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Conversion of (3*S*,4*R*)-Tetrahydrodaidzein to (3*S*)-Equol by THD Reductase: Proposed Mechanism Involving a Radical Intermediate[†]

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ABSTRACT: To elucidate the mechanism of (3*S*)-equol biosynthesis, (2,3,4-d_3)-*trans*-THD was synthesized and converted to (3*S*)-equol by THD reductase in *Eggerthella* strain Julong 732. The position of the deuterium atoms in (3*S*)-equol was determined by ^{1}H NMR and ^{2}H NMR spectroscopy, and the product was identified as (2,3,4$_\alpha$-d_3)-(3*S*)-equol. All the deuterium atoms were retained, while the OH group at C-4 was replaced by a hydrogen atom with retention of configuration. To explain the deuterium retention in this stereospecific reduction, we propose a mechanism involving radical intermediates.

Isoflavones, predominantly found in the leguminous plants, are healthy natural dietary phytoestrogens (*1*−*3*). Daidzein (4′,7-dihydroxyisoflavone) is one of the major isoflavones in soybean, which is present mostly in the glucoside form, daidzin. This compound has recently attracted a great deal of attention because of its various beneficial effects for human health, including estrogenic (*4*), anticancer (*5*), antioxidant (*6*, *7*), and cardioprotective activities (*8*). In the intestine, the glycosidic linkage of daidzin is cleaved by endogenous microorganisms, and some of the resulting daidzeins are absorbed into the bloodstream. The remaining daidzeins are further metabolized to DHD[1] (4′,7-dihydroxyisoflavanone), THD (4′,7-dihydroxyisoflavan-4-ol), and finally (3*S*)-equol [(3*S*)-4′,7-dihydroxyisoflavan], which has the most potent estrogenic effect among the daidzein-derived metabolites (Figure 1) (*9*).

Although several human intestinal bacteria that can metabolize daidzein or DHD have been described (*10*−*15*), the chemistry of the bioconversion remains poorly understood, mainly because of the extreme oxygen sensitivity of the microbes and the enzymes. Recently, the metabolic pathway and stereochemistry involved in the transformation of DHD to (3*S*)-equol by the anaerobic intestinal bacterium, *Eggerthella* strain Julong 732, were determined (*16*). Reduction of DHD produces only one of the THD stereoisomers, indisputably assigned as (3*S*,4*R*)-THD (*17*), which is then converted to (3*S*)-equol by THD reductase. Apparently, the reduction of the C-4 hydroxyl group required to form (3*S*)-equol from (3*S*,4*R*)-THD is catalyzed by a single enzyme, which requires NADPH and the other unidentified cofactor for the electron transfer (*18*).

The originally proposed biosynthetic pathway for the (3*S*)-equol biosynthesis postulated that DE (4′,7-dihydroxyisoflav-3-ene) was formed as an intermediate, probably through the *cis*-elimination reaction (Figure 2a) (*19*). However, DE could not be metabolized into (3*S*)-equol by Julong 732 (*16*), and in fact, DE was identified only from the urine sample (*20*). Recently, Kim et al. suggested a mechanism involving the formation of a carbocation intermediate to explain the unprecedented reductive isomerization reaction (*16*). In this mechanism, the Lewis acid-assisted secondary carbocation formation was proposed to facilitate the 1,2-hydride shift to form a more stable tertiary carbocation at C-3 (*21*). The carbocation mechanism could be still consistent with the newly determined absolute configuration of the substrate THD, if the subsequent hydride addition is stereospecific (Figure 2b). Alternatively, simple nucleophilic substitution of the hydroxyl group with hydride at the C-4 center would produce (3*S*)-equol (Figure 2c). However, in this instance, the assistance by a Brønsted acid is required because hydroxide is generally a poor leaving group (*22*). While two suggested mechanisms (Figure 2b,c) are possible with varying degrees of feasibility, the added hydride would end up at a different prochiral position in the (3*S*)-equol metabolites. Therefore, biotransformation of (2,3,4-d_3)-*trans*-THD by THD reductase in *Eggerthella* strain Julong 732 was performed to check the validity of the proposed mechanisms.[2]

EXPERIMENTAL PROCEDURES

Daidzein was purchased from Indofine Co. (Somerville, NJ). GAM was from Nissui Pharmaceutical Co. (Tokyo, Japan). Ammonium formate-d_5 and methol-d_4 were from CDN isotopes (Pointe-Claire, QC). Pd/C (10% Pd), DMSO, DMSO-d_6, and

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[1]Abbreviations: DE, dehydroequol; DHD, dihydrodaidzein; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; GAM, Gifu anaerobic medium; EI-MS, electron impact ionization mass spectrometry; HPLC, high-performance liquid chromatography; KCCM, Korean Culture Center of Microorganisms; NADPH, nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; SAM, *S*-adenosylmethionine; THD, tetrahydrodaidzein; UV, ultraviolet; VCD, vibrational circular dichroism.

[2]Extreme oxygen sensitivity and catalytic activity loss during the purification hindered the isolation of the enzyme.

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DMF were purchased from Aldrich (St. Louis, MO). HPLC-grade solvents of acetonitrile, ethyl acetate, methanol, and acetic acid were obtained from Fisher (Pittsburgh, PA).

EI-MS spectra of the compounds were recorded with a JMS-AX505WA instrument (JEOL, Tokyo, Japan) at 70 eV with an ion source temperature of 280 °C in positive ion mode. ^{1}H and ^{13}C NMR spectra of the compounds in DMSO-d_6 were recorded at 400 and 100 MHz, respectively, on a JNM-LA400 instrument (JEOL) at 296 K. ^{2}H NMR spectra were recorded in DMSO at 60 MHz on the same instrument.

Chemical Synthesis and Purification of (2,3,4-d$_3$)-trans-Tetrahydrodaidzein and (2,3,4,4-d$_4$)-Equol. (2,3,4-d_3)-*trans*-THD and (2,3,4,4-d_4)-equol were synthesized from daidzein by catalytic hydrogenation according to the published method with a slight modification (*23*). The reduction of daidzein was conducted in the presence of ammonium formate-d_5 and Pd/C in an inert atmosphere glovebox under nitrogen. Daidzein (260 mg, 0.90 mmol), Pd/C (255 mg), and NH_4HCO_3-d_5 (252 mg, 200 mmol) were dissolved in MeOH-d_4 (20 mL). The reaction mixture was stirred at 65 °C for 1 h, and the reaction was monitored by HPLC. For monitoring, a Prostar HPLC system (Varian, Walnut Creek, CA) equipped with a photodiode array detector (Prostar 330, Varian) operating at 280 nm and a C_{18} reversed-phase column (Spherisorb 5 μm ODS2, 4.6 mm × 250 mm, Clwyd, U.K.) were employed. The mobile phase was composed of 10% acetonitrile in 0.1% acetic acid (A) and 90% acetonitrile in 0.1% acetic acid (B). The elution profile started with an A:B ratio of 80:20 (v/v) for 3 min and then linearly changed to 20:80 (v/v) over 12 min. The flow rate was 1 mL/min. The reaction mixture was then dried on a rotary vacuum evaporator after filtration and dissolved in DMF for HPLC purification on the semipreparative C_{18} reversed-phase column (Spherisorb 5 μm ODS2, 10 mm × 250 mm). The mobile phase was composed of 100% water (A) and 100% acetonitrile (B), and the elution profile was the same as that determined via analytical HPLC. The flow rate was 3 mL/min for preprative HPLC.

(*2,3,4-d$_3$)-trans-THD*: ^{1}H NMR [$(CD_3)_2SO$, 400 MHz] δ 4.08 (s, 0.5H, H-2$_\alpha$), 4.13 (s, 0.5H, H-2$_\beta$), 5.17 (br s, 1H, OH), 6.16 (d, J = 2.4 Hz, 1H, H-8), 6.35 (dd, J = 10.8, 2.4 Hz, 1H, H-6), 6.68 (d, J = 8.4 Hz, 2H, H-3′), 7.04 (d, J = 8.4 Hz, 2H, H-2′), 7.17 (d, J = 8.4 Hz, 1H, H-5); ^{13}C NMR [$(CD_3)_2SO$, 100 MHz] δ 45.50 (C-3), 67.83 (C-2), 68.35 (C-4), 102.28 (C-8), 108.66 (C-6), 115.52 (C-3′), 118.04 (C-4a), 129.40 (C-2′), 130.36 (C-5), 130.75 (C-1′), 155.36 (C-8a), 156.47 (C-4′), 157.93 (C-7).

(*2,3,4,4-d$_4$)-Equol*: ^{1}H NMR [$(CD_3)_2SO$, 400 MHz] δ 3.86 (s, 0.5H, H-2$_\beta$), 4.10 (s, 0.5H, H-2$_\alpha$), 6.16 (d, J = 2.4 Hz, 1H, H-8), 6.27 (dd, J = 10.8, 2.4 Hz, 1H, H-6), 6.71 (d, J = 8.4 Hz, 2H, H-3′), 6.84 (d, J = 8.4 Hz, 1H, H-5), 7.09 (d, J = 8.4 Hz, 2H, H-2′), 9.15 (br s, 1H, OH), 9.27 (br s, 1H, OH).

(*3S*)-*Equol*: ^{1}H NMR [$(CD_3)_2SO$, 400 MHz] δ 2.75 (ddd, $J_{H2\alpha,4\alpha}$ = 2.3 Hz, $J_{H3,4\alpha}$ = 5.5 Hz, $J_{H4\alpha,4\beta}$ = 15.6 Hz, 1H, H-4$_\alpha$), 2.81 (dd, $J_{H3,4\beta}$ = 10.5 Hz, $J_{H4\alpha,4\beta}$ = 15.6 Hz, 1H, H-4$_\beta$), 2.97 (dddd, $J_{H2\alpha,3}$ = 3.7 Hz, $J_{H2\beta,3}$ = 10.5 Hz, $J_{H3,4\beta}$ = 10.5 Hz,

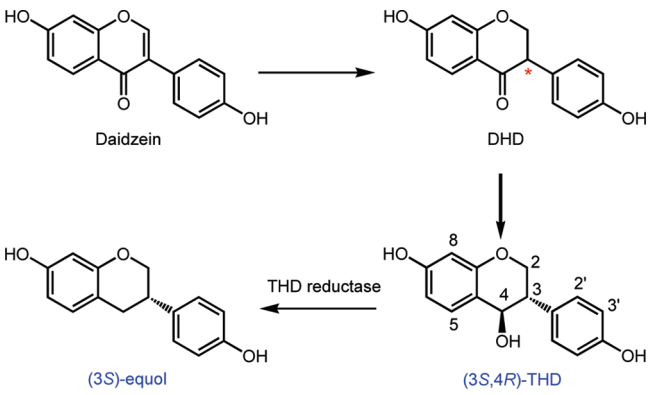

FIGURE 1: Metabolic pathway leading to the production of (3*S*)-equol from daidzein. The DHD to (3*S*)-equol pathway is metabolized by unidentified enzymes in *Eggetherlla* sp. Julong 732. The stereochemistry of DHD relevant to (3*S*,4*R*)-THD production is currently not known.

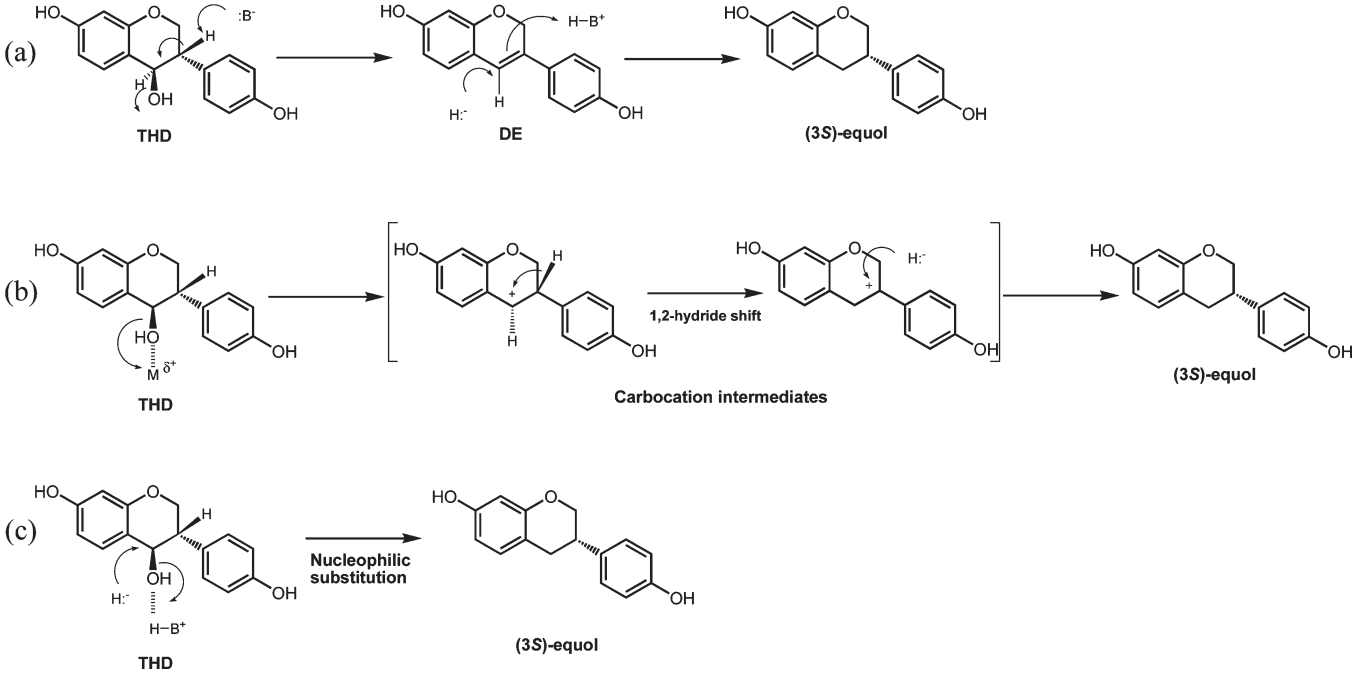

FIGURE 2: Possible mechanisms for the conversion of (3*S*,4*R*)-THD to (3*S*)-equol. (a) Base-catalyzed formation of DE and subsequent hydride addition at C-4. (b) Lewis acid-catalyzed secondary carbocation formation followed by a 1,2-hydride shift and hydride addition at C-3. (c) Direct hydride addition at C-4 by nucleophilic substitution.

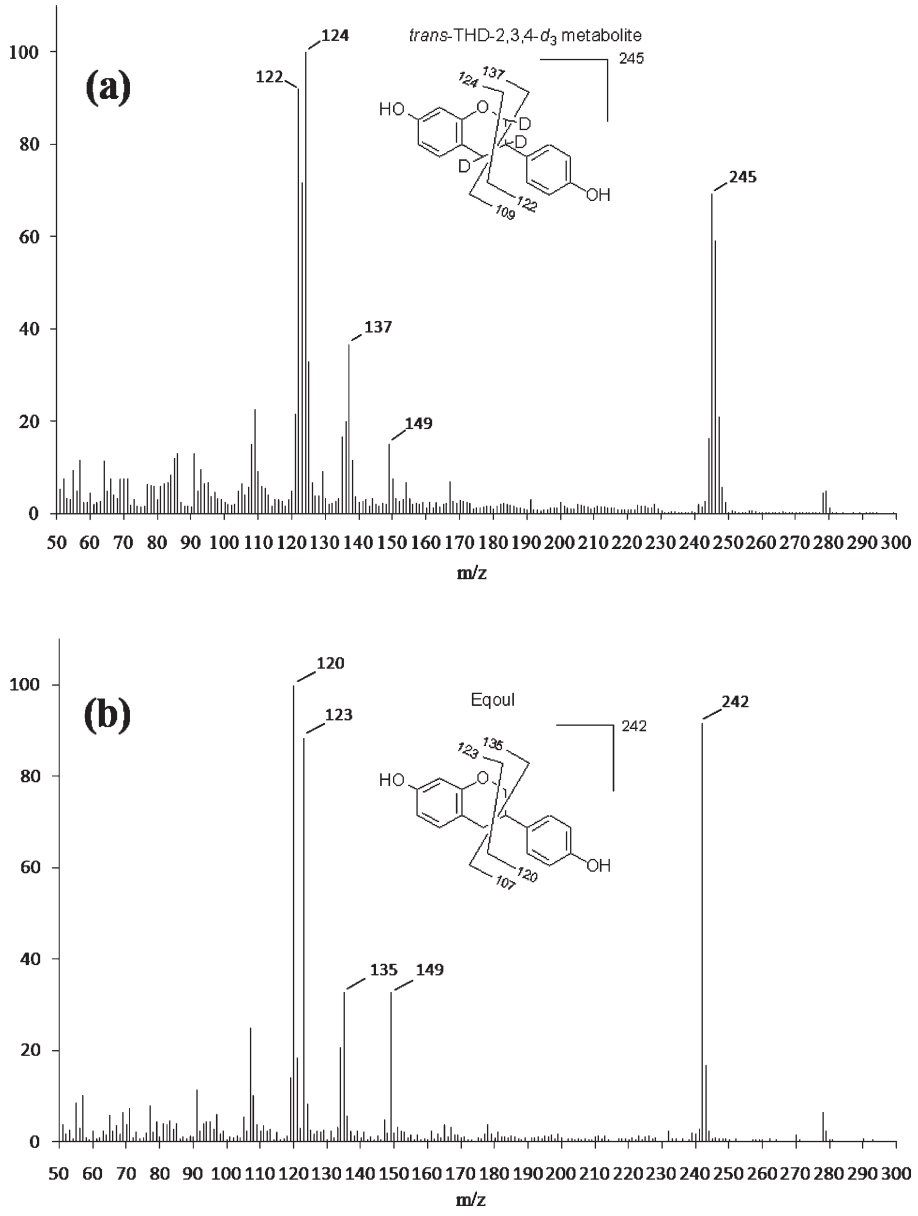

FIGURE 3: EI-MS spectra of (a) (2,3,4$_\beta$-d_3)-(3*S*)-equol and (b) equol. The insets show fragmentation patterns.

$J_{H3,4\alpha}$ = 5.5 Hz, 1H, H-3), 3.88 (dd, $J_{H2\alpha,2\beta}$ = 10.5 Hz, $J_{H2\beta,3}$ = 10.5 Hz, 1H, H-2$_\beta$), 4.12 (ddd, $J_{H2\alpha,2\beta}$ = 10.5 Hz, $J_{H2\alpha,3}$ = 3.7 Hz, $J_{H2\alpha,4\alpha}$ = 2.3 Hz, 1H, H-2$_\alpha$), 6.16 (d, J = 2.4 Hz, 1H, H-7), 6.27 (dd, J = 10.8, 2.4 Hz, 1H, H-6), 6.71 (d, J = 8.4 Hz, 2H, H-3′), 6.84 (d, J = 8.4 Hz, H, H-5), 7.09 (d, J = 8.4 Hz, 2H, H-2′), 9.13 (s, 1H, OH), 9.25 (s, 1H, OH).

Bacterial Culture and Biotransformation. The culturing of Julong 732 and biotransformations were conducted in a Concept 400 anaerobic chamber (Ruskin Technology, Leeds, U.K.) under an atmosphere of 5% CO_2, 10% H_2, and 85% N_2. The stock of *Eggerthella* sp. Julong 732 (KCCM 10490), preserved in liquid nitrogen, was thawed and incubated on a GAM agar plate (15% agar) for 4 days. A single colony was then transferred to the GAM broth. Two milliliters of the seed culture was added to 200 mL of GAM broth and incubated for 1 day, after which 40 mL of the culture was inoculated into 800 mL of GAM broth. When the optical density at 600 nm reached 0.05, the synthesized (2,3,4-d_3)-*trans*-THD substrate at 10 mM in DMF was added to the 800 mL culture to achieve a final concentration of 0.1 mM. After being incubated for 48 h, the culture was extracted with ethyl acetate and filtered, followed by evaporation to dryness on a rotary vacuum evaporator. The metabolites were then dissolved in DMF for HPLC purification on the semipreparative C_{18} reversed-phase column under the same condition described in the above section. The metabolite of (2,3,4-d_3)-*trans*-THD was dissolved in MeOH for EI-MS analysis and dissolved in DMSO-d_6 and DMSO for ^{1}H and ^{2}H NMR spectroscopy, respectively.

RESULTS

With the newly established DHD → (3*S*,4*R*)-THD → (3*S*)-equol pathway (*16*), the mechanism of THD reduction was investigated by means of a deuterium-labeled substrate. Chemical synthesis of (2,3,4-d_3)-*trans*-THD and (2,3,4,4-d_4)-equol was successfully achieved through the catalytic hydrogenation of daidzein, and the products were isolated by HPLC. The HPLC retention time and UV spectra were identical to those of the unlabeled compounds. *trans*-THD was produced as a racemic mixture of (3*R*,4*S*)- and (3*S*,4*R*)-THD. Further stereochemical resolution of the racemic *trans*-THD was not pursued, because only the (3*S*,4*R*) isomer is the substrate of THD reductase (*16*).

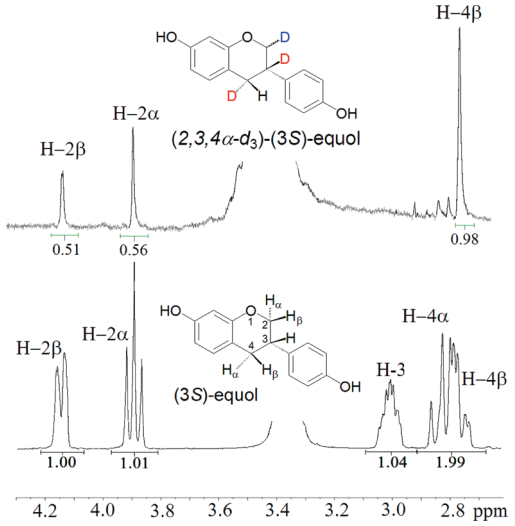

FIGURE 4: Comparison of ^{1}H NMR spectra of (2,3,4$_\alpha$-d_3)-(3*S*)-equol and (3*S*)-equol.

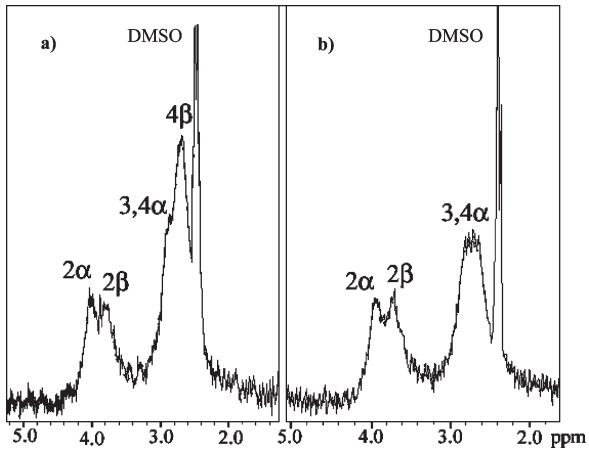

FIGURE 5: ^{2}H NMR spectra of (a) (2,3,4,4-d_4)-equol and (b) (2,3,4$_\alpha$-d_3)-(3*S*)-equol.

Comparison of the ^{1}H NMR spectra of the deuterated and unlabeled THDs allowed the identification the deuterium label positions (*24*, *25*). The ^{1}H NMR spectrum of (2,3,4-d_3)-*trans*-THD is characterized by singlet peaks for H-2$_\alpha$ and H-2$_\beta$ at δ 4.10 and 4.17, respectively, due to the nonstereospecific incorporation of a single deuterium at C-2 (that occurs during the reduction of the planar daidzein precursor) and the lack of signals for H-3 and H-4.

Deuterium-labeled (2,3,4-d_3)-*trans*-THD was reacted with *Eggerthella* sp. Julong 732 (KCCM 10490) under anaerobic conditions. The isolated (3*S*)-equol had the same retention time on HPLC and exhibited an UV−vis spectrum identical to that of the authentic standard. When the metabolite was subjected to mass spectrometry, the molecular ion peak at *m*/*z* 245 corresponding to the molecular ion of d_3-(3*S*)-equol was identified. The fragmentation pattern of the metabolite was also similar to that of (3*S*)-equol (Figure 3), implying the deuterium was positioned at C-3, C-4, and possibly C-2. The precise positions of the deuterium labels on d_3-(3*S*)-equol were determined by NMR spectroscopy (Figure 4). In the ^{1}H NMR spectrum, the proton signals for H-4$_\alpha$ at δ 2.81 and H-3 at δ 2.97 were missing, and the signals for the two H-2 protons at δ 3.88 and 4.12 were reduced in intensity by half. Therefore, the product was assigned as (2,3,4$_\alpha$-d_3)-(3*S*)-equol. Furthermore, the ^{2}H NMR spectrum of the labeled (3*S*)-equol (Figure 5) clearly showed the peaks missing from the ^{1}H NMR spectrum, finally confirming the labeling positions.

DISCUSSION

The conversion of (2,3,4-d_3)-*trans*-(3*S*,4*R*)-THD to (2,3,4$_\alpha$-d_3)-(3*S*)-equol has led us to the following conclusions. First, no deuterium labels on the C-ring of (3*S*,4*R*)-THD were lost during the reduction by THD reductase. This strongly suggests a mechanistically concerted or tightly coupled reduction of the C-4 center. Accordingly, the carbocation mechanism described in Figure 2b appears to be invalid because otherwise the (2,4,4-d_3)-(3*S*)-equol product would have been isolated. Here, we have to point out that if the secondary carbocation in Figure 2b is stable enough to prevent a 1,2-hydride shift due to the resonance stabilization of benzylic cation at C-4, then the retention of the label at C-3 would be reasonable. However, the benzylic carbocation might also be expected to undergo β-elimination to form DE, which was not observed. Second, reduction of the C-4 hydroxyl group proceeded with retention of configuration, which is inconsistent with an S_N2-type concerted hydride reduction mechanism that would produce (2,3,4$_\beta$-d_3)-(3*S*)-equol (Figure 2c). Furthermore, we could not observe any of the possible reaction intermediates that might arise from a multistep transformation, such as (3*R*)-equol, DE, or (3*S*,4*S*)-THD. Therefore, whereas the reduction of C-4 could involve either a cationic, anionic, or radical mechanism, the results of the deuterium labeling experiment make the recently described carbocation mechanism unlikely. A mechanism involving the initial formation of a benzylic carbanion intermediate also seems unlikely because abstraction of a proton at C-4 would not be energetically favorable.

However, a radical mechanism for the conversion of (3*S*,4*R*)-THD to (3*S*)-equol has a mechanistic precedent in the reactions catalyzed by ribonucleotide reductases, which are all radical enzymes (*26*, *27*). In particular, these enzymes all cleave the 3′-C−H bond of the ribose to facilitate reduction of the 2′-OH group, which occurs with retention of stereochemistry. Therefore, we propose a similar mechanism for THD reductase that accounts for the stereochemical course of the reaction.

In this mechanism, a protein-based radical (Figure 6), X$^\bullet$ (by analogy with ribonucleotide reductase this would be a thiyl radical), initially abstracts hydrogen from the C-3 position to give a relatively stable benzylic radical (**A**). The radical at C-3 facilitates the loss of the C-4 hydroxyl group to form a delocalized radical cation (**B**). Subsequent reduction of this species by a hydride donor can then occur at C-4 with retention of stereochemistry as required by the deuterium labeling pattern (**C**). Lastly, addition of the C-3 hydrogen to the same *re*-face of the molecule would complete the catalytic cycle to generate (3*S*)-equol. Interestingly, when the substrate analogue of (3*S*,4*R*)-isoflavan-4-ol, (3*S*,4*R*)-THD without the 4′,7-dihydroxyl groups, was reacted with the THD reductase under the same reaction conditions, the expected reduction product of (3*S*)-isoflavan was not isolated. Therefore, it appears the two OH groups are important for the resonance stabilization of the reaction intermediate.

In conclusion, deuterium labeling studies have uncovered the unusual stereospecific course of the (3*S*)-equol biosynthesis catalyzed by THD reductase, which is inconsistent with the previous mechanistic proposals for this enzyme. We suggest a chemically reasonable mechanism, analogous to that of ribonucleotide reductase, that involves a radical cationic intermediate

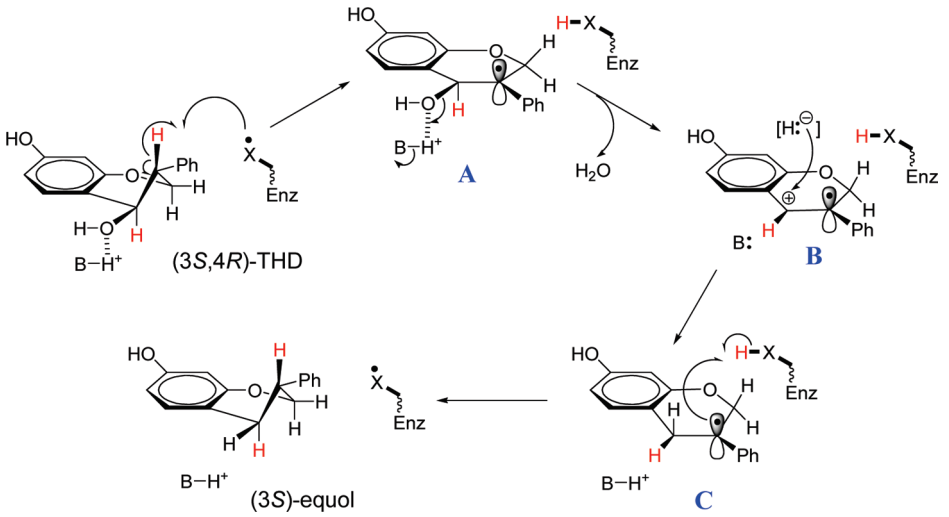

FIGURE 6: Suggested mechanism for formation of (3*S*)-equol from (3*S*,4*R*)-THD by the THD reductase. The depicted mechanism entails radical intermediates and a radical initiating cofactor, but it does not necessarily follow the SAM-utilizing ribonucleotide reductase mechanism.

to explain this unprecedented biochemical reaction. We note that many unusual microbial transformations of the natural products have been recently found to involve radical enzymes, often involving SAM (*S*-adenosylmethionine), and it seems highly plausible that THD reductase, the genes for which have yet to be cloned and sequenced, could be a member of this growing class of enzymes (*28*). Furthermore, THD reductase activity is extremely oxygen-sensitive, which is another hallmark of all radical SAM enzymes. Currently, the isolation and characterization of the THD reductase are being studied.

SUPPORTING INFORMATION AVAILABLE

HPLC chromatogram, NMR spectra of *trans*-THD and equol, and EI-MS spectrum of (2,3,4,4-d_4)-equol. This material is available free of charge via the Internet at http://pubs.acs.org.

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