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BIOTRANSFORMATION OF GERMACRONE BY SUSPENSION CULTURED CELLS*

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Key Word Index—Cell suspension culture; biotransformation; sesquiterpene; germacrone; guaiane; eudesmane; *Lonicera japonica*; *Bupleurum falcatum*; *Polygonum tinctorium*; *Solidago altissima*.

Abstract—The transformation of germacrone, a 10-membered ring sesquiterpene, by suspension cultured cells of *Lonicera japonica*, *Bupleurum falcatum*, *Polygonum tinctorium and Solidago altissima*, was investigated. Germacrone was converted into several types of sesquiterpenes, such as guaiane, eudesmane and seco-guaiane and their structures were determined from spectral and chemical evidence. The configurations of some of the products were determined by CD spectroscopy. The differences of the kinds of products among the four suspension cultured cells is discussed.

INTRODUCTION

Suspension cultured cells of plants are used for the mass production of useful secondary metabolites [1–3] and have also been thought to be potentially useful for the biotransformation of various kinds of organic compounds. The production and biotransformation of organic compounds by plant cultured cells has been studied by many researchers [4–9].

We have already reported on the transformation of germacrone (**1**), which is the key biosynthetic intermediate for some cyclic sesquiterpenes having germacrane, guaiane, pseudo-guaiane, seco-guaiane and elemane skeletons, by suspension cultured cells of *Curcuma zedoaria* [10]. The results of this study indicated the probable routes for the biogenesis (Fig. 1) of sesquiterpenes of *Curcuma* sp. plants and also the *ent*-type sesquiterpenes isolated following the transformation of **1**.

In this report, we describe the biotransformation of **1** by suspension cultured cells of *L. japonica* (Caprifoliaceae), *B. falcatum* (Umberiferae), *P. tinctorium* (Polygonaceae) and *S. altissima* (Compositae).

RESULTS AND DISCUSSION

Calli of *L. japonica*, *B. falcatum*, *P. tinctorium* and *S. altissima* were induced from young leaves of the plants and were subcultured for more than one year on Murashige and Skoog's (MS) agar medium [11]. The calli of *B. falcatum*, *P. tinctorium* and *S. altissima* were transferred into MS suspension medium supplemented with 0.1 mgl^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mgl^{-1} of 6-benzyl adenine (BA), 3 mgl^{-1} of 2,4-D and 0.2 mgl^{-1} of BA, and 1 mgl^{-1} of 2,4-D and 0.2 mgl^{-1} of BA, respectively, while the calli of *L. japonica* were transferred into Gamborg's B5 [12] suspension medium supplemented with 0.1 mgl^{-1} of 2,4-D and 0.2 mgl^{-1} of BA, which gave faster growth of *L. japonica* callus than MS medium.

Germacrone (**1**) was administered to each of the four kinds of suspension cultured cells and incubated for 10 days to produce **2**–**14** as products. The ^1H NMR and mass spectra of products **2**–**6** and **9**–**12** indicated that they were germacrone-4,5-epoxide (**2**) [13], zedoarondiol (**3**) [13], isozedoarondiol (**4**) [13], product **5** [10], germacrone-1,10-epoxide (**6**) [13], procurcumenol (**9**) [13], germacrone-1(19),10-epoxide (**10**) [10], dihydrocurcumenone (**11**) [10] and curcumenone (**12**) [13], respectively. This was confirmed by the finding that they were identical authentic samples isolated from *C. aromatica*. The epoxide **6** and diepoxide **10** were first isolated as the transformed products from **1**.

The FAB-mass and ^{13}C NMR spectrum of **8** revealed that its molecular formula was $C_{21}H_{32}O_7$. That **8** was a sesquiterpene glucoside was supported from the de-glucosyl fragment *m/z* 217 $[M+H-180]^+$ and characteristic chemical shifts for glucosyl carbons (δ62.8, 70.9, 75.2, 78.8 × 2, 101.5). The ^1H NMR spectrum of the aglycone part of **8** showed a similar signal pattern to that of **7**. So product **8** was assumed to be a glucoside of **7**. The ^1H NMR spectrum of **7** and **8** showed a sharp doublet olefinic proton (1H each, δ5.07, and 4.97, *d*, $J=10.5$ Hz) corresponding to H-1 and the presence of a carbinyl

*Part 2 in the series of 'Biotransformation of sesquiterpenes by cultured cells'. For Part 1 see ref. [10].

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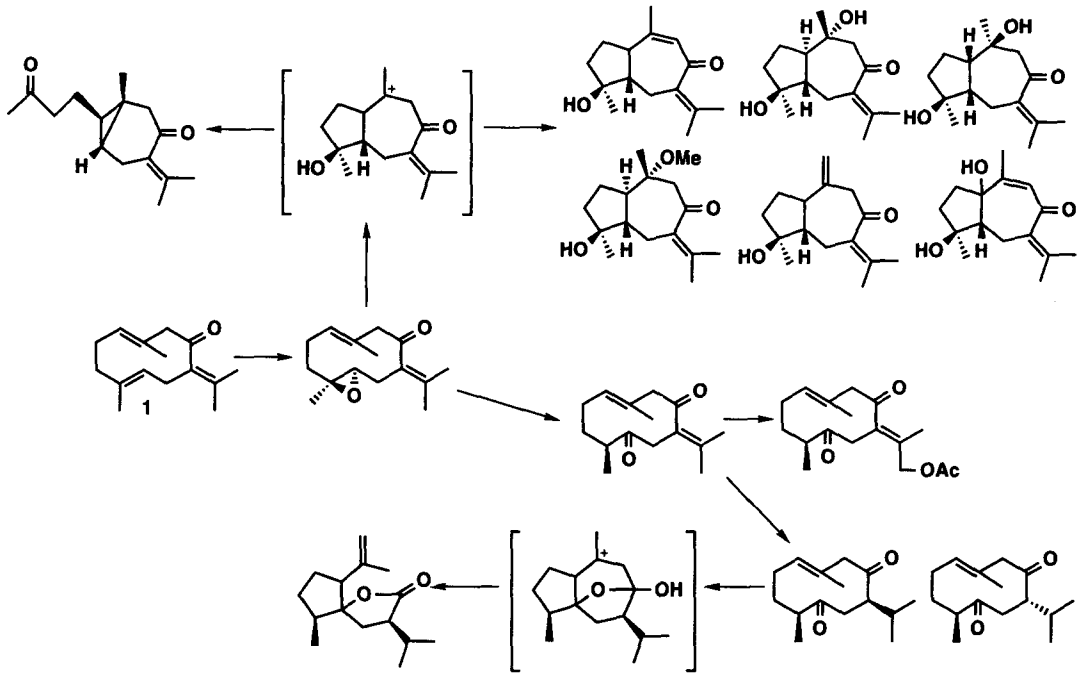

Fig. 1. Hypothetical scheme for the biogenesis of the sesquiterpenes of *Curcuma* sp.

proton [δ, 4.68 (1H, *ddd*, J = 11.0, 11.0, 6.0 Hz) and 4.83, (1H, *ddd*, J = 11.0, 10.5, 6.0 Hz), respectively] corresponding to H-2. The other signals of **7** and **8** were similar to those of **1**. So **7** and **8** were assumed to be 2-hydroxylgermacrone and its glucoside, respectively. These structures were supported from the ^{13}C NMR data of **8** (see Experimental). From the coupling constant of H-2 (*ddd*, J = 11.0, 10.5, 6.0 Hz) with H-1 and H-3, the conformation of the hydroxyl group at C-2 was determined to be equatorial and H-1 was axial. The CD spectrum of **8** showed a negative Cotton effect at 312 nm ($[\theta]$ −9276) based on the n→π* transition of a β,γ-unsaturated ketone [14, 15]. From these data, the stereochemistry of **8** was established to be as shown in Fig. 2, the 10-membered ring having CC conformation. Thus, the structure of **8** was determined to be (2*S*)-2-hydroxygermacrone-2-*O*-glucopyranoside. And **7** should have the same stereo structure with that of **8** (Fig. 2).

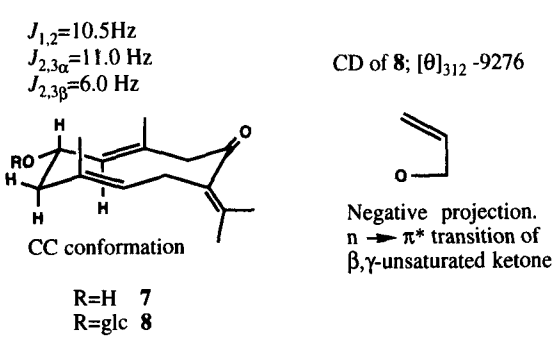

Fig. 2. Stereo structures and octanto projection of **8**.

The ^{1}H NMR spectrum of **13**, $C_{15}H_{24}O_3$, showed the presence of four singlet methyl groups, of which one methyl group (δ1.06) was assignable to an angular methyl group of the eudesmane skeleton and one (δ1.26) to a carbinyl methyl group. The other two methyl groups (δ1.85 and 2.09) were assignable to vinyl methyl groups. The ^{1}H NMR signal pattern of **13** was similar to that of **5**. This indicated that **13** was a stereoisomer of **5**. From these facts and consideration of the transannular cyclization mechanism from **6** to the eudesmane type derivatives [16], **13** was deduced to be a *cis*-eudesmane derivative. The eudesmane type products, **5** and **13**, should be formed via transition state conformations of **6a** (CC conformation) and **6b** (TC conformation) of **6** by *trans* annular cyclization, respectively (Fig. 3).

The ^{1}H NMR spectrum of **14**, $C_{15}H_{22}O_3$, exhibited only three singlet methyl groups (δ1.45, 1.70, 1.83) and two AB type doublet protons attributed to methylene protons of a hydroxymethyl group [δ4.20 (*d*, J = 12.5 Hz) and 4.31 (*d*, J = 12.5 Hz) and 4.31 (*d*, J = 12.5 Hz)]. The rest of the signal pattern of the ^{1}H NMR spectrum was similar to that of **7**. From the above facts and by correlation of the NMR data with similar compounds having a 13-hydroxy group [17], **14** was deduced to be a 13-hydroxyl derivative of **7**.

The configurations of the main products (**2**–**6** and **10**) were determined by CD spectroscopy and were compared with each other and with those of the transformed products by *C. zedoaria* and constituents of *Curcuma* sp. plants [13] (Table 1). In the suspension culture of *S. altissima*, the key intermediate, epoxide **2**, was shown to be a racemate, but the products, **3**–**5** are optically active. In the suspension culture of *P. tinctorium*, the

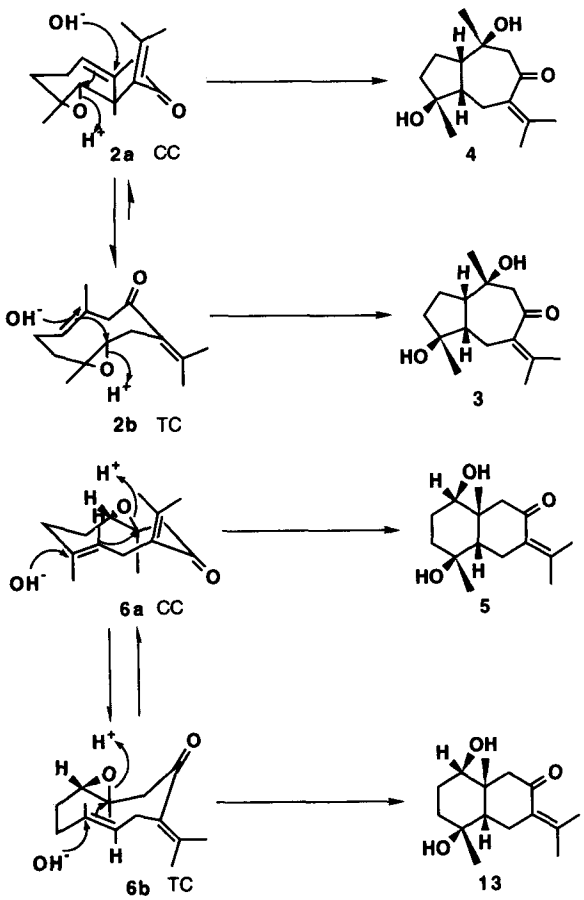

Fig. 3. Mechanisms for formation of compounds **4** and **3** from **2a** and **2b**, and of **5** and **13** from **6a** and **6b**.

Table 1. CD sign of products from the plants of *Curcuma* sp. and from cell suspension cultures of *C. zedoaria, L. japonica, B. falcatum, P. tinctorium* and *S. altissima*

	*C	CZ	LJ	BF	PT	SA
2	†+	—	‡		+	±
3	—	+	+	±	—	—
4	—	+		±		
10	—				+	—
6					+	
5		+	+	—	—	+

*C: products from the plants of *Curcuma* sp.; CZ: products by cell suspension culture of *C. zedoaria*; LJ: products by cell suspension culture of *L. japonica*; BF: products by cell suspension culture of *B. falcatum*; PT: products by cell suspension culture of *P. tinctorium*; SA: products by cell suspension culture of *S. altissima*.

†+, Positive CD; —, negative CD.

‡ Products were not isolated.

epoxide **2** and the diepoxide **10** both give rise to a positive CD curve, while CD positive **2** and CD negative **10** were isolated from *Curcuma* sp. plants. These facts indicated that the stereoselectivity of the epoxidation of **1** and the transannular cyclization of **2** and **6** are not the same in the four calli and in *Curcuma* sp. plants.

Products **3** and **5** were obtained from all incubations to which **1** was added and also from cultures of *C. zedoaria* [10]. The products having guaiane, secoguaiane and eudesmane skeletons were converted through **2** and **6** (Fig. 4), respectively. Thus the four cell cultures must have the capacity to carry out substrate epoxidation. The epoxidation of **1** to **2** or **6** is the key step, because the configuration of **2** and **6** should relate to the configurations of the sesquiterpenes which are successively transformed from **2** and **6** by transannular cyclization as shown in Fig. 3. Therefore, plants and their cultured cells must possess monooxygenase activity and the stereoselectivity of the epoxidation reaction must be different from plant-to-plant and from cultured cell-to-cultured cell.

The 2α-hydroxyl derivatives of **1**, **7** and **14**, were obtained from suspension cultures of *L. japonica* and *S. altissima*, and the glycoside **8** was obtained only from the suspension culture of *L. japonica* in a relatively high yield. Glycosides were expected to be formed by the suspension cultures of *B. falcatum*, as the mother plants like those of *L. japonica* contain saponins, however, no glycoside was obtained. It seems, therefore, that there are some differences between the biosynthetic routes of the mother plants and the cultured cells.

EXPERIMENTAL

^{1}H NMR and ^{13}C NMR: 270 and 500 MHz, and 67.8 and 125 MHz, respectively, TMS as an int. standard; FAB-MS and EI-MS: JEOL JMSOSX-102 and JEOL JMS-AX505W mass spectrometer, respectively; prep. TLC: silica gel PF_{254} (200 × 200 × 0.75 mm), hexane–EtOAc.

Plant tissue cultures. Calli of *Lonicera japonica, Bupleurum falcatum, Solidago altissima* and *Polygonum tinctorium* were induced from the young leaves of the plants of Murashige and Skoog's agar medium supplemented with 2,4-D-BA (0.1–0.2 mg l^{-1}), 2,4-D-BA (0.1–0.2 mg l^{-1}), 2,4-D-BA (3.0–0.2 mg l^{-1}) and 2,4-D-kinetin (1.0–0.1 mg l^{-1}), respectively. The calli of *B. falcatum, S. altissima* and *P. tinctorium*, after subculture for more than a year, were transferred into MS suspension cultures supplemented with 2,4-D-BA (0.1–0.2, 3–0.2 mg l^{-1}) and 2,4-D-kinetin (1–0.2 mg l^{-1}), respectively. The callus of *L. japonica* was transferred into B5 suspension medium supplemented with 2,4-D-BA (0.1–0.2 mg l^{-1}).

Biotransformation of germacrone (**1**). The substrate (**1**) (20 mg) in 0.5 ml EtOH was added to 200 ml suspension cultured cells of *L. japonica, B. falcatum, S. altissima* or *P. tinctorium* in a 500 ml conical flask through a Millipore filter. Ten flasks of each culture were treated in this way (i.e. 200 mg **1** supplied to each culture). The flasks were then incubated for a further 10 days.

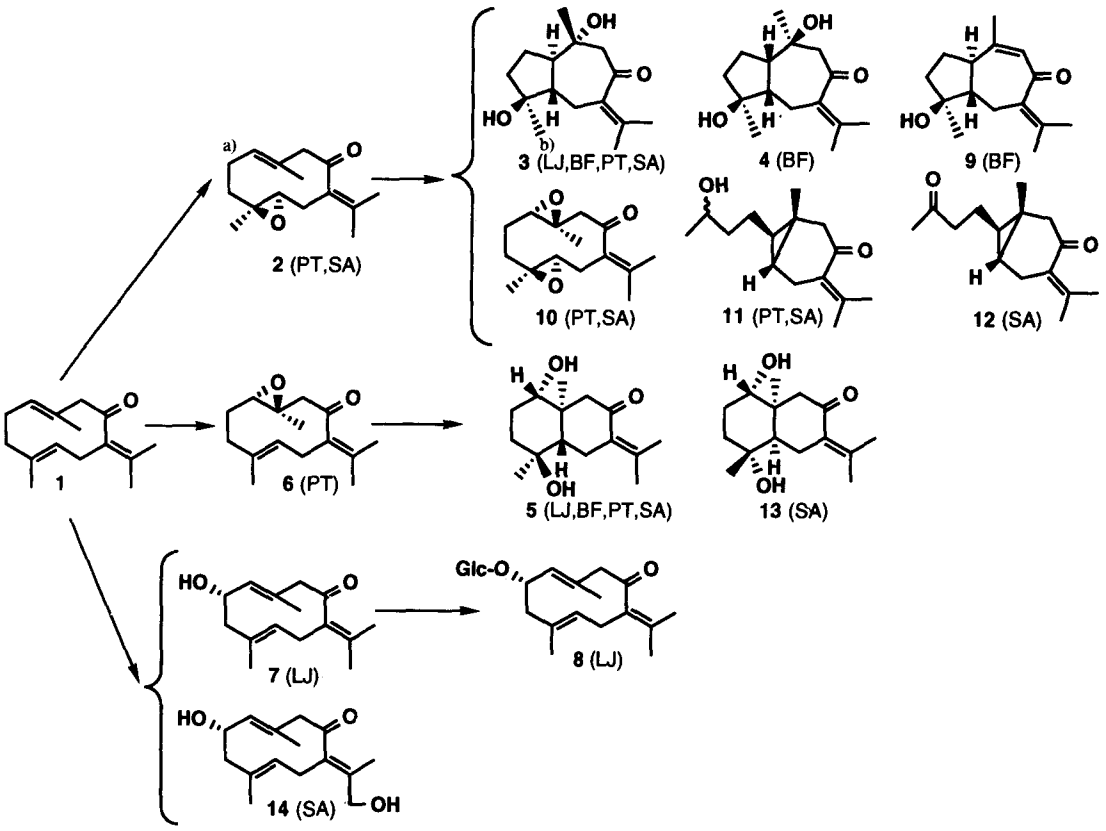

Fig. 4. Transformed products produced from **1** by cell suspension cultures of *L. japonica*, *B. falcatum*, *P. tinctorium* and *S. altissima*. (a) Only relevant configurations shown. (b) LJ; isolated from cell suspension culture of *L. japonica*; BF; isolated from cell suspension culture of *B. falcatum*; PT, isolated from cell suspension culture of *P. tinctorium*; SA, isolated from cell suspension culture of *S. altissima*.

Isolation and purification of the products from suspension cultures of L. japonica, B. falcatum, P. tinctorium and S. altissima. The suspension cultures of *L. japonica* which had been incubated with **1** were sepd into medium and cells. The pooled medium was extracted with EtOAc and *n*-BuOH, successively. After lyophilization, the pooled cells were extracted with hot EtOAc. As this extract gave no spots corresponding to sesquiterpenes on TLC, the sepn of the products was performed on the EtOAc and *n*-BuOH extracts of the incubated media. The EtOAc extract was sepd on prep. TLC, followed by HPLC using a ODS column (YMC D-ODS-7) and MeCN–H_2O as solvent to give products **2**, **3** and **5**–**8**. The *n*-BuOH extract was dissolved in water and applied to a Mitsubishi Diaion HP-20 column. After washing with H_2O, the column was eluted with MeOH. The MeOH eluate was sepd on prep. TLC and HPLC (YMC D-ODS-7) to give glucosylated product **8**. The other suspension cultures which had been incubated with **1** were treated in the same manner as those of *L. japonica* (LJ). Products **3**–**5** and **9** were isolated from the EtOAc extract of *B. falcatum* (BF), products **2**, **3**, **5**, **6**, **10** and **11** from the EtOAc extract of *P. tinctorium* (PT) and products **2**, **3**, **5** and **10**–**14** from the EtOAc extract of *S. altisima* (SA).

The yields of these products were: **2**: 1.0 mg (PT), 2.8 mg (SA); **3**: 0.3 mg (LJ), 7.7 mg (BF), 2.3 mg (PT), 2.7 mg (SA); **4**: 3.6 mg (BF); **5**: 0.2 mg (LJ), 4.1 mg (BF), 1.4 mg (PT), 7.5 mg (SA); **6**: 1.0 mg (PT); **7**: 3.6 mg (LJ); **8**: 8.6 mg (LJ); **9**: 1.8 mg (BF); **10**: 1.4 mg (PT), 1.8 mg (SA); **11**: 2.6 mg (PT), 2.0 mg (SA); **12**: 1.8 mg (SA); **13**: 1.5 mg (SA); **14**: 0.8 mg (SA).

Structures of **2**–**6**, **9**–**12**. From the ^{1}H NMR spectrum of the products, their structures were deduced to be **2**–**6** and **9**–**12**, respectively, as shown in Fig. 4. These compounds were identical with authentic samples (TLC, HPLC, MS and the ^{1}H NMR)

Product **7**. MS *m/z* 234 $[M]^+$, $C_{15}H_{22}O_2$. ^{1}H NMR ($CDCl_3$): δ1.45 (3H, *s*, Me-14), 1.69 (3H, *d*, J = 1.0 Hz, Me-15), 1.73, 1.78 (3H each, *s*, Me-12 and Me-13), 2.07 (1H, *t*, J = 11.0 Hz, H-3), 2.58 (1H, *dd*, J = 11.0, 6.0 Hz, H-3), 2.88 (1H, *dd*, J = 14.0, 3.5 Hz, H-6), 3.41 (1H, *d*, J = 10.5 Hz, H-9), 4.68 (1H, *ddd*, J = 11.0, 11.0, 6.0 Hz, H-2), 4.82 (1H, *br d*, J = 8.0 Hz, H-5), 5.07 (1H, *d*, J = 10.5 Hz, H-1).

Product **8**. FAB-MS: *m/z* 397 $[MH]^+$ $C_{21}H_{33}O_7$, 217 $[MH-C_6H_{12}O_6]^+$ $C_{15}H_{20}O$. ^{1}H NMR ($CDCl_3$); δ1.43 (3H, *s*, Me-14), 1.70 (3H, *s*, Me-15), 1.74, 1.78 (3H each, *s*, Me-12 and Me-13), 2.18 (1H, *t*, J = 11.0 Hz, H-3), 2.56 (1H, *dd*, J = 11.0, 6.0 Hz, H-3), 4.83 (1H, *ddd*, J = 11.0, 10.5,

Table 2. ^{13}C NMR data for compounds **13** and **5** ($CDCl_3$)

C	13	5	C	13	5	C	13	5
1	78.7	78.5	6	26.8	26.0	11	146.4	145.0
2	30.0	28.9	7	129.9	130.0	12	23.1[b]	22.8
3	39.7[a]	41.2	8	202.0	202.1	13	23.8[b]	22.8
4	71.0	71.9	9	55.4	56.8	14	25.9	23.5
5	47.6	50.7	10	40.3[a]	41.2	15	12.7	12.7

[a,b]Assignments may be interchanged.

6.0 Hz, H-2), 4.83 (H-5, overlapping with H-2), 4.97 (1H, *d*, $J = 10.5$ Hz, H-1). ^{13}C NMR ($CDCl_3$); δ15.5 (C-14), 16.6 (C-15), 19.7 (C-12), 22.1 (C-13), 30.2 (C-6), 45.3 (C-3), 55.7 (C-9), 62.8 (G-6), 70.9 (G-4), 71.9 (C-2), 75.2 (G-2), 78.8 × 2 (G-3 and 5), 101.5 (G-1). $[\theta]_{312}$ −9276 (MeOH; *c* 0.01).

Product **13**. MS: *m/z* 252 $[M]^+$ $C_{15}H_{24}O_3$. ^{1}H NMR ($CDCl_3$): δ1.06 (3H, *d*, $J = 1.0$ Hz, H-15), 1.26 (3H, *s*, Me-14), 1.85, 2.09 (3H each, *s*, Me-12 and Me-13), 2.08 (1H, *d*, $J = 16.0$ Hz, H-9), 2.52 (1H, *t*, $J = 16.0$ Hz, H-6), 2.61 (1H, *d*, $J = 16.0$ Hz, H-9), 2.72 (1H, *dd*, $J = 16.0$, 5.0 Hz, H-6), 3.35 (1H, *m*, H-1). ^{13}C NMR spectrum is shown in Table 2.

Product **14**. MS: *m/z* 250 $[M]^+$ $C_{15}H_{22}O_3$. ^{1}H NMR ($CDCl_3$): δ1.45 (3H, *s*, Me-14), 1.70 (3H, *d*, $J = 1.5$ Hz, Me-15), 1.83 (3H, *s*, Me-12), 2.07 (1H, *t*, $J = 11.0$ Hz, H-3), 2.59 (1H, *dd*, $J = 11.0$, 5.9 Hz, H-3), 3.44 (1H, *d*, $J = 10.0$ Hz, H-9), 4.20, 4.31 (1H each, *d*, $J = 12.5$ Hz, H-13), 4.69 (1H, *ddd*, $J = 11.0$, 11.0, 6.0 Hz, H-2), 4.78 (1H, *br t*, $J = 9.0$ Hz, H-5), 5.08 (1H, *d*, $J = 10.0$ Hz, H-1).

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