Microbial Transformation of Manool and (12Z)-Labda-8(17),12,14-triene by *Rhodococcus erythropolis* JTS-131

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Rhodococcus erythropolis JTS-131, a *cis*-abienol and sclareol transformable bacterium, converted manool and accumulated manoyl oxide in the culture broth. In the presence of metabolic inhibitors, 13 β -hydroxylabda-8(17),14-dien-18-oic acid and its methylester (a new compound) were accumulated as the transformation products. This strain also transformed (12Z)-labda-8(17),12,14-triene and accumulated (12Z)-labda-8(17),12,14-trien-18-al in the culture broth. In the presence of metabolic inhibitors, two compounds, (12Z)-labda-8(17),12,14-trien-18-ol and 4,18,19-trinor-3,4-seco-5-oxo-(12Z)-labda-8(17),12,14-trien-3-oic acid were accumulated. Based on the experiments on the degradation sequence, transformation pathways for these two labdanes by the bacterium are proposed.

In the previous paper,¹⁾ we reported the microbial transformation of *cis*-abienol [(12Z)-labda-12,14-diene] and sclareol [labd-14-en-13 β -ol] by *Rhodococcus erythropolis* JTS-131. These two labdanes were oxidized at the C-18 methyl moiety which resulted in hydroxymethyl, carboxylic acid and methylester derivatives successively. And it was considered that the biotransformation was determined by the plasmid (pCA4) in this strain.²⁾

In other papers,^{3,4)} we reported that *Nocardia restricta* JTS-162 also transformed *cis*-abienol and sclareol. With this strain, C-8 and C-17 positions of sclareol and *cis*-abienol were firstly dehydrated which resulted in labda-8(17),14-dien-13 β -ol (manool) and (12Z)-labda-8(17),12,14-triene successively. And then the C-18 methyl of these dehydrated products was oxidized stepwisely. In the transformation of *cis*-abienol, the A-ring was split after the oxidation of the C-18 methyl. It was also considered that this C-18 methyl oxidation and A-ring splitting were determined by the plasmid (pCA1) in this strain.⁵⁾

In this paper, we describe the transformation of manool and (12Z)-labda-8(17),12,14-triene, dehydrated products from sclareol and *cis*-abienol with *N. restricta* JTS-

162, respectively, by *R. erythropolis* JTS-131, together with identification of the transformation products. Possible metabolic pathways including C-18 oxidation and A-ring splitting of these two dehydrated labdanes by this strain are also reported.

MATERIALS AND METHODS

Microorganisms. *Rhodococcus erythropolis* JTS-131¹⁾ and *Nocardia restricta* JTS-162,³⁾ isolated from soil as *cis*-abienol-transformable bacteria, were used in this study. Cured strain JTS-13101²⁾ obtained from *R. erythropolis* JTS-131 was also used.

Chemicals. Manool was provided by Takasago Perfumery Co., Tokyo. (12Z)-Labda-8(17),12,14-triene was obtained as a transformation product from *cis*-abienol with *N. restricta* JTS-131.³⁾ 13 β -Hydroxylabda-8(17),14-dien-18-al (compound 8) was obtained as a transformation product from sclareol with *N. restricta* JTS-162.⁴⁾ All other reagents were commercial products of the highest quality.

Media. Medium-A, containing (g/liter) (NH₄)₂SO₄ 2.0, K₂HPO₄ 2.0, MgSO₄·7H₂O 0.2, CaCl₂·2H₂O 0.2, and FeSO₄·7H₂O 0.01, and adjusted to pH 7.2, was used as a transformation basal medium. 1-Agar, containing (g/liter) Bacto-tryptone (Difco) 10.0, Bacto-yeast extract (Difco) 5.0, NaCl 5.0, glucose 1.0 and agar 15.0, and adjusted to pH 7.0, was used as a stock medium.

Control of microbial transformation of manool and (12Z)-labda-8(17),12,14-triene with some metabolic inhibitors. A test tube containing 5 ml of medium-A, 10 mg of manool or (12Z)-labda-8(17),12,14-triene, 0.5 mg of Tween-60 and the bacterium, *R. erythropolis* JTS-131, at the concentration of 10^8 /ml was incubated at 28°C with mechanical shaking. After 12 hr incubation, one of the inhibitors in a given amount as listed in Table I (A and B) was added, and incubation was carried on for a further 48 hr. The resulting culture was acidified to pH 3.0 and extracted with ethyl acetate. The extract was analyzed by TLC and GC-MS.

Microbial transformation and isolation of transformation products. A loop of the cells grown on a L-agar slant was transferred to a 500 ml Erlenmeyer flask containing 100 ml of medium-A, 100 mg of manool or (12Z)-labda-8(17),12,14-triene, and 10 mg of Tween-60. The flask was incubated at 28°C on a rotary shaker at 210 rpm. After 12 hr incubation, ethylenediaminetetraacetic acid (EDTA) was added to the flask, which was incubated for a further 48 hr. At the end of the fermentation period, the broth was acidified with 1 N-HCl to pH 3.0 and extracted with ethyl acetate. The ethyl acetate solution was washed with 5% NaHCO₃ and then evaporated to dryness. The extract (about 1.0 g) was applied to a silicic acid column (100 mesh; Malinckrodt, 30 g, 25 mm diameter). The column was eluted with 100 ml portions of *n*-hexane containing 30, 40, 50 and 60% ethyl acetate. Each eluate was evaporated *in vacuo* and then further purified by preparative high-performance liquid chromatography (HPLC). The following were employed: Waters ALC high-performance liquid chromatograph (RI detector), μ -Polacil (4 by 300 mm), and *n*-hexane-ethyl acetate (8:2, 7:3, 6:4 and 5:5, v/v) at 1 ml/min.

Microbial transformation of conversion products. Ten mg of each compound (compounds 1, 2, 3, 4, 5, 6, 7 or 8) was added to 5 ml of medium-A in a test tube containing bacterium JTS-131 (about 10^8 /ml) and 0.5 mg of Tween-60. After 12 hr incubation at 28°C with mechanical shaking, EDTA was added at 10 mM to the test tube, which was incubated for a further 48 hr. The resulting culture was extracted with ethyl acetate. The extract was analyzed by TLC and GC-MS. The transformation of compound 2 with *N. restricta* JTS-162 was done under the same conditions as described above.

In the same way, each of the conversion products (compounds 1, 2, 3, 4, 5, 6, 7 or 8) was converted with the cured strain JTS-13101, obtained from JTS-131 by mitomycin C treatment.²⁾

Thin layer chromatography (TLC). The sample solution was spotted on a Silica Gel G plate (Merck) and developed with *n*-hexane-ethyl acetate (5:5 or 7:3, v/v). After evaporation of the organic solvents, compounds were detected by spraying with *p*-anisaldehyde (5%) and H₂SO₄

(5%) in 90% ethanol and heating at 120°C for 3 min.

Gas liquid chromatography (GLC). GLC was performed under the following conditions, using a Shimadzu-GC-4CM gas chromatograph equipped with a flame ionization detector. The column was a glass tube (1 m $\times$ 3.2 mm i.d.) packed with 10% Silicone OV-101 on Chromosorb W (AW, DMCS, 60~80 mesh) or 2% Silicone XE-60 on Chromosorb W (AW, DMCS, 60~80 mesh). The temperature was usually 230°C (isothermal); the carrier gas was He at 60 ml/min.

Spectrometry. Mass spectra were obtained with a Hitachi M-80 GC mass spectrometer, and IR spectra with a Jasco A-3 spectrophotometer. ¹³C-NMR (CMR) spectra were obtained with a JEOL-FX-100 NMR spectrometer, and ¹H-NMR (PMR) spectra with a JEOL-PS-100 NMR spectrometer (100 MHz).

Chemical preparation of labda-8(17),14-dien-13 β , 18-diol (compound 7). Compound 3 (100 mg) dissolved in dry dioxane (10 ml) was added to a suspension of LiAlH₄ (40 mg) in dry dioxane (10 ml), and then this solution was boiled under reflux for 1 hr. Chromatography of the resulting mixture on an alumina column, eluted with benzene-ether (1:1, v/v), gave the pure compound (mp. 125~126°C), which was confirmed to be compound 7 by TLC and GC-MS.

RESULTS

Microbial transformation of manool

R. erythropolis JTS-131 transformed manool to compound 1 in the absence of metabolic inhibitors (Fig. 1). The yield of compound 1 reached the maximum at 72 hr fermentation and then decreased gradually. With the addition of metabolic inhibitors, two com-

FIG. 1. Time Course of Biotransformation of Manool by *R. erythropolis* JTS-131.

Symbols: $\square$ --- $\square$, cells; $\circ$ — $\circ$, manool; $\triangle$ — $\triangle$, compound 1.

TABLE I. EFFECT OF INHIBITORS ON MICROBIAL TRANSFORMATION OF MANOOL AND (12Z)-LABDA-8(17),12,14-TRIENE

Ten mg of each substrate was added to 5 ml of medium-A containing JTS-131 (about 10^8 cells/ml). After 12 hr incubation, one of the inhibitors was added and incubation was carried on for a further 48 hr. Each inhibitor was used at the concentration of 1 or 10 mM.

A: Manool

Products	Control	EDTA	NaF	NaHSO ₃	Sodium arsenate	α,α' -Dipyridyl	Iodoacetate	<i>o</i> -Phenanthroline	8-Hydroxyquinoline	Thiourea
Compound 1	+	+	+	+	+	+	+	+	+	+
Compound 2	—	+	+	—	—	—	—	—	—	—
Compound 3	—	+	+	+	+	—	—	—	—	—

B: (12Z)-labda-8(17),12,14-triene

Products	Control	EDTA	α,α' -Dipyridyl	Iodoacetate	NaHSO ₃	<i>o</i> -Phenanthroline	8-Hydroxyquinoline	NaF	Thiourea	Sodium arsenate
Compound 4	+	+	+	+	+	+	+	+	+	+
Compound 5	—	+	+	+	—	—	—	—	—	—
Compound 6	—	+	+	+	+	+	+	—	—	—

pounds were accumulated in the culture broth (Table I, A). The addition of EDTA and NaF caused the accumulation of compounds **2** and **3**. The addition of NaHSO₃ and sodium arcenate only caused the accumulation of compound **3**.

FIG. 2. Time Course of Biotransformation of (12Z)-Labda-8(17),12,14-triene by *R. erythropolis* JTS-131.

Symbols: □---□, cells; ●—●, (12Z)-labda-8(17),12,14-triene; ▲—▲, compound **4**.

Microbial transformation of (12Z)-labda-8(17),12,14-triene

R. erythropolis JTS-131 also transformed (12Z)-labda-8(17),12,14-triene and accumulated compound **4** in the culture broth in the absence of metabolic inhibitors (Fig. 2). The yield of compound **4** reached the maximum at 60 hr fermentation and then decreased gradually. The addition of EDTA, α,α' -dipyridyl and iodoacetate caused the accumulation of compounds **5** and **6** (Table I, B). The addition of NaHSO₃, *o*-phenanthroline and 8-hydroxyquinoline only caused the accumulation of compound **6** in the culture broth.

Isolation and identification of transformation products

The transformation products were isolated by silicic acid column chromatography and HPLC. Conclusive information on these products was obtained from IR, CMR, PMR and mass spectra according to the methods de-

TABLE II. CARBON-13 CHEMICAL SHIFTS AND ASSIGNMENTS FOR COMPOUNDS **1**, **2**, **3**, **4**, **5** AND **6**^a

	Manool	Compound						(12Z)-Labda-8(17),-12,14-triene
		1	2	3	4	5	6	
C-1	38.9 t	38.9 t	38.0 t**	38.0 t*	37.5 t*	38.8 t*	33.3 t	39.3 t
C-2	19.1 t	18.4 t	19.3 t*	18.4 t	17.8 t	18.8 t	30.5 t	19.5 t
C-3	42.1 t	42.0 t	38.8 t**	37.9 t*	32.5 t	35.5 t	178.6 s	42.2 t
C-4	33.2 s	33.1 s	47.9 s	47.7 s	49.2 s	38.0 s	—	33.5 s
C-5	55.5 d	56.3 d	50.4 d	49.8 d	47.4 d	48.2 d	214.0 s	55.5 d
C-6	24.2 t	19.8 t	27.5 t	26.9 t*	26.4 t	24.0 t	28.7 t	24.3 t
C-7	38.2 t	43.1 t	38.6 t**	37.0 t	38.1 t*	38.0 t*	38.0 t	38.2 t
C-8	148.4 s	74.6 s	148.9 s	147.8 s	147.1 s	148.0 s	144.1 s	148.2 s
C-9	57.2 d	55.7d	58.0 d	57.2 d	57.2 d	57.3 d	54.8 d	57.4 d
C-10	39.7 s	36.8 s	39.8 s	39.1 s	38.4 s	39.4 s	52.0 s	39.6 s
C-11	17.6 t	15.4 t	18.5 t*	17.5 t	22.0 t	22.0 t	25.8 t	22.1 t
C-12	41.3 t	35.7 t	42.5 t	41.2 t	133.7 d	133.9 d	133.4 d	133.9 d
C-13	73.3 s	72.8 s	73.1 s	73.4 s	131.7 s	131.5 s	133.0 s	131.4 s
C-14	145.0 d	147.7 d	147.3 d	145.1 d	131.0 d	131.6 d	128.0 d	131.6 d
C-15	111.2 t	109.9 t	111.5 t	111.5 t	111.3 t	113.0 t	114.1 t	113.0 t
C-16	27.8 q	28.4 q	29.1 q	28.0 q	19.7 q	19.7 q	19.8 q	19.7 q
C-17	106.1 t	25.3 q	107.6 t	107.1 t	108.7 t	107.8 t	114.0 t	107.7 t
C-18	33.5 q	33.3 q	181.3 s	179.1 s	205.2 d	71.5 t	—	33.6 q
C-19	21.6 q	21.3 q	17.6 q	16.5 q	14.2 q	17.8 q	—	21.8 q
C-20	14.4 q	15.3 q	15.2 q	14.7 q	14.6 q	15.0 q	17.8 q	14.5 q
—O—Me				51.8 q				

^a δ -Values relative to TMS.

*** Assignment may be reversed.

scribed above. The CMR data are summarised in Table II.

Compound 1. The IR, CMR, PMR and mass spectra of compound **1** were identical to those of manoyl oxide.⁴⁾ Thus, compound **1** was identified as 8,13-epoxylabd-14-ene (manoyl oxide).

Compound 2. The IR, CMR, PMR and mass spectra of compound **2** were identical to those of 13 β -hydroxylabda-8(17),14-dien-18-oic acid.⁴⁾ Thus, compound **2** was identified as 13 β -hydroxylabda-8(17),14-dien-18-oic acid.

Compound 3. The spectral data of compound **3** were as follows: mp: 141~143°C. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3480, 2910, 2840, 1720, 1440, 1240, 1125, 910. PMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 5.91 (14-H, q, $J_{14,15}$ = 17 Hz, $J_{14,15}$ = 10 Hz), 5.19 and 5.50 (15-H₂, dd), 4.80 and 4.49 (17-H₂), 3.62 (—O—CH₃), 1.24, 1.10 and 0.67 (16-, 19- and 20-Me). MS m/z (%): 319 (2) M^+ —CH₃, 316 (6) M^+ —H₂O, 258 (8), 241 (20), 175 (18), 133 (25), 121 (100), 79 (38), 55 (34). The CMR spectrum (Table II) showed this compound had twenty one carbons. The mass spectrum gave ion peaks at m/z 319 (M^+ —CH₃) and 316 (M^+ —H₂O), in good agreement with the empirical formula, C₂₁H₃₄O₃. The IR spectrum of this compound indicated the presence of carbonyl group (1720 cm^{-1}). The CMR spectrum of this compound indicated the presence of a carbonyl carbon (δ 179.1, s) and a methyl carbon (δ 51.8, q) instead of methyl carbon (δ 33.5, q) of manool. In the PMR spectrum, *O*-methyl protons (δ 3.62, 3H, s) were observed. Furthermore, LiAlH₄ reduction of this

compound gave a hydroxymethyl derivative, compound **7**. Compound **7** had identical IR, CMR, PMR and mass spectra to those of labda-8(17),14-dien-13 β ,18-diol.⁴⁾

From these data, compound **3** was deduced to be 13 β -hydroxylabda-8(17),14-dien-18-oic acid methylester.

Compound 4. The IR, CMR, PMR and mass spectra of this compound were identical to those of (12*Z*)-labda-8(17),12,14-trien-18-al.³⁾ Thus, compound **4** was identified as (12*Z*)-labda-8(17),12,14-trien-18-al.

Compound 5. The IR, CMR, PMR and mass spectra of this compound were identical to those of (12*Z*)-labda-8(17),12,14-trien-18-ol.³⁾ Thus, compound **5** was identified as (12*Z*)-labda-8(17),12,14-trien-18-ol.

Compound 6. The IR, CMR, PMR and mass spectra of this compound were identical to those of 4,18,19-trinor-3,4-*seco*-5-oxo-(12*Z*)-labda-8(17),12,14-trien-3-oic acid.³⁾ Thus, compound **6** was identified as 4,18,19-trinor-3,4-*seco*-5-oxo-(12*Z*)-labda-8(17),12,14-trien-3-oic acid.

Microbial transformation of conversion products

To elucidate the transformation pathway of manool by *R. erythropolis* JTS-131, compounds **1**, **2**, **3**, **7** and **8** were transformed respectively by this strain in the presence of EDTA (Table III). Compound **2** was transformed to compound **3**. Compound **7** was transformed to compounds **2**, **3** and **8**. Compound **8** was transformed to compounds **2** and **3**. In the

TABLE III. BIOTRANSFORMATION OF COMPOUNDS **1**, **2**, **3**, **7** AND **8**

Ten mg of each substrate was added to 5 ml of medium-A containing JTS-131 (about 10⁸ cells/ml). After 12 hr, EDTA was added to the culture medium at the concentration of 10 mM, and incubation was continued for a further 48 hr.

Added compound	Products				
	Compound 1	Compound 2	Compound 3	Compound 7	Compound 8
Compound 1		—	—	—	—
Compound 2	—		+	—	—
Compound 3	—	—		—	—
Compound 7	—	+	+		+
Compound 8	—	+	+	—	

TABLE IV. BIOTRANSFORMATION OF COMPOUNDS
4, 5 AND 6

Ten mg of each substrate was added to 5 ml of medium-A containing JTS-131 (about 10^8 cells/ml). After 12 hr, EDTA was added to the culture medium at the concentration of 10 mM, and incubation was continued for a further 48 hr.

Added compound	Products		
	Compound 4	Compound 5	Compound 6
Compound 4		—	+
Compound 5	+		+
Compound 6	—	—	

cases of compounds 1 and 3, a further converted product was not accumulated in the culture broth. In the absence or presence of EDTA, the added substrates, compounds 1, 2, 3, 7 and 8, decreased ($>10\%$, mol/mol) or disappeared from the culture broth after 96-hr incubation.

To elucidate the transformation pathway of (12Z)-labda-8(17),12,14-triene, compounds 4, 5 and 6 were also metabolized by this strain in the presence of EDTA (Table IV). Compound 4 was transformed to compound 6. Compound 5 was transformed to compounds 4 and 6. In the case of compound 6, a metabolic product was not accumulated in the culture broth. In the presence or absence of EDTA, the added substrates, compounds 4, 5 and 6, decreased ($>10\%$, mol/mol) in the culture broth after 96 hr incubation.

In addition, compound 3 was utilized by *N. restricta* JTS-162 without accumulation of further conversion products.

Microbial transformation of conversion products by the cured strain

Manool, (12Z)-labda-8(17),12,14-triene and the conversion products (compounds 1, 2, 3, 4, 5, 6, 7 and 8) were metabolized by the cured strain, JTS-13101. Manool was transformed to compound 1 by this cured strain in the absence and presence of EDTA. However, the other compounds were not transformed by this strain under the same conditions.

DISCUSSION

In the previous papers,^{1,3,4)} we reported the microbial transformation of *cis*-abienol and sclareol by two acid-fast bacteria, *R. erythropolis* JTS-131 and *N. restricta* JTS-162. And it was indicated that *N. restricta* JTS-162 firstly dehydrated these two labdanes at the C-8 and C-17 positions, and then oxidized the C-18 methyl stepwisely and split the A-ring successively. Meanwhile, *R. erythropolis* JTS-131 oxidized the C-18 methyl without dehydration of the C-8 and C-17 positions. In this study, however, it was found that *R. erythropolis* JTS-131 transformed manool and (12Z)-labda-8(17),12,14-triene, the dehydrated products at the C-8 and C-17 positions of sclareol and *cis*-abienol.

In the transformation of manool by *R. erythropolis* JTS-131, three conversion products, 8,13-epoxylabd-14-ene (manoyl oxide, compound 1), 13 β -hydroxylabda-8(17),14-dien-18-oic acid (compound 2) and 13 β -hydroxylabda-8(17),14-dien-18-oic acid methyl ester (compound 3) were obtained. Compound 3 was a newly isolated compound, and compounds 1 and 2 were identical with the conversion products from sclareol by *N. restricta* JTS-162.⁴⁾ In order to obtain information on the transformation pathway of manool by this microorganism, these products were further transformed by this strain (Table III). From the results of these experiments the biotransformation pathway of manool by *R. erythropolis* JTS-131 is considered to be as in Fig. 3 (left).

In the transformation of (12Z)-labda-8(17),12,14-triene, three conversion products, (12Z)-labda-8(17),12,14-trien-18-al (compound 4), (12Z)-labda-8(17),12,14-trien-18-ol (compound 5) and 4,18,19-trinor-3,4-*seco*-5-oxo-(12Z)-labda-8(17),12,14-trien-3-oic acid (compound 6) were obtained. These three compounds are identical with three of the conversion products from *cis*-abienol by *N. restricta* JTS-162.³⁾ From the results of the experiments on the degradation sequence, the biotransformation pathway of (12Z)-labda-

FIG. 3. Proposed Transformation Pathways of Manool and (12Z)-Labda-8(17),12,14-triene in *R. erythropolis* JTS-131.

8(17),12,14-triene by *R. erythropolis* JTS-131 is considered to be as in Fig. 3 (right).

These two pathways are analogous to the transformation pathways of the same compounds with *N. restricta* JTS-162. The production of compound **6** in the transformation of (12Z)-labda-8(17),12,14-triene by *R. erythropolis* JTS-131 indicated that *R. erythropolis* JTS-131 has A-ring splitting ability as well as *N. restricta* JTS-162.

13 β -Hydroxylabda-8(17),14-dien-18-al (compound **8**), a transformation product from sclareol by *N. restricta* JTS-162,⁴⁾ was also transformed to compound **3** by *R. erythropolis* JTS-131. Furthermore, a methylester products, such as compound **3**, was not found in the transformation pathway of sclareol in *N.*

restricta JTS-162,⁴⁾ but compound **3** was utilized by *N. restricta* JTS-162 without accumulation of further conversion products. From these results, it may be deduced that these two acid-fast bacteria have the same C-18 oxidation and A-ring splitting pathways.

In the previous paper,²⁾ we described cured strain JTS-13101 (with no plasmid) derived from JTS-131 (with plasmid pCA4). This cured strain showed the ability to convert manool to manoyl oxide and it was indicated that this conversion ability was not determined by the plasmid as in *N. restricta* JTS-162.⁵⁾ The cured strain JTS-13101 did not metabolize compounds **4** and **6**. From the results, it is also considered that A-ring splitting ability is determined by the plasmid in *R.*

erythropolis JTS-131 as in *N. restricta* JTS-162.⁵⁾

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