

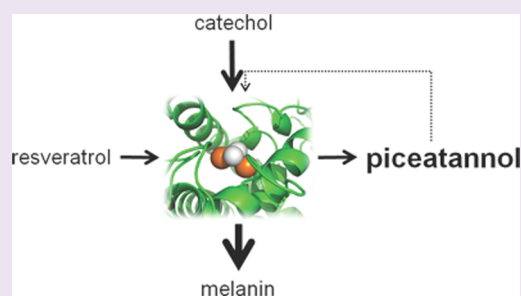
Regioselective Hydroxylation of *trans*-Resveratrol via Inhibition of Tyrosinase from *Streptomyces avermitilis* MA4680

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Supporting Information

ABSTRACT: Secreted tyrosinase from melanin-forming *Streptomyces avermitilis* MA4680 was involved in both ortho-hydroxylation and further oxidation of *trans*-resveratrol, leading to the formation of melanin. This finding was confirmed by constructing deletion mutants of *melC*₂ and *melD*₂ encoding extracellular and intracellular tyrosinase, respectively; the *melC*₂ deletion mutant did not produce piceatannol as well as melanin, whereas the *melD*₂ deletion mutant oxidized resveratrol and synthesized melanin with the same yields, suggesting that MelC₂ is responsible for ortho-hydroxylation of resveratrol. Extracellular tyrosinase (MelC₂) efficiently converted *trans*-resveratrol into piceatannol in the presence of either tyrosinase inhibitors or reducing agents such as catechol, NADH, and ascorbic acid. Reducing agents slow down the dioxygenase reaction of tyrosinase. In the presence of catechol, the regio-specific hydroxylation of *trans*-resveratrol was successfully performed by whole cell biotransformation, and further oxidation of *trans*-resveratrol was efficiently blocked. The yield of this ortho-hydroxylation of *trans*-resveratrol was dependent upon inhibitor concentration. Using 1.8 mg of wild-type *Streptomyces avermitilis* cells, the conversion yield of 100 μ M *trans*-resveratrol to piceatannol was 78% in 3 h in the presence of 1 mM catechol, indicating 14 μ M piceatannol h⁻¹ DCW mg⁻¹ specific productivity, which was a 14-fold increase in conversion yield compared to that without catechol, which is a remarkably higher reaction rate than that of P450 bioconversion. This method could be generally applied to biocatalysis of various dioxygenases.



Resveratrol is a phytoalexin naturally produced by plants such as grapes, berries, and peanuts.^{1–3} A physiological and clinical study on rats and humans showed that *trans*-resveratrol (*tRES*) has excellent antioxidizing function and can be further developed as anti-cancer, anti-inflammatory, and blood-sugar-lowering supplements and drugs.^{4–7} Extraction from natural resources and bacterial fermentation are two ways to make resveratrol in the food and pharmaceutical industries. For example, grape vines treated with cyclodextrin produce up to 5 g L⁻¹ *tRES*,⁸ and Lim *et al.* showed that 2.3 g L⁻¹ of *tRES* was obtained by combined biosynthesis of genes originating from plants and bacteria in *E. coli*.⁹

Piceatannol is a C3' hydroxylated product of resveratrol found in plants (Figure 1). It is well-known as a tyrosine kinase inhibitor and suppressor of cancer cell proliferation and growth.^{10–13} At human liver microsomes, pro-drug *tRES* is converted to piceatannol by cytochrome P450s such as CYP1B1 and CYP1A2.^{14–16} To produce a high amount of piceatannol, microbial P450s, *e.g.*, BM3 mutants from *Bacillus megaterium* are commonly used rather than recombinant CYP1A2. Although the current production yield of BM3 mutants from *B. megaterium* is quite low for industrial applications, few researchers have been successful in screening microbial P450s capable of performing its ortho-hydroxylation.¹⁷

Various *Streptomyces* strains were evaluated to identify microorganisms with such P450s to convert *tRES* into piceatannol. By measuring the decrease in initial *tRES*

concentration, *S. avermitilis* MA4680 was selected. The supernatant of its whole cell broth degraded *tRES* more significantly than the whole cell fraction, indicating that the enzymes responsible for its oxidation or degradation are secreted rather than cytoplasmic P450s. Tyrosinase was identified as the candidate enzyme for the oxidation and further degradation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and tryptic in-gel digestion followed by mass spectrometry (MS) analysis, since it was the most abundant protein among the extracellular proteins. On the basis of the *tRES* reaction profile, we hypothesized that such a tyrosinase degrades *tRES* via its ortho-hydroxylated intermediate, *i.e.*, piceatannol, and undergoes subsequent melanine synthesis via its oxidized quinone form. Yang *et al.* supported a similar idea by showing that the extracellular tyrosinase (MelC₂) of *S. avermitilis* converts phenol into quinone via catechol.¹⁸

We proposed to either clone MelC₂ of *S. avermitilis* into *E. coli* or evaluate tyrosinase deletion mutants of *S. avermitilis*. However, we were unable to express recombinant tyrosinase (MelC₂) in *E. coli*, because functional expression of the tyrosinase was strongly dependent on the helper protein

Received: May 8, 2012

Accepted: July 6, 2012

Published: July 6, 2012

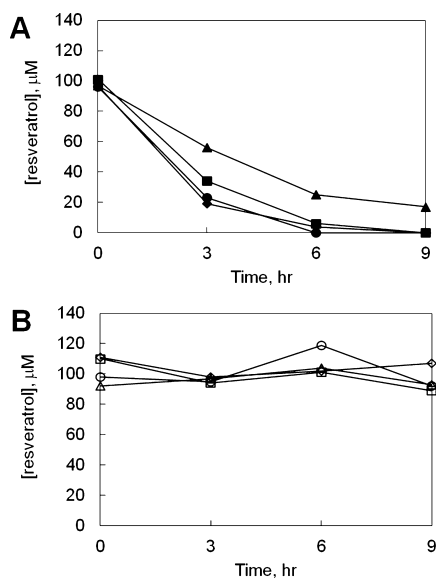


Figure 2. Comparison of consumption of 100 μM *trans*-resveratrol transformed by whole cells of *Streptomyces* spp. (A) Melanin-producing *Streptomyces* spp.: (●) *S. avermitilis*, (■) *S. griseus*, (▲) *S. pristinaespiralis*, (◆) *S. venezuelae*. (B) Non-melanin-producing *Streptomyces* spp.: (○) *S. caelestis*, (□) *S. coelicolor*, (△) *S. peustius*, (◇) *S. lividans*.

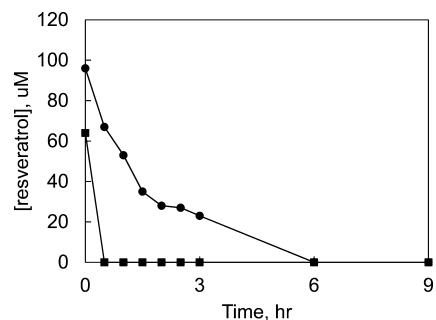


Figure 3. Time profile of 100 μM *trans*-resveratrol converted by whole cells, centrifuged, and subsequently washed twice with 50 mM phosphate buffer (pH 7.5) (●) and the supernatant broth (■) without treating with reducing agents. It was difficult to quantify the piceatannol concentration due to low peak area on the high performance liquid chromatography (HPLC) chromatogram.

between the P450s and the secreted tyrosinase from the cell pellet during the reaction. Tyrosinase deletion mutants were constructed to clarify that the extracellular tyrosinase produced not only piceatannol but also melanin.

Characterization of Tyrosinase Deletion Mutants of *S. avermitilis*. To confirm which tyrosinase is involved in piceatannol production, extracellular tyrosinase (MelC2) and intracellular tyrosinase (MelD2) deletion mutants were constructed with *S. avermitilis* (see Supplementary Data). First, the color of each mutant was compared visually to measure the degree of melanin synthesis. One half of the $\Delta melD2$ transformants were white, and the other half were brown, whereas all $\Delta melC2$ transformants were white. Brown $\Delta melD2$ deletion mutants were excluded from further experiments, because the levels of melanin synthesis of the brown $\Delta melD2$ mutants were similar to wild-type (WT), indicating that the brown color is naturally caused by the extracellular tyrosinase activity of MelC2. To confirm the proper gene

construction and expression levels of the helper proteins and tyrosinases, reverse transcription-polymerase chain reaction (RT-PCR) assays of all tyrosinases (MelC2 and MelD2), tyrosinase helper proteins (MelC1 and MelD2), and four other dioxygenases as a control were conducted with white $\Delta melC2$ and white $\Delta melD2$ mutants (Figure 4B and C). In the $\Delta melC2$

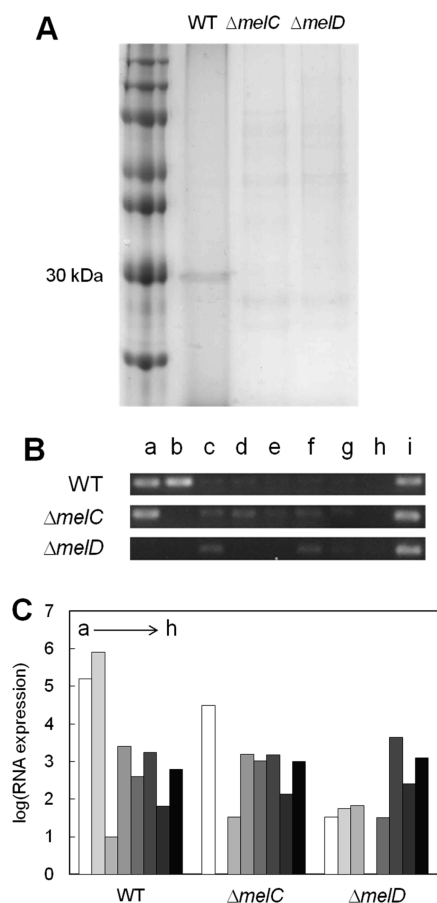


Figure 4. Characterization of *Streptomyces avermitilis* WT, $\Delta melC2$, and white $\Delta melD2$. (A) SDS-PAGE of secreted proteins concentrated 10 times. Major band of wild-type (WT) was MelC (SAV1137). (B) 1.5% (w/v) agarose gel analysis of PCR product using cDNA of tyrosinase, caddie proteins, and several dioxygenases. a: MelC1 helper protein (SAV1136), b: MelC2 tyrosinase (SAV1137), c: MelD1 helper protein (SAV5361), d: MelD2 tyrosinase (SAV5362), e: dioxygenase1 (SAV565), f: dioxygenase2 (SAV1615), g: dioxygenase3 (SAV2478), h: dioxygenase4 (SAV5671), i: 5S RNA (control). (C) Relative expression level of each mRNA compared to 5S RNA quantity.

mutant, only the *melC2* gene was disrupted and *melC* mRNA was not detected. However, in the case of $\Delta melD2$ mutant, not only the *melD2* gene was totally disrupted but also the *melC* operon decreased significantly. PCR of the two genes was carried out using the genomic DNA of the $\Delta melD2$ strain to confirm whether its genomic DNA was damaged during the deletion mutation. The band of the *melC* operon genes was observed on an agarose gel (data not shown). Compared to WT, the expression level of *melC* operon mRNA in the $\Delta melD2$ mutant was strongly suppressed approximately 10^4 -fold, whereas expression levels of the four other dioxygenases were not affected by *melC* or *melD* gene deletion alone. As shown in the SDS-PAGE gel of Figure 4A, MelC2 (30.4 kDa) secreted from the cell was not observed in either white $\Delta melC$ and

$\Delta melD$ mutants in contrast to WT. A possible explanation is that the *melC* operon is somewhat inducible and that *melC1/melC2* expression is indirectly regulated by the presence of MelD2. In the white $\Delta melD2$ mutant, not only monooxygenase activity for *tRES* hydroxylation but also dioxygenase activity for melanin synthesis were not shown. In both white *melC2* and *melD2* deletion mutants, the piceatannol peak on the HPLC chromatogram was not detected at all even after 24 h of reaction with *tRES*. At the same time, the initial *tRES* concentration did not decrease noticeably, suggesting that MelC2 is the crucial enzyme for *tRES* hydroxylation. From the result of *tRES* transformation by tyrosinase deletion mutants, we conclude that extracellular tyrosinase (MelC2) is essential for *tRES* hydroxylation, unless other *tRES* monooxygenases are amplified. Although there is some possibility that such intracellular P450s and other monooxygenases convert *tRES*, considering their k_{cat}/K_m values and expression levels in the host cells compared to those of MelC2, the monooxygenases may not significantly contribute to *tRES* hydroxylation. For example, the intracellular P450, CYP105D7 from *S. avermitilis* expressed in *E. coli* could transform daidzein to 3'-ODI (*o*-dihydroxyisoflavone) with a k_{cat}/K_m value of $0.0115 \mu\text{M}^{-1} \text{s}^{-1}$,²⁵ which is similar to that of tyrosinase for tyrosine. However, in the case of *S. avermitilis* the MelC2 expression level was much higher than that of CYP105D7, because MelC2 is the major protein in the cell supernatant and accumulated during the fermentation and bioconversion (Figure 4A). Therefore, the role of *S. avermitilis* monooxygenases in hydroxylation is minor relative to tyrosinase due to its relatively low k_{cat}/K_m value and low expression level. The MelC2 deletion mutant should be used to perform a correct evaluation and avoid a possible high background hydroxylation reaction exerted by the tyrosinase MelC2 to screen a target monooxygenase toward specific monophenolic substrate in *Streptomyces* strains.

Evaluation of Reducing Agents for Blocking Tyrosinase Activity As a Catechol Oxidase. In general, tyrosinase has monooxygenase and dioxygenase functions; hence, it catalyzes monophenolic compounds to catechol-like intermediates and to quinolic compounds sequentially. Dioxygenase activity should be suppressed to produce piceatannol from *tRES*, while maintaining its monooxygenase activity. Two strategies were attempted to suppress the second consecutive oxidation of catechol-like intermediates. First, quinolic compounds were contacted with reducing reagents such as NADH or *L*-ascorbic acid, which donate electrons to quinolic compounds and drive the reduction reaction. The other strategy is inhibiting the dioxygenase reaction by adding stronger competitive inhibitors than the catechol-like intermediates. Then, perhaps, the stronger competitive inhibitors can be oxidized by tyrosinase instead of piceatannol during the second oxidation. To examine these two strategies, 1 mM concentration of various reducing reagents or inhibitors was added with 100 μM *tRES* to 1 mL of *S. avermitilis* cells resuspended in 50 mM phosphate buffer (pH 7.5) for 3 h (Table 1). Non-phenolic inhibitors such as NADH and *L*-ascorbic acid are well-known tyrosinase reductants. Our inhibitor study showed that phenolic inhibitors such as catechol resulted in a higher yield of piceatannol than that from nonphenolic reductants. The major reason why nonphenolic reductants are less efficient than phenolic inhibitors appears to be significant consumption of the nonphenolic reductants by other oxidants and oxidases in the cell mixture. As shown in Table 1, the highest relative yield of piceatannol converted from

Table 1. Evaluation of the Ability To Accumulate Piceatannol with Various Reductants^a

| type | chemicals | relative production, % |
|--------------|-------------------------|------------------------|
| non-phenolic | without treatment | 7.2 |
| | NADH | 64.2 |
| | <i>L</i> -ascorbic acid | 63.7 |
| | glutathione | 22.2 |
| | cysteine | 42.4 |
| | hydroquinone | 97.1 |
| phenolic | 1-naphthol | 3.7 |
| | <i>p</i> -coumaric acid | 32.5 |
| | curcumin | 7.4 |
| | catechol | 100.0 |
| | pyrogallol | 32.1 |
| | ferulic acid | 6.4 |

^a100 μM *trans*-resveratrol was reacted with 1 mM reductant and whole cells of *S. avermitilis* in 50 mM Tris-HCl, pH 8.0 for 3 h.

100 μM *tRES* was obtained when hydroquinone and catechol were added to the reaction mixture at the same time that *tRES* was added. When 1 mM catechol was added to the reaction with 100 μM *tRES*, the yield of piceatannol was 1.5 fold higher than when adding nonphenolic reductants such as NADH and *L*-ascorbic acid. Catechol-like molecules slow down only the dioxygenase reaction, acting as a competitive inhibitor against dioxygenase but not against monooxygenase. In addition, the catechol-like molecules may stabilize piceatannol by donating electrons to two copper ions bridged to the oxygen atoms on the C3 and C4 positions of piceatannol. Thus, phenolic compounds become a crucial additive for the high piceatannol yield in this tyrosinase reaction.

Commercial mushroom tyrosinase was also used for piceatannol production. Its *tRES* consumption rate was similar to that of *S. avermitilis* tyrosinase. However, the ratio of monooxygenase to dioxygenase activity by mushroom tyrosinase seemed to be much lower than that of *S. avermitilis*. Further study on the comparison of the reactivity ratio of each tyrosinase should be carefully performed in depth.

High-Yield Production of Piceatannol by Adding Catechol. The results shown above suggest that catechol is the best competitive inhibitor and reducing agent for high yield production of piceatannol from *tRES*. To determine the optimum inhibitor concentration, 100 μM *tRES* was reacted with a resuspended *S. avermitilis* cell pellet in 50 mM phosphate buffer (pH 7.5) by varying the catechol concentrations from 10 to 10⁴ μM (Figure 5). The higher the catechol concentration used, the more piceatannol was yielded. However, an excessive amount of catechol slightly reduced total tyrosinase activity. Catechol at 1 mM concentration was optimal for the tyrosinase reaction with 100 μM *tRES*. Under the optimal condition of 50 mL of 50 mM Tris-HCl buffer and 1 mM catechol, the maximum yield of piceatannol reached a 78% conversion yield after a 4 h reaction (Figure 6). By using this simple inhibitor in the biotransformation of *S. avermitilis* secreting MelC2, a high yield of regiospecific hydroxylation of *tRES* was achieved. Similar to the suppression of tyrosinase activity resulting from the high catechol concentration, an excessive amount of *tRES* (>1 mM) inhibited the tyrosinase reaction due to substrate inhibition (data not shown). Based on this strategy, adding catechol to the tyrosinase reaction would be an efficient and simple way to produce ortho-hydroxylated catechol derivatives

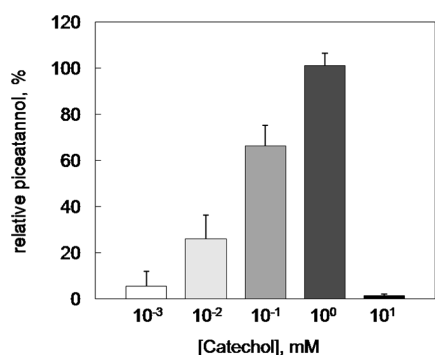


Figure 5. Relative production of accumulating piceatannol with different concentrations of catechol. From left to right: 0, 10¹, 10², 10³, and 10⁴ μ M, respectively. [resveratrol]₀ = 100 μ M; reaction volume, 50 mL; reaction time, 3 h.

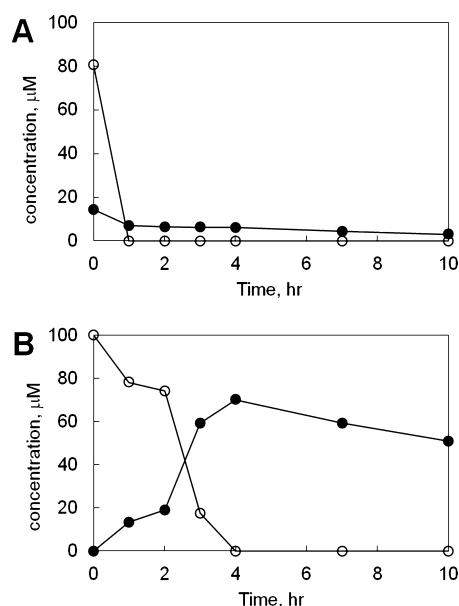


Figure 6. Comparison of piceatannol production between (A) secreted proteins and (B) washed whole cells of *Streptomyces avermitilis* treated with 1 mM catechol and 100 μ M *trans*-resveratrol in 50 mM Tris-HCl, pH 8.0: (○) *trans*-resveratrol, (●) piceatannol.

in the chemical industry. Further studies must be conducted to improve conversion yield of beneficial catechol derivatives at higher concentrations of target molecules. One such strategy is to construct a recombinant strain to overexpress functional tyrosinase.

METHODS

Chemicals. *t*RES, piceatannol, NADH, catechol, pyrogallol, 1-naphthol, *p*-coumaric acid, ferulic acid, glutathione, cysteine, L-ascorbic acid, hydroquinone, curcumin, and *N,O*-bis(trimethylsilyl)-tyrfluoroacetamide (BSTFA) were purchased from Sigma-Aldrich. All other chemicals and solvents were commercially available.

Bacterial Strains and Culture Conditions. *S. avermitilis* MA4680 and other *Streptomyces* strains were obtained from the Korea Collection for Type Cultures. All strains used are listed in the Supplementary Data. The cell culture protocol for all strains followed a general procedure.³¹ The strains were cultivated on R2YE medium, containing 10.3% (w/v) sucrose, 1% (w/v) D-glucose, 1% (w/v) MgCl₂·6H₂O, 0.025% (w/v) K₂SO₄, 0.5% (w/v) yeast extract, 0.01% (w/v) casamino acid, 0.57% (w/v) *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, 0.005% (w/v) K₂HPO₄, 0.03% (w/v)

CaCl₂·2H₂O, 0.003% (w/v) L-proline, 2 mL of trace element solution, and 5 mL of 1 N NaOH.

Plasmid Construction and Transformation of *S. avermitilis* MA4680. *S. avermitilis* genomic DNA was extracted with A G-spin Genomic DNA Extraction kit and used as a PCR template. The tyrosinase genes were amplified by PCR using a set of specific primers (see Supplementary Data). The PCR product was cloned into the pIBR25 expression vector. The shuttle plasmid was constructed with *E. coli* JM110 (Novagen) for transformation in *S. avermitilis*. Disruption of *MelC* and *MelD* was performed using a plasmid pSuperCos1 to deliver the corresponding apramycin resistance gene cassettes (see Supplementary Data). The cassette consisted of two PCR-derived flanking regions in which suitable restriction sites were introduced. All constructs were delivered into *S. avermitilis* by protoplast-mediated transformation. The transformants were selected on R2YE/apramycin medium for *melC* and *melD* deletion and further confirmed by RT-PCR.

RNA Extraction and RT-PCR. RNA extraction was carried out using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The concentration and purity of the extracted RNA were determined by measuring the A₂₆₀ and the ratio of A₂₆₀ to A₂₈₀, respectively. RT-PCR was performed using the primers and the probes of the *MelC*, *MelD*, and 5S RNA sequences to quantify the *melC* and *melD* mRNA transcripts (see Supplementary Data). The detailed experimental procedure was described in our previous work.³²

***Streptomyces* Whole Cell Reaction.** Each strain was subcultured by shaking test tubes containing 1 g of glass beads in 3 mL of R2YE medium at 200 rpm and 30 °C for 2 days. One milliliter of cultured cells was inoculated into 50 mL of fresh medium with 4 g of glass beads in a 250 mL flask, and the flask was incubated at 200 rpm and 30 °C for 24 h. The cells were centrifuged at 4000 rpm for 10 min and were washed twice with 50 mM phosphate buffer (50 mL, pH 7.5). After another centrifugation, 5 g of wet weight cells were added to 50 mL of the same phosphate buffer containing 100 μ M *t*RES. The reaction mixture was kept on the shaking incubator at 200 rpm and 30 °C. A 1 mL aliquot was extracted with 1 mL of ethyl acetate. After centrifugation at 13,000 rpm for 10 min, the upper phase was evaporated in a cold vacuum chamber. The product was analyzed by HPLC or GC-MS.

Piceatannol Accumulation by Catechol Treatment. To determine the optimal concentration of catechol for reducing piceatannol degradation, 0.1, 1, 10, and 100 mM catechol concentrations were compared. Catechol was preincubated with the whole cell mixture for 5 min before resveratrol was added. Additionally, the culture broth supernatants separated from whole cells were compared.

Analytical Method. Time-dependent consumption of resveratrol and formation of piceatannol were monitored by HPLC analysis. The analysis was performed on an Autochro-3000 (Young Lin) connected with an UV-vis detector and a C18 column (4.6 mm × 150 mm). The HPLC analysis conditions were as follows: mobile phase, 30% (v/v) acetonitrile in H₂O containing 0.1% (v/v) trifluoroacetic acid; flow rate, 1 mL min⁻¹; injection volume, 20 μ L; detection wavelength, 325 nm. GC-MS was used to identify *t*RES and its metabolites. All hydroxyl groups of the dried sample extracted with ethyl acetate were converted to TMS (trimethylsilyl) derivatives by heating for 30 min at 60 °C with BSTFA. GC-MS was performed on a Thermo Scientific Trace GC Ultra instrument connected to a Thermo Scientific ITQ1100 MS spectrometer with a TR-5 ms SQC capillary column (30 m × 0.25 mm i.d., 0.25 μ m film thickness). The GC-MS analysis was carried out as follows: injector temperature, 250 °C; temperature gradient, 150 °C hold for 1 min, 10 °C/min up to 300 °C, 300 °C hold for 5 min; MS operating mode, 70 eV electron ionization mode.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 20110020418) and WCU (World Class University) program through the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education, Science and Technology (R322011000102130).

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