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ORIGINAL ARTICLE

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# 5α-Reductase inhibitory compounds produced by polymerization of resveratrol with horseradish peroxidase

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**Abstract** To produce 5α-reductase inhibitory compounds, resveratrol was enzymatically oxidized in a horseradish peroxidase (HRP)/$H_2O_2$ system. Ethyl acetate extract of the oxidation products showed strong 5α-reductase inhibitory activity with 10%–15% organic solvents in the system, whereas without organic solvent little inhibitory activity was exhibited. The optimum pH of enzymatic oxidation for acquisition of the inhibitory activity was 4.5. The inhibitory compounds were isolated and identified as resveratrol *trans*-dehydrodimer and resveratrol *cis*-dehydrodimer by comparing with published nuclear magnetic resonance data. The two resveratrol dehydrodimers have stronger inhibitory activity than natural resveratrol dimers and trimers found in *Shorea* species.

**Key words** 5α-Reductase inhibitor · Resveratrol oligomer · Horseradish peroxidase · Resveratrol dehydrodimer · Enzymatic polymerization

## Introduction

In typical androgen target tissues, testosterone is converted to a potent androgen 5α-dihydrotestosterone (DHT) by steroid 5α-reductase.[1] Exessive production of DHT is responsible for male-pattern baldness and the pathogenesis of benign prostatic hyperplasia, acne, and female hirsutism.[2] Therefore, 5α-reductase inhibitor may be able to remedy or prevent these androgen-dependent diseases.

Recently we have shown[3] that four resveratrol trimers – vaticanol A, ampelopsin C, melapinol A, melapinol B – have strong inhibitory activity against rat liver 5α-reductase. Their inhibitory activities were almost indistinguishable from each other, but no inhibition was observed in the case of resveratrol (**1**) itself. These results suggest that a certain part of the resveratrol trimer structure that is seen only in resveratrol oligomers could play an important role in enzyme inhibition. It is worth noting that part of the structure of active natural resveratrol trimers is expected to produce the dehydrogenative polymerization of resveratrol.

In the present paper we report an attempt to produce and isolate 5α-reductase inhibitory compounds from enzymatic dehydrogenative polymerization of resveratrol with horseradish peroxidase (HRP) and hydrogen peroxide.

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## Materials and methods

The $^1$H-nuclear magnetic resonance (NMR) experiments were performed in acetone-$d_6$ with trimethylsilane (TMS) as an internal standard, using a 400-MHz spectrometer. Ultraviolet (UV) spectra were measured in MeOH. The preparative high-performance liquid chromatography (HPLC) column employed was a GL Sciences C-18 column (Inertsil PREP-ODS, 20 mm i.d. × 250 mm; eluant: $H_2O$/$CH_3CN$, 62:38; 10 ml/min).

### Materials

Horseradish peroxidase (HRP) 343 units/mg was purchased from Wako Pure Chemical Industries and was used without further purification. Resveratrol was purchased from Sigma. $H_2O_2$ (30%) was purchased from Wako. All other commercial chemicals were of the highest grade available. *Shorea laevifolia* Endert. was collected in Indonesia by Dr. Wasrin Syafii (Bogor Agricultural University, Indonesia).

### Analytical incubations for determination of conditions

Analytical incubations (2 ml) contained McIlvine buffer (pH 3–7), 1 mg HRP, 0.1 mM resveratrol, 0.1 mM $H_2O_2$, and

an organic solvent (acetone 5–20 vol%, 1,4-dioxane 10 vol%, acetonitrile 10 vol%, ethanol 10 vol%, or methanol 10 vol%). The reactions were initiated by adding $H_2O_2$. The reaction mixtures were stirred at room temperature, and the reactions were terminated by adding 1 ml of ice-cold methanol/$H_2O$ (90:10) containing 2.0 mM ascorbate according to the methods of Potter et al.[4]

### Preparation, extraction, and isolation of products

A large-scale reaction was carried out to isolate products. The reaction was initiated by adding 1 mmol $H_2O_2$ to a mixture of 200 ml McIlvine buffer (pH 4.5) containing 10 mg HRP, 2 mmol resveratrol, and 10 vol% acetone. The reaction mixtures were stirred at room temperature, and the reactions were terminated by adding 10 ml of ice-cold methanol/$H_2O$ (90:10) containing 2.0 mM ascorbate.

The reaction mixture was extracted three times with 100 ml of ethyl acetate in a 500-ml volume separatory funnel to give water-soluble and ethyl acetate-soluble fractions. The ethyl acetate solution was concentrated to dryness in vacuo. The residue (278 mg) was separated repeatedly by preparative HPLC to give resveratrol *cis*-dehydrodimer [1.9 mg, room temperature (RT), 21 min] and resveratrol *trans*-dehydrodimer (207 mg, RT, 26 min).

### Resveratrol *trans*-dehydrodimer and resveratrol *cis*-dehydrodimer

*Trans*-dehydrodimer was pale brown solid (207 mg): UV $\lambda$max (MeOH) 308 nm. $\delta_H$ 4.46 (*d*, $J$ = 8.0), 5.44 (*d*, $J$ = 8.0), 6.18 (*d*, $J$ = 2.0, 2H), 6.25 (*t*, $J$ = 2.1), 6.27 (*t*, $J$ = 2.1), 6.53 (*d*, $J$ = 2.0, 2H), 6.84 (*d*, $J$ = 8.7, 2H), 6.85 (*d*, $J$ = 8.3), 6.90 (*d*, $J$ = 16.4), 7.05 (*d*, $J$ = 16.4), 7.24 (*d*, $J$ = 8.7, 2H), 7.25 (*brs*), 7.42 (*dd*, $J$ = 1.4, $J$ = 8.3). These spectra matched well with published data.[5] The *cis*-dehydrodimer was pale brown solid (1.9 mg): UV $\lambda$max (MeOH) 281 nm. $\delta_H$ 4.40 (*d*, $J$ = 8.5), 5.36 (*d*, $J$ = 8.5), 6.12 (*d*, $J$ = 2.0, 2H), 6.20 (*t*, $J$ = 2.1), 6.23 (*t*, $J$ = 2.1), 6.30 (*d*, $J$ = 2.0, 2H), 6.34 (*d*, $J$ = 12.3), 6.47 (*d*, $J$ = 12.2), 6.75 (*d*, $J$ = 8.3), 6.85 (*d*, $J$ = 8.7, 2H), 6.93 (*brs*), 7.20 (*dd*, $J$ = 1.4, $J$ = 8.3), 7.20 (*d*, $J$ = 8.8, 2H). These spectra matched well with published data.[5]

### Isolation of natural resveratrol dimers (−)-ε-viniferin and (−)-ampelopsin A from *Shorea* heartwood

Air-dried heartwood meal of *Shorea laevifolia* Endert. (0.5 kg) was extracted with methanol for 6 days at room temperature, and the extract was concentrated to about 50 ml. After adding the same volume of water, the mixture was partitioned with 100 ml of $Et_2O$ in a 500-ml separatory funnel to give aqueous- and $Et_2O$-soluble fractions. The $Et_2O$-soluble fraction was concentrated to dryness. The residue (14 g) was separated repeatedly by silica gel column chromatography (42 mm i.d. × 105 cm) using EtOAc-*n*-hexane gradient as eluent. Separate activity-guided fractionation was repeated on a smaller scale using EtOAc-*n*-hexane (2:1) as eluent to give (−)-ε-viniferin (**4**), pale brown solid: $[\alpha]_D$ −39° in MeOH (2.1 mg); $\delta_H$ 4.48 (*d*, $J$ = 5.4), 5.42 (*d*, $J$ = 5.4), 6.23 (*brs*, 2H), 6.32 (*d*, $J$ = 2.0), 6.71 (*d*, $J$ = 16.4), 6.72 (*brs*), 6.74 (*d*, $J$ = 8.5, 2H), 6.83 (*d*, $J$ = 8.5, 2H), 6.90 (*d*, $J$ = 16.4), 7.17 (*d*, $J$ = 8.5, 2H), 7.20 (*d*, $J$ = 8.5, 2H). These spectra matched well with published data.[6] It also gave (−)-ampelopsin A (**5**), pale brown solid: $[\alpha]_D$ −167°, in MeOH (17 mg); $\delta_H$ 4.14 (*d*, $J$ = 11.5), 5.40 (*d*, $J$ = 4.9), 5.42 (*d*, $J$ = 4.9), 5.74 (*d*, $J$ = 11.5), 6.14 (*d*, $J$ = 2.0), 6.22 (*d*, $J$ = 1.9), 6.43 (*d*, $J$ = 2.1), 6.63 (*d*, $J$ = 1.9), 6.63 (*d*, $J$ = 8.7, 2H), 6.75 (*d*, $J$ = 8.7, 2H), 6.77 (*d*, $J$ = 8.7, 2H), 6.89 (*d*, $J$ = 8.8, 2H), 7.10 (*d*, $J$ = 8.8, 2H). These spectra also matched well with published data.[7]

### Preparation of rat liver microsomes

Sprague-Dawley female rat livers were removed and homogenized in medium A (0.32 M sucrose, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 6.5), as described in a previous paper.[3] The homogenate was centrifuged at 10000*g* for 10 min at 0°C. The resulting pellets were suspended in medium A and centrifuged again. The microsomes were derived from further ultracentrifugation (110000*g* for 1 h at 0°C) of the supernatants obtained by precipitation. The protein content in the microsomes was determined by a Bio-Rad protein assay kit (catalog no. 500–0006) using bovine serum albumin (BSA) as a standard.

### Determination of 5α-reductase inhibitory activity

The reaction solution contains 0.1 ml testosterone (5 mM), 1 mM dithiothreitol, 0.5 ml NADPH (1 mM), inhibitory components (50 ppm), 0.1 ml microsomes (1 mg protein), and 2.2 ml sodium phosphate (20 mM), pH 6.5, in a total volume of 3 ml. Inhibitory components were dissolved in 0.1 ml of dimethylsulfoxide (DMSO); control tubes were placed in the same volume of DMSO. The reaction was started with the rat liver microsomes addition at 37°C for 10 min of incubation. After incubation the reaction was stopped by adding 0.1 ml of $H_2O_2$ (3 M). As internal standard 0.1 ml of cholesterol acetate (1 mM) was added, and the solution was extracted with 40 ml of $Et_2O$. The $Et_2O$ fraction was concentrated to dryness and dissolved in EtOAc. Inhibitory activity was evaluated by the dihydrotestosterone (DHT)/internal standard ratio derived from gas chromatography-mass spectrometry (GC-MS) analysis selected ion monitoring (SIM) according to the method of Shimizu et al.[8]

## Results and discussion

### Influence of reaction conditions

Dehydrogenative polymerization of 4-hydroxystilbenes, including resveratrol, by the HRP/$H_2O_2$ system has been re-

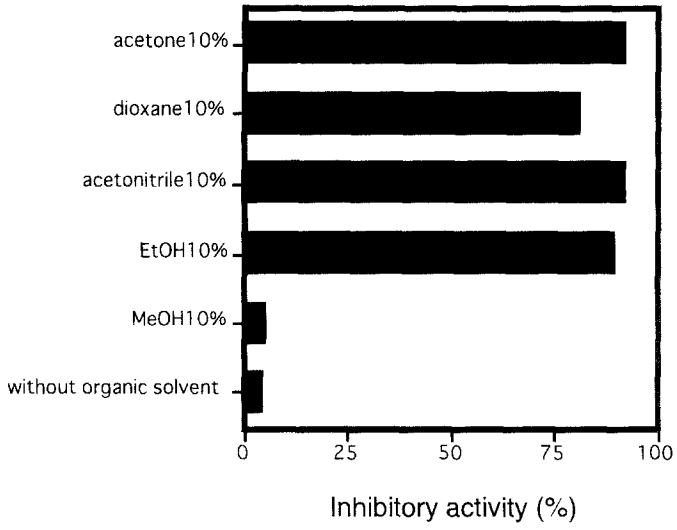


**Fig. 1.** Relations of a variety of organic solvents in the reaction system and rat liver 5α-reductase inhibitory activity on the ethyl acetate-soluble fraction of the reaction product. Buffer pH 5.0; product concentration 50 ppm in the assay system

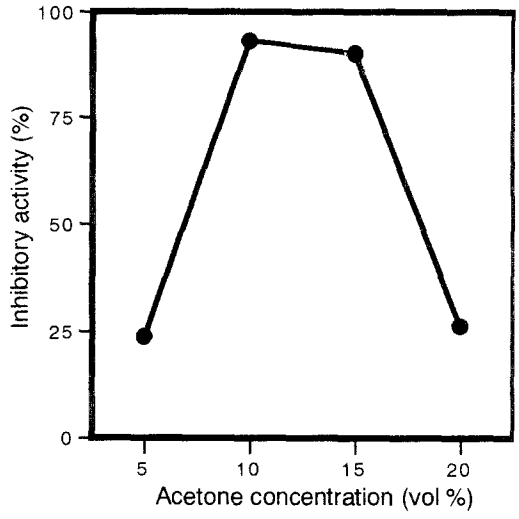


**Fig. 2.** Relation between the acetone concentration in the reaction system and rat liver 5α-reductase inhibitory activity on the ethyl acetate-soluble fraction of the reaction product. Buffer pH 5.0; product concentration 50 ppm in the assay system

ported,[9,10] so the object of the report was not to produce 5α-reductase inhibitory compounds; moreover, procedures for the enzymatic reaction are not described. We first examined the conditions of the enzymatic reaction in terms of the solvent and pH optima for producing 5α-reductase-inhibitory oligomeric compounds from resveratrol. The enzymatic reaction of resveratrol was conducted in a buffer (pH 5.0) containing various organic solvents in the same concentration (10%). Their influence on 5α-reductase inhibitory activities of reaction products are shown in Fig. 1. Strong inhibitory activity was observed when acetone, 1,4-dioxane, acetonitrile, and ethanol were employed as reaction solvents; whereas when no organic solvent was added, little inhibitory activity was exhibited. On the other hand, methanol showed about the same activity as water without organic solvent. Further experiments were performed in a buffer/acetone system because acetone is easier to remove than the other organic solvents owing to its high volatility when the reaction mixture is concentrated. The relation between the acetone concentrations in the reaction mixture and inhibitory activities of the products is shown in Fig. 2. The strongest inhibitory activities were seen at concentrations of 10%–15% acetone, and the inhibitory activity decreased at 20% concentration. This result suggests that the products of undesirable structures for 5α-reductase inhibition can be formed at higher organic solvent concentrations.

The optimum pH of the enzymatic reaction in the 10% acetone system was investigated to maximize the inhibitory activity of oxidative products from resveratrol (Fig. 3). The strongest inhibitory activities were observed when the enzymatic oxidation was performed at around pH 4.5 at 50- and 25-ppm product concentrations in the inhibitory assay. Therefore, further experiments were performed at pH 4.5 in the 10% acetone buffer system.

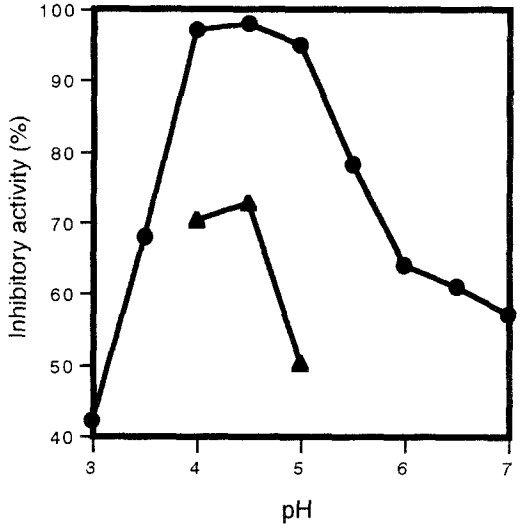


**Fig. 3.** Relation between pH in the reaction system and rat liver 5α-reductase inhibitory activity on the ethyl acetate-soluble fraction of the reaction product. Product concentration was 50 ppm (*circles*) or 25 ppm (*triangles*) in the assay system

## Structure–activity relation in resveratrol dimers

To isolate 5α-reductase inhibitory components, a larger-scale experiment was performed. 5α-Reductase inhibitory activity-guided fractionation led to the isolation of compounds **2** and **3** as inhibitory components (Fig. 4). Resveratrol *trans*-dehydrodimer (**2**) and resveratrol *cis*-dehydrodimer (**3**) were identified by comparison with NMR data in the literature.[5]

The concentrations of 50% inhibition ($IC_{50}$) of 5α-reductase inhibitory activity for these compounds are shown in Table 1, together with those of some natural

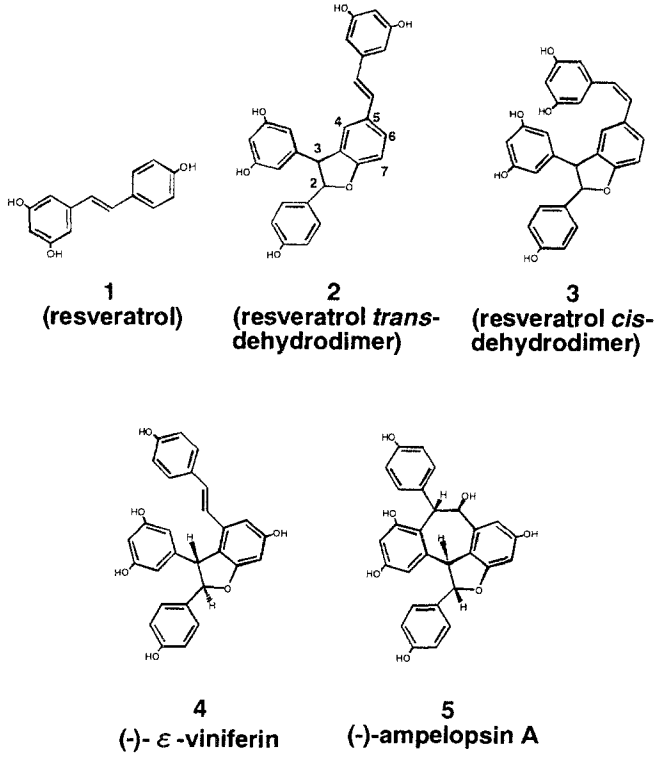


**Fig. 4.** 5α-Reductase activity-guided fractionation led to isolation of compounds **2** and **3**

**Table 1.** $IC_{50}$ values of resveratrol dehydrodimers and known inhibitors of rat liver 5α-reductase

| Compound | $IC_{50}$ (μM) |
|---|---|
| Melapinol A | 49.4 |
| Melapinol B | 44.5 |
| Vaticanol A | 59.1 |
| Ampelopsin C | 52.2 |
| Resveratrol *trans*-dehydrodimer (**2**) | 8.6 |
| Resveratrol *cis*-dehydrodimer (**3**) | 38.1 |
| (−)-ε-Viniferin (**4**) | 20.1 |
| (−)-Ampelopsin A (**5**) | 48.8 |
| α-Linolenic acid | 116 |
| Finasteride | 0.73 |

$IC_{50}$, inhibitory concentration of 50%

products and a synthesized compound (finasteride) as a positive control. $IC_{50}$ values of 8.6 and 38.1μM were observed for the resveratrol *trans*- and *cis*-dehydrodimers, respectively. In regard to the structure–activity relation in 2,3-diaryl-2,3-dihydrobenzofuran derivatives, the *trans*-oriented phenylethene side chain (**2**) had more potent inhibitory activity than the *cis*-oriented one (**3**). The *trans*-oriented phenylethene side chain attached to the 5-position of the 2,3-diaryl-2,3-dihydrobenzofuran skeleton (**2**) also had more potent inhibitory activity than did the 4-phenylethene derivative (**4**). On the other hand, the ring closure type (**5**) led to a loss of inhibitory activity. Inhibitory activity of the resveratrol *trans*-dehydrodimer, the most potent inhibitor of these resveratrol dimers, was five- to sevenfold stronger than that of the resveratrol trimers isolated from melapi heartwood.[3] These results suggest that the 2,3-diaryl-2,3-dihydrobenzofuran skeleton is available for the lead compound of 5α-reductase inhibitor, and that orientation and configuration of the phenylethene side chain play an important part.

The inhibitory activity of resveratrol *trans*-dehydrodimer was about 14-fold stronger than that of α-linolenic acid, reported to be a nonsteroidal 5α-reductase inhibitor.[11] On the other hand, compared with finasteride,[12] a commercial steroidal 5α-reductase inhibitor used for therapy of symptomatic benign prostatic hyperplasia and male-pattern baldness, the inhibitory activity of resveratrol dehydrodimers was more than 10-fold lower.

This is the first report of 5α-reductase inhibitory compounds synthesized by HRP-catalyzed polymerization. Resveratrol *trans*-dehydrodimer was first reported by Liangcake and Pryce[9] as analogous to that of ε-viniferin, which was discussed as phytoalexin from grapevines. However, the paper did not describe the preparation method. Resveratrol *cis*-dehydrodimer was also reported[5] as a metabolite of resveratrol produced by *Botrytis cinerea*, the organism responsible for gray mold.

Resveratrol dehydrodimers had rat liver 5α-reductase inhibitory activity, which is stronger than that of trimers but weaker than that of finasteride. Nevertheless, resveratrol dehydrodimer is a possible leading compound for new nonsteroidal 5α-reductase inhibitors.

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