accumulating mutant (B-11M). The conversion activities of these mutants are summarized in Table II. As seen in Table II, the carbomycin A and B-accumulating mutant, M-1382, was found to lack the reductase of the C-9 position. Leucomycin-accumulating mutant, A23-6, had strong reductase activity of C-9, but the epoxidation activity of C-12 and 13 was hardly observed. Both leucomycin and maridomycin producer, B-11M, had strong activity of the reduction of C-9 and moderate activity of the epoxidation of C-12 and 13.

From these data, it may be concluded that maridomycin II is synthesized *via* two pathways in the fermentation, that is, (i) carbomycin B $\rightarrow$ leucomycin A₃ $\rightarrow$ maridomycin II, or (ii) carbomycin B $\rightarrow$ carbomycin A $\rightarrow$ maridomycin II.

TABLE II. COMPARISON OF CONVERSION ACTIVITY BY THE WASHED CELLS OF SEVERAL MUTANTS

Cells used	Ratio of bioconverted product (%)					
	CM B→ CM A	CM A→ CM B	CM B→ LM A ₃	CM A→ MDM II	LM A ₃ → MDM II	MDM II→ LM A ₃
A23-6	ND	ND	100	100	ND	ND
M-1382	75	ND	ND	ND	ND	ND
B-11M	ND	ND	80	100	10	ND
No. BH 5050-HA	ND	ND	50 ^a	100	50	ND

The experimental methods are the same as described in the footnote of Table I. The reaction pH is 6.5.

^a One half of LM A₃ formed is considered to be converted to MDM II, because no CM B remains.

TABLE III. COMPARISON OF CONVERSION ACTIVITY BY 48 HR CULTURED-CELLS AND 120 HR CULTURED-CELLS

Cells used	Cultured time (hr)	Ratio of bioconverted product (%)					
		CM B→ CM A	CM A→ CM B	CM B→ LM A ₃	CM A→ MDM II	LM A ₃ → MDM II	MDM II→ LM A ₃
No. BH 5050	48	ND	ND	50 ^a	100	50	ND
-HA	120	ND	ND	90	98	5	ND
M-1382	48	75	ND	ND	ND	ND	ND
	120	5	ND	ND	ND	ND	ND

The experimental methods and ^a mark are the same as described in the footnote of Table II.

Changes of conversion activities during the fermentation

Effect of cell age on the conversion activities was examined with 48-hr cultured cells and 120-hr cultured cells. Table III shows the results obtained. The epoxidation activities of leucomycin A₃→maridomycin II or carbomycin B→carbomycin A were observed with 48 hr-cultured cells, but hardly observed with 120 hr-cultured cells. The intact cells of M-1382 had not the epoxidation activity of leucomycin A₃→maridomycin II. So, it may be considered that the epoxidase for leucomycin A₃→maridomycin II is not the same that for carbomycin B→carbomycin A. The reduction activity of C-9 was constant during the course of fermentation.

To confirm the conversion of these 16-membered macrolides, fermentation experiments were carried out. Table IV shows the results. The addition of leucomycin A₃, carbomycin A or B to the culture at 48 hr enhanced the production of maridomycin II. The macrolides added to the culture were almost completely converted to maridomycin

TABLE IV. CONVERSION OF 16 MEMBERED MACROLIDES IN THE FERMENTATION BY *S. hygroscopicus* No. B-5050-HA

Macrolide added	Addition time (hr)	Marido-mycin produced (μg/ml)	Conversion ratio (%)
LM A ₃	48	6750	97.5
	120	4900	5.0
CM B	48	6680	94.0
	120	4820	1.0
CM A	48	6800	100.0
	120	6720	96.0
MDM II	48	6820	—
	120	6800	—
None	—	4800	—

Each macrolide was added to the fermentation medium at the concentration of 2000 μg/ml.

II. The conversion of maridomycin II to other macrolides was hardly observed when it was added to the culture. However, when the additions were made at 120 hr, only carbomycin A was converted to maridomycin II. The addition of carbomycin B to the culture of strain M-1382 at 48 hr enhanced the production of carbomycin A. The conversion of

carbomycin A to carbomycin B, when the former was added to the culture at 48 hr, was not observed. Carbomycin B added to the culture at 120 hr was not converted to carbomycin A.

These findings agree with the facts observed with the intact cells.

DISCUSSION

Omura *et al.*, have investigated the origin of the skeletal carbons in the lactone ring of the 16-membered macrolide antibiotics, and clarified that the aglycone of leucomycin is derived from 5 acetate, 1 propionate, 1 butyrate and 1 unknown precursor corresponding to two carbons. Furumai *et al.*⁴⁻⁷⁾, have proposed the biosynthetic pathway of platenomycins, a

family of the 16-membered macrolide antibiotics belonging to leucomycin-maridomycin group, and presented the three biosynthetic routes from platenolide to platenomycin. However, up to now, nobody attempted to clarify the biosynthetic interrelation among the 16-membered macrolide antibiotics belonging to leucomycin-maridomycin group. So, we attempted to make clear the relation among these four macrolides, that is, leucomycin A₃, carbomycin A, carbomycin B and maridomycin II. The experimental results described in this paper lead to conclusion that maridomycin II is biosynthesized as follows;

The stages indicated by broken lines are hypothetical

FIG. 2. Tentative Scheme to Illustrate the Possible Course of Maridomycin II Biosynthesis.

On the basis of the experimental results of this paper and the reports of Furumai *et al.*, and Omura *et al.*, the biosynthetic pathway of the 16-membered macrolide antibiotics belonging to leucomycin-maridomycin group can be drawn as shown in Fig. 2. As is evident from Fig. 2, maridomycin is considered to be the end product of the macrolides described above. Until now, many strains such as *S. halstedii*, *S. kitasatoensis*, *S. platenensis*, *S. hygroscopicus*, *etc.*, have been reported as the 16-membered macrolide antibiotics producers. *S. halstedii* (carbomycin A and B producer) is considered to be a blocked mutant of C-9 reductase of the aglycon. *S. kitasatoensis* (leucomycin producer) is considered to be a blocked mutant of C-12 and -13 epoxidase. *S. hygroscopicus* No. B-5050-HA (maridomycin producer) is considered to be a strain having strong C-9 reductase and C-12, -13 epoxidase. Therefore, *S. hygroscopicus* No. B-5050-HA produces a large amount of maridomycin.

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