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Journal of Molecular Catalysis B: Enzymatic 32 (2004) 7–13
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Peroxidase-catalyzed synthesis of neolignan and its anti-inflammatory activity

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Received 27 August 2004; received in revised form 21 September 2004; accepted 25 September 2004
Available online 28 October 2004

Abstract

Different peroxidases were used to catalyze the synthesis of neolignan. Horseradish peroxidase (type I, Sigma) was employed successfully to catalyze the formation of magnolol by the phenolic oxidative coupling of allyphenol. The effects of organic co-solvents, pH, addition rate of H_2O_2, and the ratio of enzyme/substrate on the yields of dimer were studied. After the peroxidase-catalyzed reaction was performed, different neolignans were isolated and identified as magnolol (**3**), the trimeric *ortho–ortho*-coupled product dunnianol (**4**), the *ortho-O*-coupled product isomagnolol (**5**), and the Pummerer's ketone derivative (**6**). The inhibitory activities of magnolol against cyclo-oxygenase and lipoxygenase were also reported.

Keywords: Neolignan; Enzymatic synthesis; Peroxidase; COX; LOX

1. Introduction

Magnolol (**3**) is a significant bioactive constituent isolated from the bark of *Magnoliae officinalis*, or *M. obovata*. Recently, magnolol (**3**) has been demonstrated to possess potent antioxidative activity [1–3], anxiolytic [4], anti-inflammatory [5], and anti-cancer [6] activities. Magnolol (**3**) can reduce myocardial ischemia/reperfusion injury [2], inhibit thromboxane and leukotriene synthesis [5], and have cytotoxicity against A459 cell-line (human non-small lung cell cancer), SK-MEL-2 (human melanoma), SK-OV-3 (ovarian cancer), XF498 (CNS cell-line), HCT-15 (colon cancer), and HepG-2 (liver cancer) [6–9].

Magnolol (**3**) belongs to a group of neolignan. It has been proposed that phenolic oxidative coupling reaction is a key step for the biosynthesis of this group of natural products [10]. Magnolol (**3**) has been successfully obtained by chemical syntheses such as Stille's coupling [11]. Chemically catalyzed phenolic oxidative coupling of 4-allylphenol (**2**) in the presence of Fe(III) has also been used successfully to synthesize five different neolignans including magnolol (**3**) [12].

Peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) have been successfully employed to catalyze oxidative coupling of phenols and aromatic amines [13–15]. These compounds are oxidized by peroxidase in the presence of hydrogen peroxide to form dimeric, oligomeric or polymeric products.

Recently, peroxidase-catalyzed the oxidative coupling have been used successfully to synthesize the dimer derivatives of naphthol [16,17], tyrosine [18–21], hydroxyphenylglycine [22,23], eugenol [24] and coniferyl alcohol [25] with satisfactory yields. Peroxidases catalyzed polymerization of phenols or aromatic amines have also been applied for the removal of phenols from wastewater [26,27].

The present study employed peroxidase to catalyze phenolic coupling for the biomimetic synthesis of magnolol (**3**). 4-Allylphenol (**2**) was used as the starting material. Since there is no *ortho*-substituent next to the phenolic hydroxyl group, polymerization due to *ortho–ortho* C–C coupling

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doi:10.1016/j.molcatb.2004.09.009

or *ortho–ortho* C–O coupling can be expected. This study demonstrated the effects of different peroxidases, pH, organic solvents/buffers, and different concentrations of and rates of addition of H_2O_2 on the yield of the *ortho–ortho* C–C dimer of 4-allylphenol (**2**) formation.

2. Materials and methods

2.1. General

HPLC analysis was conducted with LabAlliance liquid chromatography equipped with a Series 1500 HPLC pump, Model 500 Variable UV–vis detector (State College, PA, USA). Reversed-phase column (15 cm × 4.6 mm, 5 μm; Veropak Inertsil 5 ODS) was used throughout this study. Analyses were carried out using methanol–water (8:1, v/v) as the mobile phase at a flow rate of 1.0 ml/min and detected at 288 nm. The chromatographic data was handled by using SCIC software. 4-Allylphenol (**2**) and magnolol (**3**) eluted at 5.3 and 12.5 min, respectively. Authentic sample of magnolol (**3**) was used as the external standard to calibrate the amount of magnolol and other side products in the reaction mixtures.

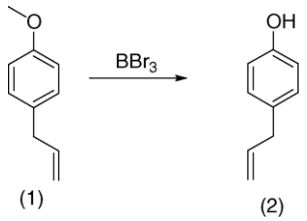
Structures were confirmed with a 500 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) spectrometer with TMS as an internal reference.

2.2. Materials

All the organic solvents were of HPLC grade and purchased from Merck (Düren, Germany). Enzymes including peroxidase: such as horseradish peroxidase (type I, HRP-I, 148 units/mg, 1 unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C), horseradish peroxidase (type VI, HRP-VI, 987 units/mg, 1 unit will oxidized 1 μmol of ABTS/min at 5 °C at pH 5), soybean peroxidase (SBP, 54 unit/mg, 1 unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C), and mushroom tyrosinase (1,2-benzenediol:oxygen oxidoreductase, EC 1.10.3.1, 2590 units/mg, 1 unit = ΔA_{280} of 0.001 min^{-1} at pH 6.5 at 25 °C in 3 ml reaction mixture containing L-tyrosine) were purchased from Sigma (St. Louis, MO, USA). Indomethacin and other biochemicals were obtained from (St. Louis, MO, USA). Silica gel 60 (230–400 mesh) was obtained from Merck (Düren, Germany). Borane tribromide–dimethylsulphide complex and 4-allylanisole (**1**) were purchased from Aldrich (Milwaukee, WI, USA). All the enzymes, reagents and solvents were used as received without further purification. Authentic magnolol sample was obtained from the Pharmaceutical Industry Technology and Development Center (Taipei, ROC).

2.3. Preparation of 4-allylphenol

To the solution of 4-allylanisole (**1**) (1 g, 6.75 mmol) in dichloroethane (10 ml) was added borane tribromide-dimethylsulphide complex (1.1 equiv.). After the reaction mixture was refluxed for 1 h under nitrogen atmosphere, the reaction was quenched with water. The aqueous phase was separated and extracted with dichloroethane (10 ml × 3 ml). The combined organic solution was dried over sodium sulfate and concentrated under reduced pressure. After separation by column chromatography (silica gel, hexane/ethyl acetate, 4:1), the desired product, 4-allylphenol (**2**) 0.89 g, 98%), was obtained as a colorless syrup. ^{1}H NMR of 4-allylphenol (**2**) δ_H(acetone-d_6): 6.97 (2H, d, J = 8.35 Hz, H-3/5), 6.73 (2H, d, J = 8.45 Hz, H-2/6), 5.92 (1H, m, H-8), 5.02 (1H, dd, J = 18.7, 1.8 Hz, H-9), 4.97 (1H, dd, J = 9.8, 1.8 Hz, H-9), 3.26 (2H, d, J = 6.15 Hz, H-7) (Scheme 1).



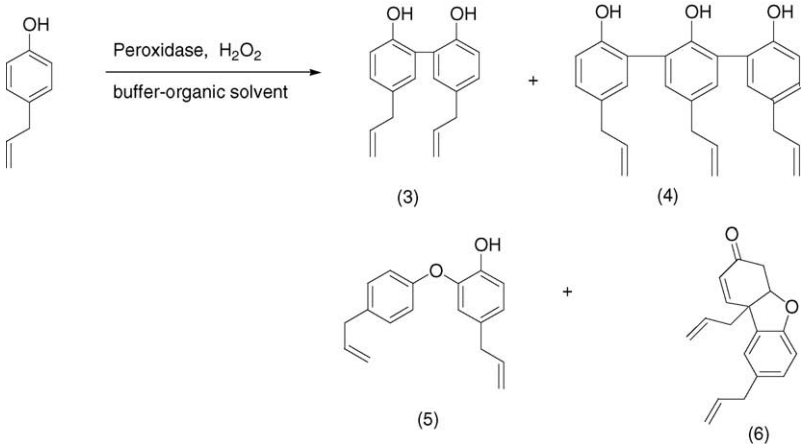
Scheme 1. Preparation of 4-allylphenol.

2.4. General procedure for the study of the oxidative coupling conditions of 4-allylphenol catalyzed by peroxidase

To the different buffer solutions (50 mM phosphate buffer, pH 4.0, 6.0 or 7.0) or (50 mM borate buffer, pH 9.0 or 9.5) were added 4-allylphenol **2** (0.1 mmol) in different water-miscible organic solvents (acetonitrile, dioxane, or methanol, 5, 10, or 30% (v/v) of the total reaction mixtures) or water-immiscible organic solvents (dichloroethane, ethyl acetate or *tert*-butyl methyl ether, 1:1 (v/v)). After the addition of enzymes (horseradish peroxidase (HRP), soybean peroxidase (SBP), or tyrosinase, 100–2000 units/mmol substrate), different concentrations of hydrogen peroxide were added slowly (final amount: 0.02–0.12 mmol). The reaction mixture was vigorously stirred at room temperature. Aliquots (50 μl) of reaction mixture were withdrawn and the reaction was stopped by the addition of saturated sodium bisulphite solution following by extraction with ethyl acetate. The progress of reaction was monitored by HPLC.

2.5. Typical procedure for the preparation of magnolol (3)

To the solution of 4-allylphenol 2 (140 mg, 1 mmol) in 5 ml of methanol and 45 ml of phosphate buffer (50 mM, pH 6.0) was added HRP (Sigma, type I, 1000 units). Hydrogen peroxide (1.0% (v/v) in water) was added slowly to initiate the reaction. The reaction mixture was vigorously stirred at room temperature for 15 min (0.5 mmol of hydrogen peroxide was added). The reaction was quenched by the addition of sodium bisulphite. After acidification, the reaction mix-



Scheme 2. Peroxidase-catalyzed oxidative coupling of 4-allylphenol.

ture was extracted with ethyl acetate. The combined organic solution was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The reaction mixture was separated by silica gel column chromatography (hexane:ethyl acetate = 10:1). The desired product, magnolol (**3**) (38 mg, yield = 27.3 mol% based on 4-allylphenol), was obtained as a colorless syrup and all the other fractions were pooled together. ^{1}H NMR of magnolol (**3**) δ_H(acetone-d_6): 7.09 (2H, d, J = 2.04 Hz, H-5), 7.04 (2H, dd, J = 8.2, 2.1 Hz, H-3), 6.90 (2H, d, J = 8.2 Hz, H-2), 5.97 (2H, m, H-8), 5.07 (2H, dd, J = 17.0, 1.8 Hz, H-9), 4.99 (2H, dd, J = 10.1, 1.8 Hz, H-9), 3.33 (4H, d, J = 6.7 Hz, H-7) (Scheme 2).

The first fraction from the previous column chromatography was further purified by another column chromatography (silica gel, hexane:ethyl acetate = 20:1). The first fraction was identified as Pummerer's ketone derivative (**6**) (13.4 mg, 5 mol% based on 4-allylphenol) and the second fraction was identified as isomagnolol (**5**) (10.3 mg, yield = 3.9 mol% based on 4-allylphenol). The third fraction from the previous column chromatography was further purified by preparative HPLC (Luna C18, 25 cm × 10 mm, 5 μm, Phenomenex, 80% methanol, 5 ml/min). The fractions eluted at 8–9 min were collected and concentrated under reduced pressure. The compound was identified as (**4**) (1.5 mg, 0.4 mol% based on 4-allylphenol).

Dunnianol (**4**) δ_H(acetone-d_6): 7.17 (2 H, d, J = 2.0 Hz, H-5), 7.09 (2 H, s, H-3′/5′), 6.90 (2 H, dd, J = 8.1, 2.0 Hz, H-3), 6.71 (2 H, d, J = 8.1 Hz, H-2), 6.00 (3 H, m, H-8/8′), 5.08 (2 H, dd, J = 17, 1.7 Hz, H-9 or 9′), 5.06 (2 H, dd, J = 17, 1.8 Hz, H-9 or 9′), 4.96 (3 H, d, J = 10, H-9), 3.33 (6 H, m, H-7/7′).

Isomagnolol (**5**) δ_H(acetone-d_6): 8.1 (1H, OH), 7.14 (2H, d, J = 8.5 Hz, H-3′/5′), 6.93 (1 H, d, J = 8.2 Hz, H-2), 6.88 (1H, dd, J = 8.2, 1.6 Hz, H-3), 6.83 (2H, d, J = 8.5 Hz, H-2′/6′), 6.77 (1H, d, J = 1.6 Hz, H-5), 5.95 (2H, m, H-8/8′), 5.05 (4H, m, H-9/9′), 3.32 (2H, dd, J = 6.7 Hz, H-7′), 3.27 (2H, d, J = 6.6 Hz, H-7).

Pummerer's ketone (**6**) δ_H(acetone-d_6):7.25 (1H, s, H-5), 7.01 (1H, dd, J = 8.15, 1.5 Hz, H-3), 6.72 (1H, d, J = 8.1 Hz, H-2), 6.49 (1H, dd, J = 10.3, 1.75 Hz, H-5′), 5.91 (1H, d, J = 10.2 Hz, H-6′), 5.92 (2H, m, H-8/H-8′), 5.26 (1H, d, J = 17 Hz, H-9 or H-9′), 5.16 (1H, J = 10.85 Hz, H-9 or H-9), 5.06 (1H, dd, J = 17, 1.5 Hz, H-9 or H-9′), 5.01 (1H, d, J = 10.5 Hz, H-9 or H-9′), 4.90 (1H, m, H-3′), 3.34 (2H, d, J = 6.7 Hz, H-7), 2.89 (1H, dd, J = 17.25, 3.9 Hz, H-2′), 2.86 (1H, dd, J = 13.3, 2.7 Hz, H-7′), 2.83 (1H, dd, J = 17.45, 2.75 Hz, H-2′), 2.74 (1H, dd, J = 13.6, 7.7 Hz, H-7′).

2.6. Lipoxygenase inhibition studies

The lipoxygenase inhibitory activity of magnolol was assayed according to the methodology developed by Kondo et al. [28]. Briefly, arachidonic acid (100 mM, 50 μL) and magnolol (**3**) (different concentrations in methanol) or vehicle alone was added to the Tris–HCl buffer solution (0.05 M, pH 10.5, 0.04% Tween 20) in a polystyrene cuvette. The reaction mixture was then analyzed by a luminometer (Bio-Orbit Luminometer 1251, Turku, Finland). Luminol (1 mM, 0.1 ml) and soybean lipoxygenase (0.1 ml, 100 units) were then added via a microprocessor-controlled dispenser (Bio-Orbit Oy). The total volume of the reaction mixture was 1.0 ml. The light emitted from the sample was measured monitored. Luminescence above baseline was then plotted against time and quantified as the area under the curve. Nordihydroguaiaretic acid (NDGA) was used as the positive control.

2.7. Cyclooxygenase inhibition studies

The COX-2 inhibitory activity of magnolol was assayed according to the methodology developed by Forghani et al. [29]. Briefly, to the Tris–HCl buffer solution (0.05 M, pH 6.5, 0.04% Tween 20) in a polystyrene cuvette was added 0.1 ml of rhCOX-2 (Calbiochem, Darmstadt, Germany). Arachidonic acid (100 mM, 50 μL) and magnolol (different concentrations in methanol) or vehicle alone were added and then the enzyme mixture was preincubated at 35 °C for 15 min. The reaction mixture was then analyzed by a luminometer (Bio-Orbit Luminometer 1251, Turku, Finland). Luminol (1 mM, 0.1 ml) was then added via a microprocessor-controlled dis-

penser (Bio-Orbit Oy). The total volume of the reaction mixture was 1.0 ml. The light emitted from the sample was measured at 0.5 s intervals for 90 s. Luminescence above the baseline was then plotted against time and quantified as the area under the curve. Indomethacin was used as the positive control.

3. Results and discussion

Among the three different peroxidases (HRP-I, HRP-VI, SBP) and an oxidase (tyrosinase) that were used to catalyze the dimerization of 4-allylphenol (**2**) in the preliminary study, horseradish peroxidase (type I, HPR-I)-catalyzed reaction gave the highest yields of the dimer (**3**) (Table 1). Under the same reaction conditions (pH 6.0; methanol, 10% (v/v); H_2O_2 addition rate: 0.2 equiv./min), the yield of magnolol (**3**) obtained from HRP-I catalyzed reaction was about 12 mol% (yield based on the starting material, 4-allyl-phenol (**2**)) while the yields obtained by the oxidative coupling in the presence of HRP-VI, SBP were less than 3 mol%. Although tyrosinase has been successfully employed to catalyze polymerization of tyrosine [30], the yield of magnolol (**3**) obtained in the presence of tyrosinase was nearly undetected. Thus, the HRP-1 was used throughout this study.

Since the reaction rate was also regulated by the addition rate and concentration of hydrogen peroxide, the effects of different rates of addition were also examined. The yield obtained by adding hydrogen peroxide with the addition rate of 0.03 equiv./min gave the highest yield of dimer (**3**) (Table 2, Fig. 1). If the addition of hydrogen peroxide was faster (0.2 equiv./min as in the preliminary study), the yield of dimer (**3**) was lower (12.1 mol%). If the reaction prolonged, the yield of the dimer (**3**) decreased caused by further reactions. Similar condition for the synthesis of bis-eugenol according to the literature was also studied [24], hydrogen peroxide (0.005 equiv./min) was added to the reaction mixture for 60 min (final amount of hydrogen peroxide: 0.3 equiv.) and the reaction progress was monitored after 15 h. It was obvious that if the reaction was too slow, only 4.8 mol% of magnolol was obtained and 48% of the starting material remained.

Table 1
Effects of different enzymes on the yield of magnolol[a]

Enzyme	Yield[b] (%)	H_2O_2[c] (equiv.)	Unreacted allylphenol (**2**) (%)
HRP type VI	2.4	1.0	20.1
SBP	1.7	1.0	36.7
Tyrosinase	0.0	–	–
HRP type I	12.1	0.4	28.3

[a] A 10% of methanol in phosphate buffer (pH 6.0), enzymes (1000 units/mmol), H_2O_2 addition rate: 0.2 equiv./min.
[b] Percentage of the yield of magnolol was based on allylphenol and determined by comparing with the authentic sample of magnolol according to the HPLC analysis.
[c] Final total amount of hydrogen peroxide in the reaction mixture.

Table 2
Effects of addition rate of H_2O_2 on the yield of magnolol[a]

H_2O_2 (equiv./min)	Yield[b] (%)	H_2O_2 (equiv.)[c]	Unreacted allylphenol (**2**) (%)
0.2	12.1	0.40	28.3
0.05	26.2	0.50	2.1
0.03	40.4	0.54	2.4
0.005	4.5	0.30	47.8

[a] A 10% of methanol in phosphate buffer (pH 6.0), HRP-1 (1000 units/mmol), H_2O_2 addition rate: as designated in the table.
[b] Percentage of the yield of magnolol was based on allylphenol and determined by comparing with the authentic sample of magnolol according to the HPLC analysis.
[c] Final total amount of hydrogen peroxide in the reaction mixture.

Phenolic oxidative coupling catalyzed by horseradish peroxidase (HRP) in aqueous organic co-solvents (such as dioxane, methanol, THF, acetonitrile) has been studied extensively [31]. Not only the stability of HRP was highly affected by the kinds and amount of organic solvents, but also the yields of the desired product were subjected to different organic solvents. Different water-miscible organic solvents (acetonitrile, methanol, dioxane, or DMF, 10% (v/v) in phosphate buffer of pH 6) were tried in this study, and the yields of magnolol obtained in the presence of dioxane or methanol were higher (28 and 40 mol%, respectively) than the yields obtained from the reactions in the presence of acetonitrile or DMF (Table 3). Although 28% of unreacted 4-allylphenol remained in the reaction mixture when the yield of magnolol reached 28 mol%, the yield of magnolol decreased if the reaction prolonged. The rate of consumption of magnolol for the formation to other side products was higher than the rate of the formation of magnolol in the presence of dioxane than other co-solvent system. The yields stated in the tables represented the highest yields obtained under the designated conditions. If the addition of hydrogen peroxide into the reaction mixture continued, the yields of magnolol (**3**) decreased.

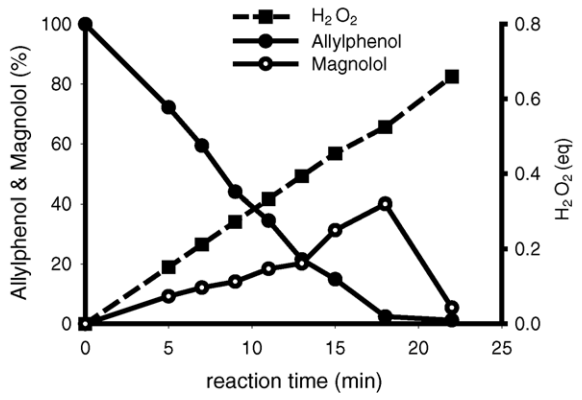


Fig. 1. Time-course of the HRP-1-catalyzed phenolic coupling of 4-allylphenol (**2**) (●) for the synthesis of magnolol (**3**) (○). The dashed line represented the amount of H_2O_2 (■) added in to the reaction mixture. Reaction condition: methanol (10%, v/v) in phosphate buffer (pH 6.0), HRP-1 (1000 units/mmol substrate), H_2O_2 addition rate (0.03 equiv./min).

Table 3
Effect of different organic co-solvents on the yield of magnolol[a]

Solvent (10%, v/v)	Yield[b] (%)	H_2O_2 (equiv.)[c]	Unreacted allylphenol (**2**) (%)
Acetonitrile	15.8	0.50	1.9
Methanol	40.4	0.55	2.4
Dioxane	28.2	0.27	20.6
DMF	26.0	0.43	9.4

[a] A 10% of organic solvent in phosphate buffer (pH 6.0), HRP I (1000 units/mmol), addition rate of H_2O_2: 0.03 equiv./min.
[b] Percentage of the yield of magnolol was based on allylphenol and determined by comparing with the authentic sample of magnolol according to the HPLC analysis.
[c] Final total amount of hydrogen peroxide in the reaction mixture.

We envisioned that water-immiscible organic solvents could extract the dimer (**3**) from the aqueous phase of the reaction mixture immediately after its occurrence and prohibit further polymerization. Different water-immiscible organic solvents were studied under similar conditions (phosphate buffer, pH 6.0, addition rate of H_2O_2: 0.03 equiv. H_2O_2/mol of substrate) as the reaction in the presence of methanol. If only 0.45 equiv. of H_2O_2 were added into the reaction mixtures containing different co-solvents, 4, 8 and 12 mol% of magnolol (**3**) were obtained in the reaction mixtures and 33, 38, and 34% of starting material remained in the presence of dichloroethane, *tert*-butyl methyl ether and ethyl acetate respectively. If the addition of H_2O_2 continued, the highest yield of magnolol (**3**) obtained in the presence of ethyl acetate, *t*-butyl methylether and dichloroethane were 22, 13 and 10 mol%, respectively (Table 4). If the reactions prolonged, lower yields of magnolol was obtained. The lower yields in heterogeneous system might be caused by less starting material in the aqueous phase to participate the enzymatic reactions in the two-phase reaction mixtures than in the homogeneous water-miscible co-solvent system and the heterogeneous systems promoted the consumption of magnolol.

When different percentages of methanol in phosphate buffer (pH 6.0) were studied, magnolol could be obtained in 40 mol% yields if 10% (v/v) methanol was used as the co-solvent (Table 5). However, if a higher content of methanol (30%, v/v) was used, the yield of magnolol was lower (12.6 mol%) and nearly 30% of unreacted starting material could be recovered as the result of enzyme denaturation due to a higher concentration of methanol.

Table 4
Effect of different organic solvents on the yield of magnolol[a]

Organic solvent	Yield[b] (%)	H_2O_2 (equiv.)	Unreacted allylphenol (**2**) (%)
Dichloroethane	9.5	0.75	10.4
tert-Butyl methyl ether	12.5	1.50	6.6
Ethyl acetate	21.7	1.08	10.9

[a] Organic solvent in phosphate buffer (pH 6.0) (1:1 v/v), HRP I (1000 units/mmol), addition rate of H_2O_2: 0.03 equiv./min.
[b] Percentage of the yield of magnolol was based on allylphenol and determined by comparing with the authentic sample of magnolol according to the HPLC analysis.

Table 5
Effects of methanol content on the yield of magnolol[a]

Methanol content (%, v/v)	Yield[b] (%)	H_2O_2 (eqiv.)	Unreacted allylphenol (**2**) (%)
5	15.2	0.45	13.4
10	40.4	0.55	2.4
15	26.4	0.48	5.3
20	27.6	0.42	19.0
30	12.6	0.36	31.5

[a] Methanol in phosphate buffer (pH 6.0), HRP I (1000 units/mmol), addition rate of H_2O_2: 0.03 equiv./min.
[b] Percentage of the yield of magnolol was based on allylphenol and determined by comparing with the authentic sample of magnolol according to the HPLC analysis.

Borate was well known to form ester complex with diol under basic condition. We envisioned that the dimer (**3**) would not continue to polymerize if the dimer could form borate ester complex so that no free OH group could form phenoxyl radical. Thus, borate buffer (pH 9.5) was tried in the presence of dioxane or methanol (10%, v/v) and the yield of magnolol obtained from both conditions were close to 27–29 mol%. However, when the borate buffer (pH 9.0) was used in the presence of dioxane, the yield was lower (11 mol%) (Table 6). The results suggested that pH played a more important role to obtain a higher yield of (**3**) than the formation of any diol borate ester.

Different enzyme/substrate ratios have been reported to affect the yields of tyrosine dimers [19]. When the amount of HRP-I increased from 100 units/mmol of substrate to 500 units/mmol of substrate, the yields of dimer increased from 6 to 18 mol% and 58% and 10% of the unreacted 4-allylphenol (**2**) remained respectively. However, when the amount of enzyme increased to 1500 or 2000 units/mmol, the yields of the dimer (**3**) obtained from the reaction mixtures decreased to 23 and 13–16% of starting material remained (Table 7). Based on the above observation, it was obvious that low concentration of peroxidase caused slow progress in the reaction and prolonged reaction caused enzyme denaturation before dimerization. On the other hand, if the amount of

Table 6
Effects of different organic solvents/buffer and pH on the yield of magnolol[a]

Solvent	Yield[b] (%)	H_2O_2 (equiv.)	Unreacted allylphenol (**2**) (%)
Phosphate buffer (pH 6.0)			
MeOH (10%, v/v)	40.4	0.54	2.4
Dioxane (10%, v/v)	27.3	0.25	27.4
Borate buffer (pH 9.0)-dioxane (10%, v/v)	11.2	0.25	24.1
Borate buffer (pH 9.5)			
Dioxane (10%, v/v)	29.2	0.40	29.4
MeOH (10%, v/v)	33	0.45	17.5

[a] A 10% of organic solvent in buffer, HRP I (1000 units/mmol), addition rate of H_2O_2: 0.03 equiv./min.
[b] Percentage of the yield of magnolol was based on allylphenol and determined by comparing with the authentic sample of magnolol according to the HPLC analysis.

Table 7
Effects of different amounts of HRP-I on the yields of magnolol[a]

HRP-I (units/mmol of substrate)	Yield[b] (%)	H_2O_2 (equiv.)	Unreacted allylphenol (**2**) (%)
100	5.8	0.45	58.3
500	17.9	0.45	9.5
1000	40.4	0.54	2.4
1500	22.7	0.45	16.3
2000	23.0	0.42	13.1

[a] Methanol (10%, v/v) in phosphate buffer (pH 6.0), HRP I (units/mmol of substrate designated in the table), addition rate of H_2O_2: 0.03 equiv./min.
[b] Percentage of the yield of magnolol was based on allylphenol and determined by comparing with the authentic sample of magnolol according to the HPLC analysis.

enzyme increased, the lower yields of the dimer might cause by consumption of dimer for further reactions.

A mmol-scale peroxidase-catalyzed oxidative coupling of 4-allylphenol (**2**) has been conducted (10% (v/v)methanol, pH 6.0, 18 min) and the products have been purified and identified (Scheme 2). The yields of dimeric *ortho–ortho* C–C-coupled derivative, magnolol (**3**) was about 30 mol% based on 4-allylphenol (**2**). The side products obtained by peroxidase-catalyzed oxidative coupling of 4-allylphenol (**2**) in the present studies were similar to the results obtained by Fe(III)-catalyzed oxidative coupling (Table 8) [12]. The yields of trimeric *ortho–ortho* C–C-coupled product dunnianol (**4**) obtained by peroxidase-catalyzed oxidative coupling of 4-allylphenol was 2.1%, the yield of the *ortho-O*-coupled product isomagnolol (**5**) was 7.4%, and the yield of the Pummerer's ketone derivative (**6**) was 9.6%. According to [12], employing $FeCl_3$ or $K_3Fe(CN)_6$ as the oxidative catalyst for phenolic coupling of 4-allylphenol (**2**) produced magnolol (**3**) in only 8–15 mol% yields. The yield of magnolol isolated from the HRP-1-catalyzed reaction mixture in the present study was higher than the yields of magnolol isolated from Fe(III)-catalyzed reaction mixtures. Since the profile of side-products obtained from the enzymatic reaction was similar to the side products obtained from the chemical reaction (Table 8), peroxidase might act as the free radical initiator and the phenoxyl radical might react with other phenolic compounds outside of the enzyme pocket. The oxidative coupled products obtained from 4-allylphenol, including magnolol, dunnianol, and Pummerer's ketone derivative have been reported isolated from the genus *Illicium* [12]. The *ortho-O*-coupling product, isomagnolol, has also been isolated from *Magnolia* bark.

Table 8
Comparison of the yields of different products obtained by chemically[a] and enzymatically[b] oxidative coupling of 4-allylphenol in the literature[a] and the present study[b]

	(**3**) (%)	(**4**) (%)	(**5**) (%)	(**6**) (%)	Unreacted (**2**) (%)
$K_3Fe(CN)_6$[a]	8.0	11.0	12.0	5.0	13.0
$FeCl_3$[a]	15.0	2.0	1.0	NA	58.0
HRP-1[b]	40.4	0.7	10.9	16.0	2.4
HRP-1[c]	26.0	0.7	12.5	11.2	9.4
HRP-1[d]	28.2	4.2	13.1	13.8	20.6
HRP-1[e]	15.0	0.2	3.4	9.9	4.4
HRP-1[f]	21.7	0.8	3.6	5.8	10.9

[a] Previous report: Ref. [12].
[b] Present study: methanol (10%, v/v) in buffer (pH 6.0), HRP I (1000 units/mmol).
[c] Present study: DMF (10%, v/v) in buffer (pH 6.0), HRP I (1000 units/mmol).
[d] Present study: dioxane (10%, v/v) in buffer (pH 6.0), HRP I (1000 units/mmol).
[e] Present study: TBME (10%, v/v) in buffer (pH 6.0), HRP I (1000 units/mmol).
[f] Present study: ethyl acetate (10%, v/v) in buffer (pH 6.0), HRP I (1000 units/mmol).

It is well established that the expression of cyclooxygenase-2 (COX-2) is induced by pro-inflammatory stimuli such as cytokines, bacterial lipopolysaccharide, growth factors, and tumor-promoting agents [31]. Selective COX-2 inhibitors are believed to avoid the gastrointestinal ulcer caused by the traditional non-selective non-steroid anti-inflammatory drugs [31]. Another arachidonate metabolic pathway is catalyzed by lipoxygenase (LOX) which converts arachidonate to leukotrienes. Leukotrienes can sustain the inflammatory process. One of lipoxygenase inhibitors, Zileuton, has been marketed to control the onset of asthma [32].

Magnolia bark has been used as an anti-inflammatory herb in the traditional Chinese medicine and magnolol has been identified as the principle anti-inflammatory constituent of *Magnoliae*. Recently, using the isolated rat peripheral neutrophile suspension, magnolol (3.7 μM) was demonstrated to suppress the A23187-induced thromboxane B2 (TXB2) and LTB4 formation [33]. By using the commercially available pure enzymes in this study, the inhibition of lipoxygenase (soybean) and cyclo-oxygenase (recombinant human COX-2) by magnolol were assayed by chemiluminescence. The activity of magnolol against soybean lipoxygenase (IC_{50}: 7.73 μM) was lower than the activity obtained by the well-known lipoxygenase inhibitor (NDGA, IC_{50}: 0.09 μM). Nevertheless, the activity of magnolol against human recombinant COX-2 (IC_{50}: 2.08 μM) was close to the activity of anti-arthritis drug, indomethacin (IC_{50}: 1.49 μM).

4. Conclusion

The neolignans can be obtained by peroxidase-catalyzed phenolic coupling. The yield of the dimer (**3**) obtained by peroxidase-catalyzed oxidative coupling of 4-allylphenol (**2**) was higher than that obtained by chemically catalyzed reactions. Magnolol (**3**) has a significant anti-inflammatory activity and its anti-COX-2 activity was close to the clinically used non-steroid anti-inflammatory drug, indomethacin.

Acknowledgement

This work was supported by the National Science Council of the Republic of China under Grant NSC 91-2113-M-019-001.

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