HETEROCYCLES, Vol. 69, 2006, pp. 429 - 436. © The Japan Institute of Heterocyclic Chemistry
Received, 18th May, 2006, Accepted, 3rd July, 2006, Published online, 6th July, 2006. COM-06-S(O)12

OXIDATIVE CYCLIZATION REACTION OF DIBENZYLBUTANOLIDES WITH COMBINED UTILIZATION OF SYNTHETIC CHEMISTRY AND BIOTECHNOLOGICAL METHODS

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Abstract – We reported plant cell culture (as an enzyme source)-catalyzed oxidative cyclization reaction of dibenzylbutanolides (**1a**, **b**, **d**, **e**) to cyclic product (**2a**, **b**, **d**, **e**) via a hypothetical quinone methide intermediate.

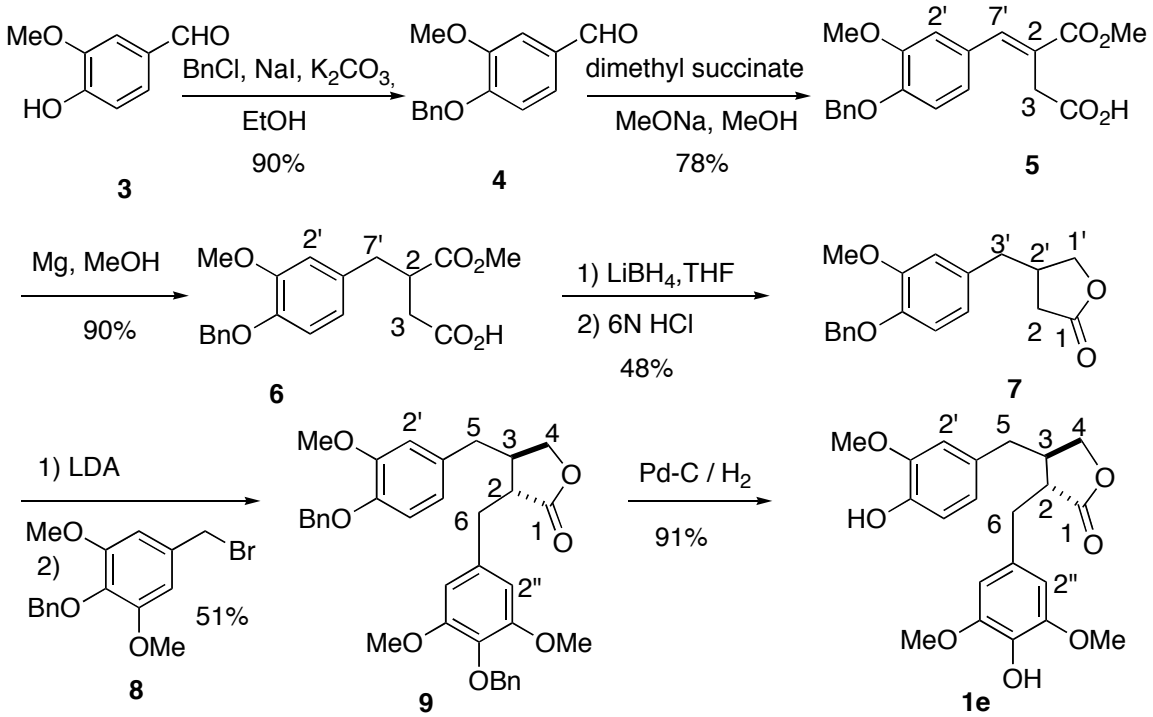
INTRODUCTION

Lignans have attracted much attention for their variation of structures together with the important pharmacological activities.[1] Lignans are generally accessible only through multistep synthesis and/or direct isolation from the living plant. Our synthetic approach to lignans is directed toward the utilization of a combination of biotechnological and chemical methods. We had reported horseradish peroxidase (HRP)-H_2O_2 catalyzed carbon-carbon bond formation with phenolic systems: enzyme-catalyzed oxidative cyclization reaction of dibenzylbutanolides.[2] However, the addition of H_2O_2 to the reaction mixture resulted in red-brown darkening of the reaction mixture which decreased the chemical yield. We have found that plant cell culture is an efficient source of peroxidase enzymes as 'reagents' in organic synthesis and a substantial amount of H_2O_2 is produced in plant cell cultures by the addition of foreign substrates.[3] Then, our attention paid to the subject, whether plant cell cultures catalyze oxidative cyclization reaction of dibenzylbutanolides.

RESULTS AND DISCUSSION

We studied oxidative cyclization reaction of dibenzylbutanolides (**1a**-**e)** with plant cell cultures. Dibenzylbutanolides (**1a**-**d)** were synthesized according to the reported procedure (**1a**[4],**1b**[4], **1c**[4], **1d**[5]).

Dibenzylbutanolide (**1e**) was prepared as shown in Scheme 1. Benzyl ether (**4**), which was synthesized from 4-hydroxy-3-methoxybenzaldehyde (**3**) with benzyl chloride and potassium carbonate,[6] was subjected to Stobbe condensation with dimethyl succinate to give the conjugated hemiester (**5**) in 78% yield. Spectral data indicated that only one geometric isomer (probably *cis*) was present, as expected, by comparison with the results reported for an analogous reaction with piperonal.[7] The resulting double bond of the hemiester (**5**) was reduced with magnesium in methanol to afford the unconjugated hemisuccinate (**6**) in 90% yield. The ^{1}H-NMR spectrum of **6** showed proton signals at C2, C3, and C7' as multiplets due to the creation of a chiral center at C2. The reductive lactonization of the hemisuccinate (**6**) was treated with lithium borohydride to afford butanolide (**7**) directly in 48% yield. The ^{1}H-NMR spectrum of **7** showed two new methylene protons at C1' as doublets of doublets at δ 4.03 and δ 4.31, respectively. Alkylation of **7** with bromide (**8**)[5] gave dibenzylbutanolide (**9**) in 51% yield. The ^{1}H-NMR spectrum of the product (**9**), in comparison with that of **7**, showed the loss of one signal of C2 but the expected *trans* orientation of the two substituents with respect to the lactone ring could not be confirmed owing to the close proximity to the complex signals at C2 and C3. To remove the *O*-benzyl group, **9** was hydrogenated over Pd on carbon to give bis(hydroxybenzyl)butanolide (**1e**) in 91% yield.

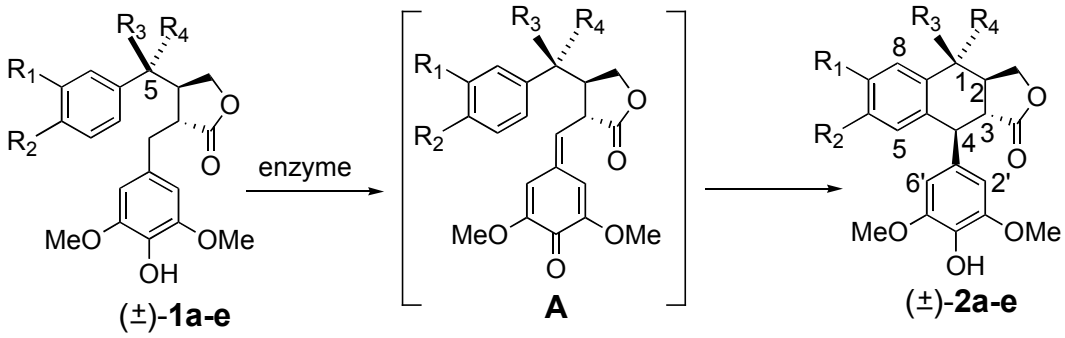


Scheme 1. Synthesis of dibenzylbutanolide (**1e**)

We then proceeded to investigate oxidative cyclization reaction of dibenzylbutanolides (**1a**-**e**) with plant cell cultures. In this work, we used suspension-cultured cells which had originally been isolated from *Nicotiana* (*N.*) *tabacum* "Bright Yellow-2", *Daucus* (*D.*) carota, *Camellia* (*C.*) *sinensis* and *Catharanthus* (*C.*) *roseus*. These cell cultures (*N. tabacum*, *D. carota*, *C. roseus* and *C. sinensis*) were prepared as

described in our previous papers.[8-11] Peroxidase activity (POD) was assayed by the published procedure[3] and was shown to vary with the subcultural time of cells. The highest level of POD was found; *C. sinensis* cell (18 days, 15.5 U/mL), *N. tabacum* cell (22 days, 9.6 U/mL), *D. carota* (12 days, 8.0 U/mL), *C. roseus* cell (8 days, 0.5 U/mL). In this work, we used suspension-cultured cells which had the highest POD. An oxidative cyclization reaction was performed by the methods with freely suspended plant cells in the stationary phase after incubation (*C. sinensis* 18 days, *N. tabacum* 22 days, *D. carota* 12 days, *C. roseus* 8 days). A substrate (50 mg) was added to the freely suspended *C. roseus* (B-5 medium, pH 5.5), *N. tabacum* "Bright Yellow-2" (MS medium, pH 5.8), *D. carota* (MS medium, pH 5.8), and *C.sinensis* (B-5 medium, pH 5.8). The mixture was shaken at 25 °C in a rotary shaker (110 rpm) in the dark. POD-catalyzed oxidative cyclization reaction of **1a-e** was examined as shown in Table 1. When **1a** was subjected to *C. sinensis* cell culture for 6 h, racemic butanolide (**1a**) was cyclized to the desired ring-closed product (**2a**) quantitatively via a hypothetical quinone methide intermediate A in the absence of foreign H_2O_2 as a cofactor (Entry 1). Increasing reaction time promoted the decomposion of **2a** to decrease yield (Entry 2). We next surveyed a variety of plant cell cultures, and these results are shown in Table 1. These cell cultures showed lower POD than *C. sinensis*. Therefore, lower peroxidase activity resulted in incomplete conversion of **1a** to lower yield and required longer reaction time than *C. sinensis* (Entries 3—5). For the sake of comparison, the biotransformation of **1a** was investigated with commercial horseradish peroxidase in the presence of hydrogen peroxide. The yield of **2a** was 18%, thereby revealing that *C. sinensis* is far superior for the present study (Entry 6). A control experiment was also performed in order to determine if H_2O_2, in the absence of horseradish peroxidase or plant cell culture, was able to oxidize **1a** under the conditions used in the biotransformation. Extraction of the reaction mixture gave 82% recovery of **1a** (Entry 7). Biotransformation of **1b** proceeded with *C. sinensis* for 6h to afford **2b** in 82% yield (Entry 8). In the case of **1a** or **1b** where the hydroxyl group (C5) is the β or α isomer, their reactivities were the same. But biotransformation of **1c** was unsuccessful with *C. sinensis* (Entry 9). When **1d** and **1e** were added to *C. sinensis* cell culture for 0.5 h, racemic butanolides (**1d**) and (**1e**) were cyclized to the desired (trans relationship of the lactone ring) products (**2d**) and (**2e**) quantitatively in the absence of foreign H_2O_2 as a cofactor (Entries 10, 14). Dibenzylbutanolides (**1d**) and (**1e**) were consumed very nearly within 0.5 h under a very similar rate. Even a prolonged incubation failed to convert **1d** and **1e** completely into **2d** and **2e**. The color of the reaction mixture changed from an initial pale yellow to red within 1 h, suggesting extensive oxidation of the starting material. Organic extracts were poor and it was clear that extensive decomposition occurred even in 1 h. The biotransformation of **1d** and **1e** was unsuccessful with other plant cell cultures (Entries 11—13, 15—17). No ring-closed products (**2d**) and (**2e**) were given, and moreover recovery of the material as organic extracts was low. The structures of ring-closed products (**2a**),[4] (**2d**)[5] and (**2e**)[3] were confirmed by a comparison of the ^{1}H-NMR and FAB-MS data with that reported. The

Table 1. Biotransformation of dibenzylbutanolide (**1a-e**) to cyclic product (**2a-e**) by peroxidase enzymes



a; R_1=OH, R_2=$OCH(CH_3)_2$, R_3=OH, R_4=H **b**; R_1=OH, R_2=$OCH(CH_3)_2$, R_3=H, R_4=OH

c; R_1=OH, R_2=$OCH(CH_3)_2$, R_3=SPh, R_4=SPh **d**; R_1=OH, R_2=OMe, R_3=H, R_4=H

e; R_1=OMe, R_2=OH, R_3=H, R_4=H

Entry	Substrate	Enzyme	Time (h)	Product **2a-e** (%)	Recovery **1a-e** (%)
1	**1a**	*C. sinensis*	6	quant	0
2	**1a**	*C. sinensis*	24	10	0
3	**1a**	*C. roseus*	168	37	23
4	**1a**	*N. tabacum*	168	14	66
5	**1a**	*D. carota*	168	12	53
6	**1a**	HRP(H_2O_2)	0.5	18	15
7	**1a**	H_2O_2	6	0	82
8	**1b**	*C. sinensis*	6	82	0
9	**1c**	*C. sinensis*	24	0	92
10	**1d**	*C. sinensis*	0.5	85	0
11	**1d**	*C. roseus*	71	0	8
12	**1d**	*N. tabacum*	71	0	12
13	**1d**	*D. carota*	71	0	7
14	**1e**	*C. sinensis*	0.5	78	0
15	**1e**	*C. roseus*	45	0	18
16	**1e**	*N. tabacum*	45	0	25
17	**1e**	*D. carota*	45	0	21

structure of cyclic product (**2b**) was confirmed by analysis of the ^{1}H-NMR spectrum. The *trans* relationship of the lactone ring (stereochemistry of C3 and C4) was confirmed from the proton signal at C3 (doublets of doublets at 2.91 ppm with coupling constants of 14.0 and 11.3 Hz) and the proton signal at C4 (a doublet at 4.05 ppm with coupling constant of 11.3 Hz). The oxidative cyclization reaction can be highly efficient if the substrate, as in the case of **1a** or **1b**, possesses a hydroxyl group at C(5) of the dibenzylbutanolide system. Since **1d** and **1e** react very readily with peroxidase, the biotransformation was conducted under milder conditions than those employed for **1a** and **1b**. In this study, we used *C. sinensis* cell cultures, in

which cell wall peroxidases rapidly metabolize a substantial amount of H_2O_2 produced by the addition of foreign substrates. We succeeded in the oxidative cyclization reaction of dibenzylbutanolide (**1a**, **1b**, **1d** and **1e**) with *C. sinensis* cell culture in the absence of foreign H_2O_2 as a cofactor. The development of enzymes for oxidation reactions aimed at green chemistry is very important. This synthetic method has some advantageous features such as mild reactions, easy work-up, and safety; therefore, it is a valuable alternative to the oxidative cyclization reaction by HRP.

EXPERIMENTAL

General Experimental Procedures. ^{1}H-NMR spectra were measured at 270 MHz on a JEOL JNM-EX 270 FT NMR spectrometer. Chemical shifts are quoted in ppm with tetramethylsilane as an internal standard, and coupling constants (*J*) are given in Hz. FAB-MS was taken on a JEOL JMS-SX 102 mass spectrometer.

Preparation of 2-(4-benzyloxy-3-methoxybenzylidene)butanedioic acid 1-methyl ester (5) Sodium methoxide (4.6 g, 85.9 mmol) was carefully added to dry methanol (20 mL) under Ar. A solution of benzyl ether (**4**) (5.0 g, 20.6 mmol) in dimethyl succinate (4.3 g, 29.2 mmol) was added dropwise over 40 min during reflux. After an additional 5.5 h of stirring during reflux, the bulk of the solvent was removed *in vacuo*. The suspension was cooled to 0°C and acidified with 6 M HCl. The solids were removed by filtration and the filtrate was extracted with CH_2Cl_2 (2×50 mL). The solids were added to the organic extract, washed with brine (100 mL), dried over $MgSO_4$, filtered, and evaporated *in vacuo* to yield an oily yellow solid. The solid was subjected to silica gel column chromatography using CH_2Cl_2–MeOH (20:1) to afford the hemiester (**5**) (5.7 g, 78%) as an amber resin.

5: FAB-MS *m/z* 356 (M^+), HR-MS *m/z*: 356.1267 (calcd. for $C_{20}H_{20}O_6$: 356.1260).

^{1}H-NMR ($CDCl_3$) δ: 3.61 (2H, s, 3-H), 3.81 (3H, s, -OMe), 3.86 (3H, s, -COOMe) , 5.16 (2H, s, -OCH_2Ph), 6.80-7.00 (3H, m, 2'-H, 5'-H and 6'-H), 7.25—7.44 (5H, m, -OCH_2Ph), 7.84 (1H, s, 7'-H).

Preparation of (±)-2-(4-benzyloxy-3-methoxybenzyl)butanedioic acid 1-methyl ester (6)

The hemiester (**5**) (5.7 g, 15.9 mmol) was added to a suspension of Mg shavings (8.0 g, 329 mmol) in dry methanol (100 mL) under Ar. After a few minutes of stirring, the reaction vessel was immersed in an ice bath and stirred at 0°C for 5.5 h. The suspension was acidified with 6 M HCl and the remaining solids were removed by filtration. The filtrate was extracted with CH_2Cl_2 (3× 60 mL), washed with brine (100 mL), dried over $MgSO_4$, filtered, and evaporated *in vacuo* to afford the hemisuccinate (**6**) (5.1 g, 90%).

6: ^{1}H-NMR ($CDCl_3$) δ: 2.43 (1H, dd, *J* = 17.0, 4.6 Hz, 3-H), 2.64—2.74 (2H, m, 2-H and 3-H), 2.94—3.02 (2H, m, 7'-H), 3.66 (3H, s, -COOMe), 3.86 (3H, s, -OMe), 5.12 (2H, s, -OCH_2Ph), 6.60-6.68 (2H, m, 2'-H and 6'-H), 6.78 (1H, d, *J*=8.2 Hz, 5'-H), 7.26—7.44 (5H, m, -OCH_2Ph). MS *m/z*: 358 (M^+).

Preparation of 2'-(4-benzyloxy-3-methoxybenzyl)butanolide (7)

$LiBH_4$ (320 mg, 6.2 mmol) in dry THF (50 mL) was carefully added to a solution of the hemisuccinate (**6**)

(5.1 g, 14.1 mmol) in THF (80 mL) during reflux under Ar. The solution was stirred for 5 h during reflux and then cooled to rt. Water (2 mL) and 6 M HCl (3 mL) were added and the solution was stirred at rt for 15 h. The bulk of the solvent was removed *in vacuo* and the resultant mixture was extracted with ether (50 mL). The organic extract was washed with saturated aqueous $NaHCO_3$ (3×20 mL) and water (20 mL) before being dried over $MgSO_4$, filtered, and evaporated *in vacuo*. The residue was subjected to silica gel column chromatography using CH_2Cl_2 –MeOH (40:1) to afford β-butanolide (**7**) (2.1 g, 48%). The analytical sample was recrystallized from AcOEt–hexane as a white powder (mp 76—79°C).

7: *Anal. Calcd* for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 72.81; H, 6.51. ^{1}H-NMR ($CDCl_3$) δ: 2.28 (1H, dd, *J* = 17.5, 6.6 Hz, 2-H), 2.60 (1H, dd, *J*=17.5, 7.9 Hz, 2-H). 2.68—2.84 (3H, m, 2'-H and 3'-H), 3.88 (3H, s, -OMe), 4.03 (1H, dd, *J* = 5.9, 8.9 Hz, 1'-H), 4.31 (1H, dd, *J* = 6.3, 8.9 Hz, 1'-H), 5.13 (2H, s, -OCH_2Ph), 6.61—6.75 (2H, m, 2'-H and 6'-H), 6.82 (1H, d, *J*=8.3Hz, 5'-H), 7.26—7.45 (5H, m, -OCH_2Ph).

Preparation of *trans*-2-(4-benzyloxy-3,5-dimethoxybenzyl)-3-(4-benzyloxy-3-methoxybenzyl)-butanolide (9)

To a solution of diisopropylamine (0.53 g, 5.0 mmol) in dry THF (20 mL) at -78°C was added *n*-BuLi (3.0 mL, 4.3 mmol) and stirring was continued for 30 min. β-Butanolide (**7**) (1.1 g, 3.5 mmol) in THF (15 mL) was added, and the bright yellow solution was stirred for 90 min prior to the addition of bromide (**8**) (1.4 g, 4.0 mmol) in THF (15 mL) and further stirred for 16 h at -78°C. The solution was warmed to 0°C, acidified with 1 M HCl, and extracted with CH_2Cl_2 (2×100 mL). The combined organic extracts were washed with water (80 mL), dried over $MgSO_4$, filtered, and evaporated *in vacuo*. The residue was subjected to silica gel column chromatography using hexane–AcOEt (4:1), hexane–AcOEt (2:1), and CH_2Cl_2 to afford dibenzylbutanolide (**9**) (1.0 g, 51%) as an yellow oil.

9: FAB-MS *m/z* 568 (M^+), HR-MS *m/z*: 568.2445 (calcd. for $C_{35}H_{36}O_7$: 568.2462). ^{1}H-NMR ($CDCl_3$) δ: 2.23 (1H, dd, *J* = 14.2, 8.3 Hz, 6-H), 2.40—2.75 (3H, m, 2-H, 3-H, and 6-H), 2.92 (1H, dd, *J* = 14.5, 5.3 Hz, 5-H), 3.76 (6H, s, -OMe), 3.82 (3H, s, -OMe), 4.10 (1H, dd, *J* = 9.3, 6.9 Hz, 4-H), 4.90 (2H, s, -OCH_2Ph), 5.11 (2H, s, -OCH_2Ph), 6.25—6.90 (5H, m, 2'-H, 5'-H, 6'-H, 2"-H and 6"-H), 7.25—7.43 (10H, m, -OCH_2Ph).

Preparation of *trans*-2-(3,5-dimethoxy-4-hydroxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)-butanolide (1e)

Pd-C (5%, 1.0 g) and **9** (0.9 g, 1.6 mmol) was suspended in AcOEt–EtOH (1:3) (30 mL) and stirred under hydrogen at atmospheric pressure for 2.0 h. The catalyst was filtered off and the solvent was evaporated *in vacuo* to yield bis(hydroxybenzyl)butanolide (**1e**) (0.6 g, 91%) as an amorphous white solid. The analytical sample was recrystallized from ether-petroleum ether as a white powder (mp 35—40°C).

1e: FAB-MS *m/z* 388 (M^+), HR-MS *m/z*: 388.1507 (calcd. for $C_{21}H_{24}O_7$ 388.1522). ^{1}H-NMR ($CDCl_3$) δ: 2.40—2.72 (4H, m, 2-H, 3-H and 6-H), 2.90 (1H, dd, *J* = 5.3, 10.9 Hz, 5-H), 3.78—3.87 (10H, m, -OMe

and 4-H), 4.17 (1H, dd, *J* = 7.3, 3.2 Hz, 4-H), 6.25—6.90 (7H, m, 2”-H, 6”-H, 2’-H, 5’-H, 6’-H, 4’-OH and 4”-OH).

Cultivation of *C. roseus* cells

Suspension cells of *C. roseus* were subcultured every 7 days by transferring a 1-week culture (8 mL) into B5 medium (80 mL) containing 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/L) and 2% sucrose (pH 5.5) on a rotary shaker (110 rpm) at 25°C in the dark.

Cultivation of *D. carota* cells

Suspension cells of *D. carota* were subcultured every 7 days by transferring a 1-week culture (8 mL) into MS medium (80 mL) containing 2,4-D (2 mg/L) and 3% sucrose (pH 5.8) on a rotary shaker (110 rpm) at 25°C in the dark.

Cultivation of *N. tabacum* cells

Suspension cells of *N. tabacum* were subcultured every 7 days by transferring a 1-week culture (1.3 mL) into MS medium (80 mL) containing 2,4-D (2 mg/L) and 3% sucrose (pH 5.8) on a rotary shaker (110 rpm) at 25°C in the dark.

Cultivation of *C. sinensis* cells

Suspension cells of *C. sinensis* were subcultured every 10 days by transferring a 1-week culture (10 mL) into B5 medium (80 mL) containing 2,4-D (1.25 mg/L) and 5% sucrose (pH 5.8) on a rotary shaker (110 rpm) at 25°C in the dark.

Biotransformation of substrates (1a-e) with plant cell cultures

A substrate (**1a**-**e**) (50 mg) was added to the freely suspended *C. roseus* (10 g of cells and 40 mL broth, B-5 medium, pH 5.5, 8 d old), *N. tabacum* ‘ Bright Yellow-2’ (10 g of cells and 40 mL broth, MS medium, pH 5.8, 22 d old), *D. carota* (10 g of cells and 40 mL broth, MS medium, pH 5.8, 12 d old), and *C. sinensis* (10 g of cells and 40 mL broth, B-5 medium, pH 5.8, 18 d old). The mixture was shaken at 25°C on a rotary shaker (110 rpm) in the dark. At the termination of the reaction, the incubation mixture was filtered, and the filtered cells were washed with AcOEt. The filtrates and washings were combined and extracted with AcOEt. The AcOEt layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to silica gel column chromatography (**2a** and **2b**: MeOH–CH_2Cl_2 (1:20), **2d** and **2e**: ether–hexane (30:1). The reaction time and the chemical yield are listed in Table 1.

2b: FAB-MS *m/z* 430 (M^+), ^{1}H-NMR (CD_3COCD_3) δ: 1.08 (3H, d, *J* = 6.0, $CH(CH_3)_2$), 1.18 (3H, d, *J* = 6.0, $CH(CH_3)_2$), 2.57—2.64 (1H, m, 2-H), 2.91 (1H, dd, *J* = 11.3, 14.0 Hz, 3-H), 3.75 (6H, s, OMe), 4.05 (1H, d, *J* = 11.3 Hz, 4-H), 4.15 (2H, m, 2α-H), 4.23—4.29 (1H, m, $CH(CH_3)_2$), 4.60 (1H, s, OH, D_2O exchangeable), 4.93 (1H, d, *J* = 7.5 Hz, 1-H), 6.38 (1H, s, 5-H), 6.54 (2H, s, 2’-H, 6’-H), 6.95 (OH, s, D_2O exchangeable), 7.14 (1H, s, 8-H), 7.31 (1H, s, OH, D_2O exchangeable).

Biotransformation of dibenzylbutanolide (1a) with HRP

Dibenzylbutanolide **1a** (50 mg) was dissolved in a mixture of pH 6.4 phosphate buffer-water (1:22 v/v, 40 mL), which contained 5 mg HRP. 1.3 mL of 0.5% H_2O_2 was added during a period 0.5 h while shaking (110 rpm). The resulting reaction mixture was extracted with AcOEt. The AcOEt layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to silica gel column chromatography [MeOH–CH_2Cl_2 (1:20)] to afford **2a**. The chemical yield is listed in Table 1.

Reaction of dibenzylbutanolide (1a) with H_2O_2

Dibenzylbutanolide (**1a)** (50 mg) was dissolved in a mixture of pH 6.4 phosphate buffer-water (1:22 v/v, 40 mL). 1.3 mL of 0.5% H_2O_2 was added during a period 0.5 h while shaking (110 rpm). The resulting reaction mixture was shaken for 6 h and extracted with AcOEt. The AcOEt layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to silica gel column chromatography [MeOH–CH_2Cl_2 (1:20)] to afford **2a**. The chemical yield is listed in Table 1.

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