Synthesis of Lyoniresinol with Combined Utilization of Synthetic Chemistry and Biotechnological Methods

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We have synthesized lyoniresinol with the combined utilization of synthetic chemistry and biotechnological methods, specifically using plant cell cultures as an "enzyme source."

Key words lyoniresinol; plant cell culture; oxidative coupling; enzyme; peroxidase; dibenzylbutanolide

Lignans of the 4-aryltetralin series, *e.g.*, lyoniresinol (**1**), have attracted considerable attention recently for their antioxidative and antimutagenic activities (Chart 1). Lignans are generally accessible only through multistep syntheses and/or direct isolation from the living plant. Lyoniresinol (**1**) was isolated *via* extraction of the *Lyonia ovalifolia* var. *elliptica* plant.[1,2] Compound **1** was also obtained by enzymatic hydrolysis of lyoniresinol glucoside with cellulase.[3]

In this paper, we report the synthesis of **1** with the combined utilization of synthetic chemistry and biotechnological methods, specifically using plant cell cultures as an "enzyme source." The biosynthetic pathway generally postulated for the podophyllotoxins (lignans in general), as shown in Chart 2, involves enzyme-catalyzed oxidative coupling of dibenzylbutanolide to the cyclic lignan in the later steps of the pathway (from **2** to **3**).[4,5] Such ring-closure reactions (phenol oxidative coupling) are considered to be achieved through peroxidase (POD) enzymes present in the plant. Kutney *et al.* reported horseradish peroxidase (HRP)–H_2O_2 catalyzed ring-closure reactions with phenolic systems: enzyme-catalyzed ring-closure reaction of dibenzylbutanolides suitable for biotransformation to lignans as potential intermediates.[6] However, the addition of H_2O_2 to the reaction mixture resulted in red-brown darkening of the solvent which decreased the chemical yield. Recently, we have found that *Camellia sinensis* cell culture is an efficient source of POD as "reagents" in organic synthesis and that a huge amount of H_2O_2 is produced in plant cell cultures with the addition of foreign substrates.[7] Thus we planned to construct the skeleton of **1** using *C. sinensis* cell culture (as an enzyme source)-catalyzed ring-closure reaction of dibenzylbutanolide (**11**) as the target synthetic intermediate to cyclic product **12** *via* a hypothetical quinone methide intermediate (A), as shown in Chart 3.

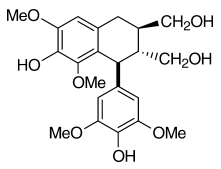

Chart 1. (±)-Lyoniresinol (**1**)

Results and Discussion

Butanolide (**11**) as the target synthetic intermediate for the synthesis of **1** was prepared according to the method shown in Chart 4. Benzyl ether (**5**), which was synthesized from 4-hydroxy-3,5-dimethoxybenzaldehyde (**4**) with benzyl chloride and potassium carbonate (reflux 6 h, 92%), was subjected to Stobbe condensation with dimethylsuccinate to give the conjugated hemisuccinate **6** in 69% 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.[8] The resulting double bond of the hemisuccinate **6** was reduced with magnesium in methanol to afford the unconjugated hemisuccinate **7** in 73% yield. The ^{1}H-NMR spectrum of **7** 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 **7** was treated with lithium borohydride to af-

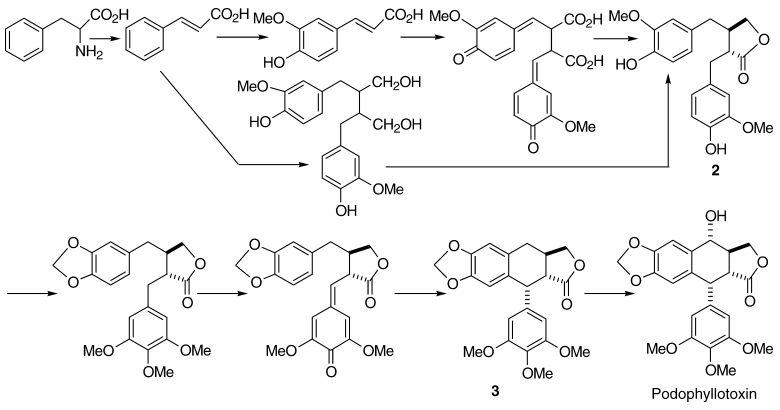

Chart 2. Proposed Biosynthetic Pathway of Podophyllotoxin

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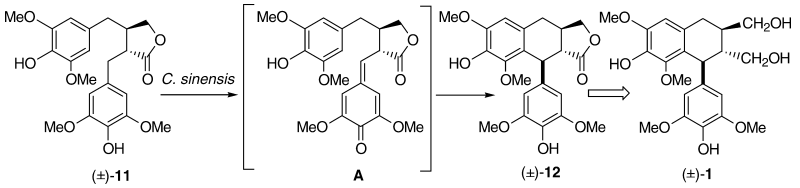


Chart 3

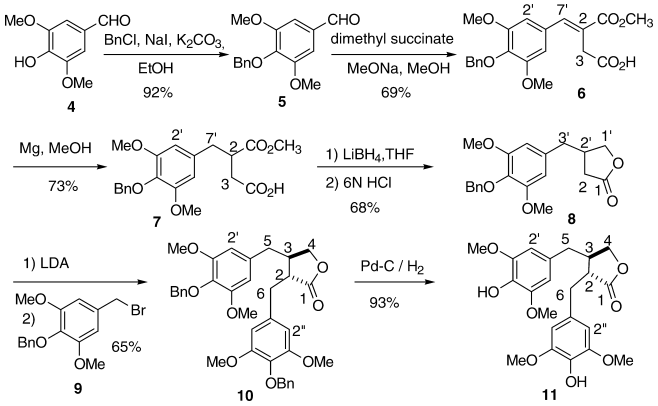
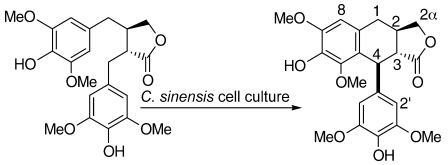


Chart 4. Synthesis of Dibenzylbutanolide **11**

ford butanolide (**8**) directly in 68% yield. The ^{1}H-NMR spectrum of **8** showed two new methylene protons at C1′ as doublets of doublets at δ 4.05 and δ 4.35, respectively. Alkylation of **8** with the bromide **9** gave the dibenzylbutanolide **10** in 65% yield. The ^{1}H-NMR spectrum of the product **10**, in comparison with that of **8**, 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 benzyl group, **10** was hydrogenated over Pd on carbon to give bis(hydroxybenzyl)butanolide **11** in 93% yield.

Next, POD-catalyzed oxidative coupling of **11** was examined with *C. sinensis* cell culture[9] which produced POD [15.5 units (U)/ml].[7] The oxidative coupling reaction of the dibenzylbutanolide **11** to cyclic product **12** was performed in small-scale experiments (250 mg of **11**) with freely suspended cells in the stationary phase after 30 d of incubation (50 g of cells and 200 ml of broth). To derive optimum conditions for the biotransformation of **11** to **12** using *C. sinensis*, we evaluated the biological and reaction parameters (the age of *C. sinensis*). Oxidative coupling was performed using cells of various ages (20—52 d old), as shown in Table 1 (entries 1—4). The optimum age of *C. sinensis* was 30 d (entry 2). A longer reaction time (24 h) promoted the decomposition of **12**. Then the reaction was carried out for different times using *C. sinensis* (30 d) (entries 5—7). The optimum reaction time was 10 h (entry 7). It was concluded that 30-d-old *C. sinensis* and a reaction time of 10 h would give optimum yields of **12**. The structure of cyclic product **12** was confirmed by analysis of the ^{1}H-NMR spectrum, which revealed only aromatic proton singlets (δ 6.30 and 6.51 for protons at C2′, C6′, and C8). The *trans* relationship of the lactone ring (stereochemistry of C3 and C4) was confirmed from the proton signal at C3 (doublets of doublets at 2.40 ppm with coupling constants of 15.4 and 11.6 Hz) and

Table 1. Biotransformation of Dibenzylbutanolide **11** to Cyclic Product **12** by *C. sinensis* Cell Culture



Entry	Plant age	Time (h)	Product **12** (%)	Recovery **11** (%)
1	20	24	3	0
2	30	24	22	0
3	42	24	8	10
4	52	24	10	0
5	30	1	0	86
6	30	5	34	42
7	30	10	48	19

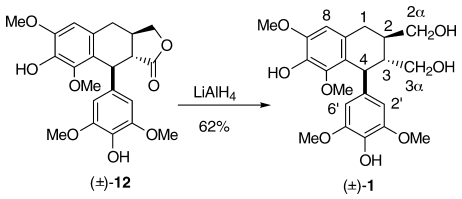


Chart 5

proton signal at C4 (a doublet at 4.10 ppm with coupling constant of 11.6 Hz).

Next, we performed a large-scale biotransformation with a bioreactor using *C. sinensis* cell culture under optimum conditions. The bioreactor was fitted with a peristaltic pump and a filter. Substrate **11** (1 g in 8 ml ethanol) was added to the freely suspended *C. sinensis* cell culture (200 g of cells and 800 ml of broth, 30 d old) in the bioreactor for 10 h to afford **12** in 62% yield.

Finally, **12** thus obtained was reduced with lithium aluminum hydride (LAH) in THF to give **1** in 62% yield as shown in Chart 5. The structure of synthetic lyoniresinol (**1**) was confirmed by a comparison of the ^{1}H-NMR and FAB-MS data with that reported.[3]

Conclusion

In this study, we used *C. sinensis* cell cultures, in which cell wall peroxidases rapidly metabolize a huge amount of H_2O_2 produced by the addition of foreign substrates. We succeeded in the oxidative coupling of the dibenzylbutanolide **11** with *C. sinensis* cell culture in the absence of foreign hydrogen peroxide as a cofactor. The development of enzymes for oxidation reactions aimed at green chemistry is very im-

portant. HRP is a commercially available metalloporphyrin oxidative enzyme and has been established as an effective biocatalyst for organic reactions using H_2O_2. However, in the oxidative coupling of dibenzylbutanolide, the addition of H_2O_2 to the reaction decreases chemical yield. 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 coupling by HRP.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus without correction. ^{1}H-NMR spectra were recorded on a JEOL JNM-EX 270 FT NMR (270 MHz). Chemical shifts (δ) are given in ppm with tetramethylsilane as an internal standard, and coupling constants (J) are given in Hz. FAB-MS were recorded on a JEOL JMS-SX 102 mass spectrometer, and HR-MS on a JEOL JMS-DX 300 mass spectrometer. Thin-layer chromatography (TLC) was performed on silica gel (Kieselgel 60F_{254} on aluminum sheets, Merck). All compounds were located by spraying the TLC plates with a 10% solution of phosphomolybdic acid in ethanol and heating it on a hot plate. Preparative TLC was performed on preparative layer chromatography plates (Kieselgel 60F_{254}, 2 and 0.5 mm, Merck). Column chromatography was performed on silica gel (Kieselgel 60, 70—230 mesh, Merck).

Cultivation of *C. sinensis* Cells Suspensions of *C. sinensis* cells were subcultured every 14 d by transferring a 2-week culture (10 ml) into B5[10] 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 11 with *C. sinensis* Cell Cultures Compound **11** (250 mg) in ethanol (2 ml) was added to the freely suspended *C. sinensis* (50 g of cells and 200 ml of broth, pH 5.8). The mixture was shaken at 25 °C on a rotary shaker (110 rpm) in the dark. Upon 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 with hexane–AcOEt (2 : 1). The reaction time and the chemical yield are listed in Table 1.

4-Benzyloxy-3,5-dimethoxybenzaldehyde (5) A suspension of 4-hydroxy-3,5-dimethoxybenzaldehyde (**4**, 4.5 g, 24.6 mmol), potassium carbonate (3.7 g, 28.5 mmol), benzyl chloride (3.7 g, 29.5 mmol), sodium iodide (1.0 g, 6.5 mmol), and ethanol (250 ml) was refluxed for 6 h while stirring with a mechanical stirrer. Water (10 ml) was added and the ethanol removed *in vacuo*. The slurry was poured into a mixture of 1 M sodium hydroxide (20 ml) and ice (8 g). The solids were filtered off, washed with ice-cold water (3×20 ml), and dried *in vacuo*. The residue was subjected to silica gel column chromatography using hexane–AcOEt (7 : 1), hexane–AcOEt (5 : 1), and hexane–AcOEt (4 : 1) to afford **5** (6.2 g, 92%). ^{1}H-NMR ($CDCl_3$) δ: 3.90 (6H, s, –OMe), 5.13 (2H, s, –OCH_2Ph), 7.11—7.48 (7H, m, aromatic), 9.86 (1H, s, –CHO).

4-Benzyloxy-3,5-dimethoxybenzyl Bromide (9) Sodium borohydride (0.9 g, 24.6 mmol) was carefully added to a solution of benzyl ether **5** (6.0 g, 22.0 mmol) in EtOH (200 ml). The mixture was stirred for 4 h. Aqueous NH_4Cl (50 ml) was added and the solution was extracted with ether (2×100 ml). The ether extracts were dried over $MgSO_4$, filtered, and evaporated *in vacuo* to afford benzyl alcohol (5.7 g, 95%). PBr_3 (1.8 g, 6.7 mmol) was carefully added to a solution of benzyl alcohol (1.8 g, 6.6 mmol) in ether (200 ml) under Ar at 0 °C. The mixture was stirred for 1 h at 0 °C. $NaHCO_3$ was added and the solution was neutralized. The solution was extracted with ether (2×100 ml). The ether extracts were dried over $MgSO_4$, filtered, and evaporated *in vacuo* to afford the benzyl bromide **9** (1.4 g, 61%). ^{1}H-NMR ($CDCl_3$) δ: 3.83 (6H, s, –OMe), 4.46 (2H, s, –CH_2Br), 5.00 (2H, s, –OCH_2Ph), 6.61 (2H, s, 2-H, 6-H), 7.26—7.50 (5H, m, –OCH_2Ph).

2-(4-Benzyloxy-3,5-dimethoxybenzylidene)butanedioic Acid 1-Methyl Ester (6) Sodium methoxide (4.6 g, 85.0 mmol) was carefully added to dry methanol (20 ml) under Ar. A solution of benzyl ether **5** (5.7 g, 21.0 mmol) in dimethyl succinate (4.3 g, 29.0 mmol) was added dropwise over 40 min during reflux. After an additional 4.5 h of stirring during reflux, the bulk of the solvent was removed *in vacuo*. The suspension was cooled to 0 °C and acidified with hydrochloric acid 6 M. The solids were removed by filtration and the filtrate was extracted with dichloromethane (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 (40 : 1) to afford the hemisuccinate **6** (5.6 g, 69%) as an amber resin that solidified at 0 °C (mp 122—130 °C). *Anal.* Calcd for $C_{21}H_{22}O_7$: C, 65.28; H, 5.74. Found: C, 65.26; H, 5.65. ^{1}H-NMR ($CDCl_3$) δ: 3.62 (2H, s, 3-H), 3.82 (6H, s, –OMe), 3.85 (3H, s, –COOMe) , 5.05 (2H, s, –OCH_2Ph), 6.64 (2H, s, 2′-H and 6′-H), 7.26—7.49 (5H, m, –OCH_2Ph), 7.85 (1H, s, 7′-H).

(±)-2-(4-Benzyloxy-3,5-dimethoxybenzyl)butanedioic Acid 1-Methyl Ester (7) The hemisuccinate **6** (5.2 g, 13.5 mmol) was added to a suspension of magnesium shavings (5.0 g, 208 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 h. The suspension was acidified with hydrochloric acid 6 M and the remaining solids were removed by filtration. The filtrate was extracted with dichloromethane (3×50 ml), washed with brine (100 ml), 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 the hemisuccinate **7** (3.8 g, 73%). The analytical sample was recrystallized from AcOEt–hexane as a yellow powder (mp 102—105 °C). *Anal.* Calcd for $C_{21}H_{24}O_7$: C, 64.94; H, 6.23. Found: C, 64.72; H, 5.72. ^{1}H-NMR ($CDCl_3$) δ: 2.49 (1H, dd, J=17.0, 4.6 Hz, 3-H), 2.65—2.76 (2H, m, 2-H, 3-H), 2.97—3.10 (2H, m, 7′-H), 3.68 (3H, s, –COOMe), 3.80 (6H, s, –OMe), 4.98 (2H, s, –OCH_2Ph), 6.35 (2H, s, 2′-H, 6′-H), 7.28—7.49 (5H, m, –OCH_2Ph).

3-(4-Benzyloxy-3,5-dimethoxybenzyl)butanolide (8) Lithium borohydride (294 mg, 5.7 mmol) in dry THF (50 ml) was carefully added to a solution of the hemisuccinate **7** (3.3 g, 8.5 mmol) in THF (80 ml) during reflux under Ar. The solution was stirred for 4 h during reflux and then cooled to room temperature. Water (2 ml) and hydrochloric acid 6 M (3 ml) were added and the solution was stirred at room temperature for 15 h. The bulk of the solvent was removed by evaporation *in vacuo* and the resultant mixture was extracted with ether (50 ml). The organic extract was washed with saturated sodium bicarbonate (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 (20 : 1) to afford β-butanolide **8** (2.0 g, 68%). The analytical sample was recrystallized from AcOEt–hexane as a white powder (mp 104—108 °C). *Anal.* Calcd for $C_{20}H_{22}O_5$: C, 70.16; H, 6.48. Found: C, 70.03; H, 6.52. ^{1}H-NMR ($CDCl_3$) δ: 2.30 (1H, dd, J=17.5, 6.6 Hz, 2-H), 2.55—2.92 (4H, m, 2-H, 2′-H, 3′-H), 3.81 (6H, s, –OMe), 4.05 (1H, dd, J=5.9, 9.2 Hz, 1′-H), 4.35 (1H, dd, J=6.9, 9.2 Hz, 1′-H), 4.99 (2H, s, –OCH_2Ph), 6.34 (2H, s, 5′-H, 9′-H), 7.26—7.49 (5H, m, –OCH_2Ph).

***trans*-2-(4-Benzyloxy-3,5-dimethoxybenzyl)-3-(4-benzyloxy-3,5-dimethoxybenzyl)butanolide (10)** To a solution of diisopropylamine (1.3 g, 13.0 mmol) in dry THF (20 ml) at −78 °C was added *n*-BuLi (7.7 ml, 11.0 mmol) and stirring was continued for 30 min. β-Butanolide **8** (3.0 g, 8.0 mmol) in THF (15 ml) was added, and the bright yellow solution was stirred for 90 min prior to the addition of the bromide **9** (3.4 g, 10.0 mmol) in THF (15 ml) and further stirred for 16 h at −78 °C. The solution was warmed to 0 °C, acidified with hydrochloric acid 1 M, and extracted with dichloromethane (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 (1 : 1) to afford dibenzylbutanolide **10** (3.3 g, 65%) as a yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 2.42—2.65 (4H, m, 2-H, 3-H, 6-H), 2.95 (2H, dd, J=14.0, 8.4 Hz, 5-H), 3.76 (6H, s, –OMe), 3.78 (6H, s, –OMe), 3.88 (2H, dd, J=9.0, 8.4 Hz, 4-H), 4.97 (2H, s, –OCH_2Ph), 4.99 (2H, s, –OCH_2Ph), 6.19, 6.38 (2H each, s, s, 2′-H, 6′-H, 2″-H, 6″-H), 7.24—7.47 (10H, m, –OCH_2Ph). FAB-MS m/z 598 (M^+), HR-MS m/z: 598.2589 (Calcd for $C_{36}H_{38}O_8$: 598.2567).

***trans*-2-(3,5-Dimethoxy-4-hydroxybenzyl)-3-(4-hydroxy-3,5-dimethoxybenzyl)butanolide (11)** Palladium-on-charcoal (5%, 1.5 g) and **10** (1.4 g, 2.3 mmol) were suspended in AcOEt–EtOH (1 : 3) (30 ml) and stirred under hydrogen at atmospheric pressure for 3.5 h. The catalyst was filtered off and the solvent evaporated *in vacuo* to yield bis(hydroxybenzyl)butanolide **11** (0.9 g, 93%) as an amorphous white solid. The analytical sample was recrystallized from diethyl ether/petroleum ether as a white powder (mp 60—63 °C). FAB-MS m/z 418 (M^+), HR-MS m/z: 418.1642 (Calcd for $C_{22}H_{26}O_8$: 418.1628). ^{1}H-NMR ($CDCl_3$) δ: 2.42—2.63 (4H, m, 2-H, 3-H, 6-H), 2.91 (2H, dd, J=5.6, 11.6 Hz, 5-H), 3.82—3.85 (13H, m, –OMe, 4-H), 4.21 (1H, dd, J=6.9, 8.9 Hz, 4-H), 5.42 (2H, s, –OH), 6.18 (2H, s, 2″-H, 6″-H), 6.32 (2H, s, 2′-H, 6′-H).

(3,5-Dimethoxy-4-hydroxyphenyl)-6,8-dimethoxy-7-hydroxy-3-hydroxymethyl-1,2,3,4-tetrahydro-2-naphthoic Acid γ-Lactone (12) Compound **11** (1 g in 8 ml ethanol) was added to the freely suspended *C. sinensis*

cell culture (200 g of cells and 800 ml of broth, 30 d old) for 10 h in a bioreactor fitted with a peristaltic pump and a filter. The mixture was reacted at 25 °C (110 rpm) in the dark. Upon 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 with hexane–AcOEt (1 : 2) to afford **12** (617 mg) in 62% yield. FAB-MS *m*/*z* 416 (M^+). HR-MS *m*/*z*: 416.1463 (Calcd for $C_{22}H_{24}O_8$: 416.1471). ^{1}H-NMR ($CDCl_3$) δ: 2.40 (1H, dd, J=15.4, 11.6 Hz, 3-H), 2.70—2.95 (2H, m, 1-H, 2-H), 3.10—3.20 (1H, m, 1-H), 3.83 (6H, s, –OMe), 3.91 (6H, s, –OMe), 3.98 (1H, dd, J=11.6, 7.3 Hz, 2α-H), 4.10 (1H, d, J=11.6 Hz, 4-H), 4.26 (1H, dd, J=11.6, 7.3 Hz, 2α-H), 5.37 (1H, br s, OH), 5.41 (1H, br s, OH), 6.30 (2H, s, 2′-H, 6′-H), 6.51 (1H, s, 8-H).

Lyoniresinol (1) A solution of **12** (500 mg, 1.2 mmol) in THF (10 ml) was added to a suspension of $LiAlH_4$ (36.3 mg, 0.96 mmol) in THF (15 ml) at 0 °C, and the mixture was stirred at room temperature for 1 h. The mixture was extracted with ether (50 ml). The organic extract was evaporated *in vacuo*. The residue was subjected to silica gel column chromatography using hexane–AcOEt (1 : 2) to afford **1** (313 mg, 62%) as a white amorphous solid. FAB-MS *m*/*z* 420 (M^+). HR-MS *m*/*z*: 420.1796 (Calcd for $C_{22}H_{28}O_8$: 420.1784). ^{1}H-NMR ($CDCl_3$): δ: 1.72 (1H, m, 2H), 1.90 (1H, m, 3-H), 2.53—2.71 (2H, m, 1-H_2), 3.29 (3H, s, OMe), 3.50—3.77 (4H, m, 2α-H_2, 3α-H_2), 3.78 (6H, s, OMe), 3.87 (3H, s, OMe), 3.99 (1H, d, J=7.6 Hz, 4-H), 5.44 (2H, br s, OH), 6.34 (2H, s, 2′-H, 6′-H), 6.44 (1H, s, 8H).

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