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BIOTRANSFORMATION OF THE GERMACRANE TYPE SESQUITERPENE CURDIONE BY SUSPENSION CULTURED CELLS OF *LONICERA JAPONICA*

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Key Word Index—*Lonicera japonica*; biotransformation; suspension cultured cells; curdione; sesquiterpene; 2-hydroxycurdione; 1,10-epoxycurdione.

Abstract—Curdione, a sesquiterpene with a germacrane skeleton, was incubated with suspension cultured cells induced from calli of *Lonicera japonica* for 10 days. The ethyl acetate-soluble fraction of the culture medium gave a number of products, of which six were shown to be (2*S*)-2-hydroxycurdione, (2*R*)-2-hydroxycurdione, (8*S*)-6-hydroxycurdione, (2*R*, 8*S*)-8-hydro-2-hydroxycurdione, (1*S*, 10*S*)-1,10-epoxycurdione and (1*R*, 10*R*)-1,10-epoxycurdione, respectively. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Many types of sesquiterpene have been isolated from *Curcuma* sp. [1–6]. Curdione (**7**) is one of the constituents with a germacrane skeleton. It was first isolated from *C. zedoaria* Roscoe [7] and is believed to be an important intermediate in the biosynthesis of curcuma lactone, procurcumenol and curcumenol. It is the main constituents of *C. aromatica* Salisb. [5].

We previously reported on the biotransformation of germacrone into several guaiane-type sesquiterpenes by suspension cultured cells induced from *C. zedoaria* [8], *Lonicera japonica* Thunb. [9], *Bupleurum falcatum* L. [9], *Polygonum tinctorium* Lour [9] and *Solidago altissima* L. [9], respectively. Germacrone is a key precursor of germacrane, guaiane, seco-guaiane and eudesmane type sesquiterpenoids of *Curcuma* sp. Curdione is transformed into a number of hydroxylated derivatives by the fungus *Aspergillus niger* [10]. This paper reports on the biotransformation of curdione by suspension cultured cells of *L. japonica* [9].

RESULTS AND DISCUSSION

Callus, induced from the shoot apex of *L. japonica*, was transferred to suspension culture and cultured for 4 weeks. After which time, it was incubated with curdione for 10 days. The culture medium was treated as given in the Experimental to give six products (**1**–**6**).

Product **1** was assigned the molecular formula $C_{15}H_{24}O_3$ (mass spectrometry and ^{13}C NMR data). The ^1H NMR spectrum of compound **1** (Table 1) showed the presence of three secondary methyl groups, an olefin proton and a newly formed secondary hydroxyl. The carbinyl proton was shown to couple with the H-1 olefin proton by H–H COSY. The remaining signals were similar to those of curdione (**7**). The assignment of all of the ^1H NMR signals of compound **1** was achieved by H–H COSY (Table 1). From these data, the structure of compound **1** was determined to be 2-hydroxycurdione. The ^{13}C NMR data showed the presence of newly formed carbinyl carbon (δ 66.9) and also supported the structure. The carbon signals were assigned as shown in Table 2 with the support of a C–H COSY experiment. The NOE difference experiment showed that the conformation of **1** was T, T (Fig. 1) and the hydroxyl group at C-2 was pseudo-equatorial. Product **1** was thus shown to be (2*S*)-2-hydroxycurdione.

Product **2** was assigned the same molecular formula ($C_{15}H_{24}O_3$) as product **1** from the mass spectral and ^{13}C NMR data. The ^1H NMR spectrum of compound **2** showed the presence of three secondary methyl groups, an olefin proton and a newly formed secondary hydroxyl group (Table 1). This proton was coupled with the doublet signal of the H-1 olefin proton. The remaining ^1H NMR signals were similar to those of **7**. These data suggested that **2** was the C-2 epimer compound of **1**. All of the proton signals of compound **2** were completely assigned (Table 1). The

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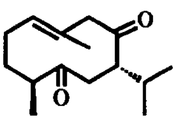
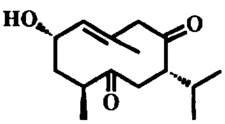


Curdione (7)



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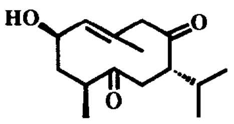

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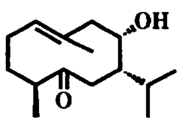

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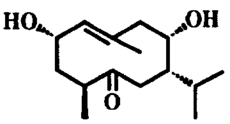

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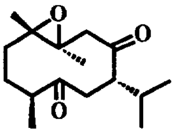

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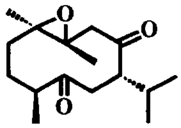

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Table 1. ^{1}H NMR data of products **1**–**6** (δ (ppm) in $CDCl_3$)

H	**1**	**2**	**3**	**4**	**5**	**6**
1	5.39, *d* (6.8)	5.08, *dq* (10.0, 1.3)	4.98, *br d* (9.0)	4.90, *d* (10.3)	2.71, *dd* (10.5, 2.2)	2.88, *dd* (10.0, 3.5)
2	4.53, *br t* (6.6)	4.46, *dt* (5.7, 10.0)	2.23, *dq* (5.0, 12.5)	4.50, *dt* (5.7, 10.3)	2.04, *ddt* (14.3, 2.2, 4.8)	1.33, *m*
			2.35–2.40 overlap		1.20–1.30 overlap	2.06, *m* overlap
3	1.62, *dt* (14.5, 1.9)	1.83, *dt* (12.0, 5.5)		2.24, *dt* (10.5, 12.4)	1.56, *ddt* (13.8, 2.0, 5.3)	2.06, *m*
	2.36, *ddd* (14.5, 10.5, 4.6)	2.17, *m*		1.88–1.96 overlap	2.2–2.3 overlap	1.66, *m*
4	2.85, *qdd* (7.0, 10.5, 1.9)	2.30, *dt* (7.0, 11.5)		2.46, *m*	2.4–2.5 overlap	2.80, *m*
6	3.17, *dd* (16.7, 11.3)	2.4, *m*	3.01, *dd* (17.5, 5.0)	2.90, *dd* (7.8, 5.1)	2.78, *dd* (15.4, 8.6)	3.06, *dd* (18.5, 12.0)
	2.55, *dd* (16.7, 3.0)		2.35–2.40 overlap	1.89–1.96 overlap	2.41, *dd* (15.4, 1.9)	2.79, *dd* (18.5, 4.0)
7	2.64, *dd* (11.3, 8.0, 3.0)	2.8, *m*		1.89–1.96 overlap	2.92, *dt* (1.9, 8.6)	2.66, *ddd* (12.0, 9.0, 4.0)
8			4.19, *br s*	4.22, *br s*		
9	3.28, *d* (11.9)	3.16, *d* (10.5)	2.12, *dd* (14.0, 3.0)	2.12, *dd* (13.5, 3.0)	2.80, *d* (10.3)	2.69, *d* (11.0)
	2.69, *d* (11.9)	2.83, *d* (10.5)	2.35–2.40 overlap	2.39, *dd* (13.5, 4.9)	2.23, *d* (10.3)	2.22, *d* (11.0)
11	1.83, *m*	1.83, *m*		1.63, *m*	1.91, *m*	1.70, *m*
12	0.86, *d* (7.0)	0.85, *d* (6.5)	0.94, *d* (7.0)	0.94, *d* (6.5)	0.88, *d* (6.8)	0.86, *d* (7.0)
13	0.94, *dd* (7.0)	0.92, *d* (6.5)	0.93, *d* (7.0)	0.93, *d* (6.5)	0.95, *d* (7.0)	0.96, *d* (6.5)
14	0.97, *d* (7.00)	0.98, *d* (6.5)	0.97, *d* (7.0)	0.99, *d* (7.0)	1.09, *d* (7.0)	1.06, *d* (7.0)
15	1.46, *s*	1.74, *s*	1.90, *s*	1.98, *s*	1.30, *s*	1.16, *s*

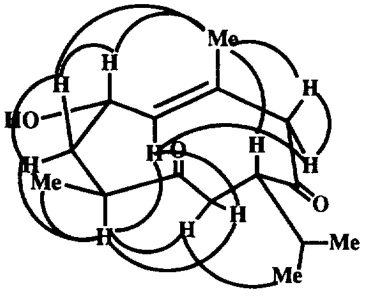
^{13}C NMR spectrum of compound **2** (Table 2) also showed the presence of two carbonyl groups, two olefin carbons and a newly formed carbinyl carbon (68.2 ppm). The NOE difference experiment showed that compound **2** had the same stable C, C conformation as compound **7** and equatorial substituents at C-2, C-4 and C-7 (Fig. 1). From these facts, product **2** was shown to be (2*R*)-2-hydroxycurdione.

The CD spectrum of **1** showed a negative Cotton effect ($\Delta\varepsilon = -12.91$ (304 nm, methanol)) based on the $n \rightarrow \pi^*$ transition of the β, γ-unsaturated ketone [7], i.e. the opposite effect to that given by compound **7**. As expected, the CD spectrum of compound **2** showed a positive Cotton effect ($\Delta\varepsilon = +5.46$ (312 nm, MeOH)) based on the $n \rightarrow \pi^*$ transition of the β, γ-unsaturated ketone, which is the same as that of compound **7**. These CD data support the results for the conformation of compounds **1** and **2** mentioned above. In particular, that the absolute configurations at C-2 of compounds **1** and **2** are 2*S* and 2*R*, respectively (Fig. 2). The absolute configurations at C-2 of compounds **1** and **2** were further confirmed by

Table 2. ^{13}C NMR data of products **1**–**6** and curdione (**7** (δ (ppm), in $CDCl_3$)

C	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	135.3	133.8	130.9	132.9	62.5	64.2	131.5
2	66.9	68.2	27.4	68.0	25.8	24.8	26.4
3	43.0	42.5	37.7	45.7	29.1	29.6	34.1
4	41.5	44.3	47.1	43.5	46.8	44.5	46.8
5	212.7[a]	215.1[a]	217.0	217.1	211.9[a]	209.0[a]	211.0
6	44.6	44.4	40.4	40.8	42.5	42.7	44.2
7	54.9	54.1	44.0	44.4	55.3	50.8	53.5
8	209.6[a]	210.4[a]	72.4	72.5	214.7[a]	213.1[a]	214.2
9	53.4	56.4	48.1	48.2	55.9	56.0	55.9
10	131.6	130.9	133.7	136.9	58.1	58.9	129.8
11	30.5	29.9	32.6	32.6	30.5	30.1	30.0
12	21.0[b]	19.8[b]	20.5[b]	20.4[b]	19.8[b]	21.1[b]	19.9
13	20.3[b]	21.1[b]	20.6[b]	20.6[b]	20.8[b]	20.6[b]	21.1
14	18.7	18.5	19.0	18.8	18.2	19.0	18.5
15	17.7	17.0	19.2	19.6	17.3	16.1	16.6

[a,b] May be interchanged in each vertical column.



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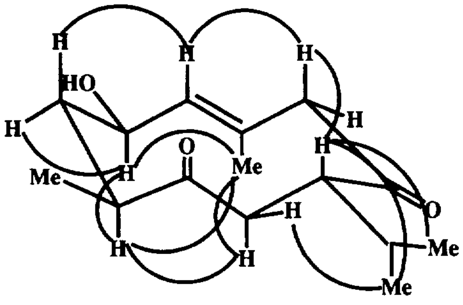

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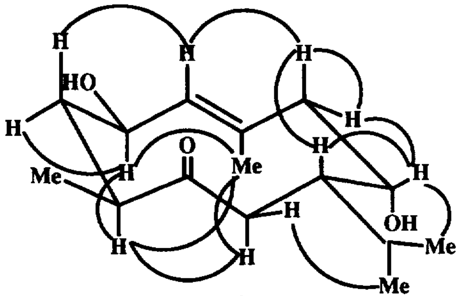

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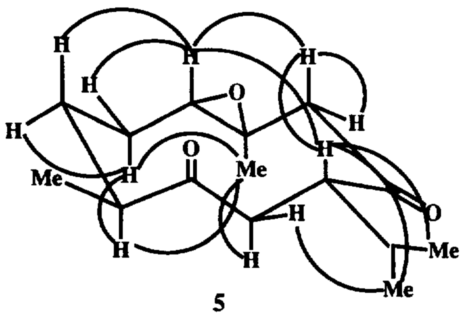

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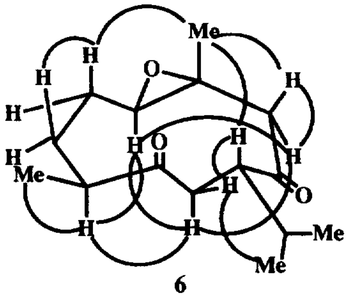

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Fig. 1. Difference NOE of products **1**, **2**, **4**, **5** and **6**.

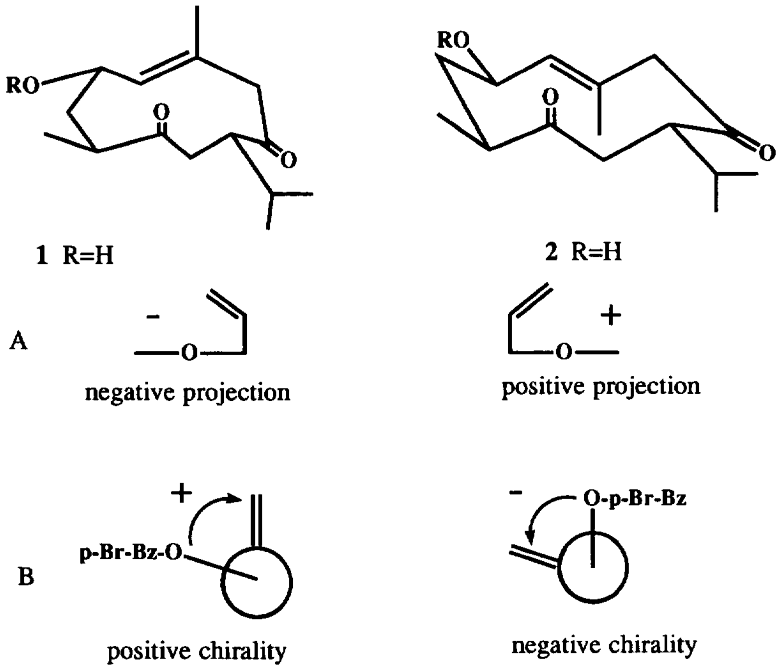

Fig. 2. Projections for $n \rightarrow \pi^*$ transitions of β,γ-unsaturated ketones of compounds **1** and **2** (A), and chiralities of their allyl *p*-bromobenzoates.

application of the exciton chilarity method to the allyl benzoate [12]. The CD spectrum of the *p*-bromobenzoate of compound **1** showed a positive first Cotton effect ($\Delta\varepsilon = +3.2$ (245 nm, shoulder)), indicating that the absolute configuration at C-2 of compound **1** was *S*. The CD spectrum of the *p*-bromobenzoate of compound **2** showed a negative first Cotton effect ($\Delta\varepsilon = -6.3$ (245 nm)) indicating that the absolute configuration at C-2 of compound **2** was *R* (Fig. 2).

Product **3** was assigned the molecular formula $C_{15}H_{26}O_2$ (mass spectrometry and ^{13}C NMR). The ^1H NMR spectrum of compound **3** (Table 1) showed the presence of a newly formed secondary hydroxyl group (δ 4.19; *br s*) and a methylene group (δ 2.12 (1H, *dd*, J = 14.0, 3.0 Hz), 2.38 overlap) coupling with an adjacent vicinal proton instead of an isolated methylene at C-9 as in **7**. These facts indicated that **3** was the C-8 reduction product of compound **7**. The ^{13}C NMR spectrum of compound **3** showed the absence of one of two carbonyl carbons and the appearance of a carbinyl carbon (δ 72.4). The other signals were similar to those of compound **7** (Table 2). From the broad singlet of the carbinyl proton at C-8, the configuration of the hydroxyl group at C-8 was assigned to the α-pseudo-axial position. Product **3** was therefore (8*S*)-8-hydrocurdione.

Product **4** was assigned the molecular formula $C_{15}H_{26}O_3$ (mass spectrometry and ^{13}C NMR). The ^1H NMR spectrum of compound **4** (Table 1) showed the presence of two newly formed secondary hydroxyl groups (δ 4.22 (*br s*) and 4.50 (*dt*, J = 5.7, 10.3 Hz)), the absence of an isolated methylene group at C-9, and the appearance of a methylene group with a vicinal coupling to the carbinyl proton at C-8, which was confirmed by a H–H COSY experiment. This indicated that one of two secondary hydroxyl group was located at C-8. The H–H COSY experiment further suggested that the second secondary hydroxyl group was located at C-2. From these data, product **4** was clearly the 2-hydroxy derivative of compound **3**. This was supported by the ^{13}C NMR spectrum, in which one of the two carbonyl groups of compound **7** was absent and two secondary carbinyl groups were present (δ 68.0 at C-2 and δ 72.5 at C-8). The stereochemistry at C-2 and C-8 of compound **4** was deduced from the coupling constants of carbinyl protons. The coupling constant at C-2 was similar to that of compound **2** (δ 4.50, *dt*, J = 5.7, 10.3 Hz) and the signal shape at C-8 was similar to that of compound **3** (δ 4.22 *br.s*). The ^{13}C NMR chemical shifts of compound **4** were almost the same (Table 2) as those of compound **2**. This indicated that compounds **2** and **4** have the same C, C conformation. This was confirmed by measurement of the difference NOE (Fig. 1). From these data, the structure of product **4** was determined to be (2*R*, 8*S*)-8-hydro-2-hydroxycurdione.

Product **5** was assigned the molecular formula $C_{15}H_{24}O_3$ (mass spectrometry and ^{13}C NMR). The ^1H NMR spectrum compound of **5** (Table 1) showed the disappearance of the olefin proton and the presence of a carbinyl proton that resonated at higher field (δ 2.71, 1H, *dd*, J = 10.5, 2.2 Hz) and a tertiary methyl group at δ 1.30 (*s*). The ^{13}C NMR spectrum of compound **5** showed the presence of two carbonyl groups (δ 214.7 and 211.9) and two carbinyl carbons (δ 62.5 and 58.1), characteristic of epoxide carbons, and the absence of olefin carbons. These facts suggested that compound **5** was the C-1,10 epoxide of compound **7**.

Product **6** was assigned the molecular formula $C_{15}H_{24}O_3$ (mass spectrometry and ^{13}C NMR). The ^1H NMR spectrum of compound **6** (Table 1) showed the disappearance of the olefin proton and the appearance of a carbinyl proton (δ 2.88, 1H, *dd*, J = 10.0, 3.5 Hz), which resonated at higher field, and a tertiary methyl group (δ 1.16). The ^{13}C NMR spectrum of compound **6** (Table 2) also showed the disappearance of olefin carbons and presence of two carbinyl carbons (δ 64.2, 58.9). These data indicated that compound **6** was also an epoxide of compound **7** and a stereoisomer of compound **5**.

In order to confirm the structures of compounds **5** and **6**, curdione was epoxidated with *m*-chloroperbenzoic acid to give two reaction products. These products were identical to the two biotransformed products, **5** and **6**, respectively.

From the NOE experiment and the coupling constants of H-1, H-2α and H-7, compound **5** was shown to be in the C, C conformation and to have 1*S*, 10*S*-configuration (Fig. 1). From the determination of the absolute configuration at C-1 and C-10 of compound **5**, it followed that the absolute configuration of C-1 and C-10 of compound **6** was 1*R*, 10*R*. This was supported from difference NOE experiments, as shown in Fig. 1. Therefore, compound **5** was an epoxidation product from the *si-re* site of **7** and compound **6** was from the *re-si* site of **7**.

In conclusion, curdione was biotransformed by suspension cultured cells induced from *L. japonica* to give several products, six of which were isolated as pure compounds. Most of these were oxidized products of curdione, so the same kinds of monooxygenases are involved in the metabolism of the substrates [13].

Asakawa *et al.* [10] reported that the biotransformation of curdione by *Aspergillus niger* gave 2α-hydroxycurdione and 2β-hydroxycurdione. 2α-hydroxycurdione, as reported by Asakawa *et al.* [10], was not identical to compound **1**, but was identical to compound **2** in terms of NMR data. 2β-hydroxycurdione, as reported by Asakawa *et al.* [10], was not identical to compounds **1** or **2**. In the reported ^{13}C NMR data of the 2β-hydroxycurdione, the chemical shift of C-1 showed a higher field shift and C-4 showed a lower field shift compared with those of compound **7**. This does not fit with the values anticipated from hydroxylation at C-2, but does fit with the values anticipated from hydroxylation at C-3 of compound **7**, in which C-1 and C-14 are shifted to higher field, and C-2 and C-4 to lower field, respectively. From these facts, the structures of 2α-hydroxycurdione and 2β-hydroxycurdione reported by Asakawa must be revised to be a 2β-hydroxycurdione and 3-hydroxycurdione, respectively.

EXPERIMENTAL

General. ^1H and ^{13}C NMR: 270 and 500 MHz, and 67.8 and 125 MHz, respectively, TMS as an int. standard; EI-MS: JEOL JMS-AX505W mass spectrometer; MS: JEOL JMS-AX505W.

Plant tissue culture. Calli of *L. japonica* Thunb. was induced from young leaves of the plant on Murashige and Skoog's agar medium supplemented with 2,4-D-BA (0.1–0.2 mg l^{-1}) [9]. The calli were transferred into Gamborg B5 suspension medium supplemented with 2,4D-BA (0.1–0.2 mg l^{-1}).

Biotransformation of curdione (**7**). The substrate (**7**) (30 mg) in a 0.5 ml EtOH was added through a Millipore filter to 200 ml of suspension culture cells of *L. japonica* pre-cultured for 4 weeks in a 500 ml conical flask. In total 300 mg of compound **7** was incubated for 10 days.

Isolation and purification of the products from the suspension culture medium. The suspension culture medium was incubated with compound **7** for 10 days then extracted with EtOAc. The extract was then resuspended with H_2O and further extracted with EtOAc. The EtOAc-soluble extract (500 mg) was sepd by HPLC using an ODS column (YMC D-ODS-7) and 70% MeCN–H_2O to give 7 frs. Fr. 1 was further purified by HPLC (45% MeCN-H_2O) to give compound **1** (32 mg), **2** (38 mg) and **4** (7 mg). Frs 2 and 3 were also purified using 50% MeCN–H_2O to give compounds **5** (13 mg) and **6** (7 mg), respectively. Fr. 5 was purified on HPLC using 55% MeCN-H_2O to give compound **3** (11 mg). Compound **7** (140 mg) was recovered from fr. 6.

Product 1 (**1**). Viscous oil; EI-MS: *m/z* 252 $[M]^+$ $C_{15}H_{24}O_3$; $[\alpha]_D -398.9^\circ$ (c = 0.8, MeOH); $\Delta\varepsilon$ = +5.46 (312 nm, MeOH), ^1H and ^{13}C NMR: Tables 1 and 2.

Product 2 (**2**). Viscous oil; EI-MS: *m/z* 252 $[M]^+$ $C_{15}H_{24}O_3$; $[\alpha]_D + 185.6^\circ$ (c = 0.6, MeOH); $\Delta\varepsilon$ = −12.91 (304 nm, MeOH), ^1H and ^{13}C NMR: Tables 1 and 2.

Product 3 (**3**). Oil; EI-MS: *m/z* 238 $[M]^+$ $C_{15}H_{26}O_2$; ^1H and ^{13}C NMR: Tables 1 and 2.

Product 4 (**4**). Oil; EI-MS: *m/z* 254 $[M]^+$ $C_{15}H_{26}O_3$; ^1H and ^{13}C NMR: Tables 1 and 2.

Product 5 (**5**). Viscous oil; EI-MS: *m/z* 252 $[M]^+$ $C_{15}H_{24}O_3$; $[\alpha]_D + 89.8^\circ$ (c = 0.4, MeOH); ^1H and ^{13}C NMR: Tables 1 and 2.

Product 6 (**6**). Viscous oil; EI-MS: *m/z* 252 $[M]^+$ $C_{15}H_{24}O_3$; $[\alpha]_D - 320.7^\circ$ (c = 0.5, MeOH); ^1H and ^{13}C NMR: Tables 1 and 2.

Epoxidation of curdione (**7**). To a soln of compound **7** (60 mg) in $CHCl_3$ (5 ml), *m*-chloroperbenzoic acid (120 mg) in $CHCl_3$ (5 ml) was added at room temp. The reaction soln was stirred at room temp. for 3 hr. Then poured into H_2O and extracted with $CHCl_3$. The $CHCl_3$ soln was evapd to give a mixt. which was purified by prep. TLC to give compounds **5** (25 mg) and **6** (18 mg). These synthetic products were identical with those of the biotransformed products in all respects.

p-*Bromobenzoylation of* **2**. One mg each of **1** and **2** and excess *p*-bromobenzoyl chloride in pyridine was allowed to stand overnight at 80°. The reaction mixt. was poured into H_2O and extracted with *n*-hexane.

The *n*-hexane was evapd and the product was purified by HPLC (analytical scale) and the product peak was collected. The purified product was examined by FAB-MS (*m/z* 435 and 437 $[MH]^+$) and the UV and CD of each soln of product was measured. The concn was calculated from the UV absorption and the calcd *A*-value of the first Cotton effect, $\Delta\varepsilon = +3.2$ (245 nm, shoulder) and $\Delta\varepsilon = -6.3$ (245 nm), respectively.

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