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Efficient Synthesis of Theaflavin 3-gallate by a Tyrosinase-Catalyzed Reaction with (-)-Epicatechin and (-)-Epigallocatechin Gallate in a 1-Octanol/Buffer Biphasic System

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Abstract

Theaflavins, the orange-red pigments contained in black tea, have attracted attention due to their health-promoting effects. However, their synthetic preparation, in which the enzymatic oxidation of catechol-type catechin is followed by the quinone-induced oxidative dimerization of selectively combined catechol- and pyrogallol-type catechins, provides only a low yield. In the present study, we found that a 1-octanol/buffer biphasic system improved the yield of theaflavin 3-gallate in a tyrosinase-catalyzed synthetic reaction with (-)-epicatechin and (-)-epigallocatechin gallate. When the enzymatic reaction proceeded in a buffer solution, oxidized (-)-epigallocatechin gallate was preferentially used for self-dimerization. However, self-dimerization was suppressed in the octanol phase, allowing oxidized (-)-epigallocatechin gallate to participate in coupling with (-)-epicatechin quinone leading to effective production of theaflavin 3-gallate. Furthermore, the preferential localization of theaflavin 3-gallate in the octanol phase prevented (-)-epicatechin quinone-induced degradation.

Key words

theaflavin; tyrosinase; enzymatic synthesis; biphasic system; 1-octanol

Introduction

Theaflavins, the characteristic orange or orange-red pigments in black tea leaves and their exudates, are produced during the fermentation of *Camellia sinensis* leaves, in which endogenous polyphenol oxidase (PPO) and peroxidase (POD) are involved.[1, 2] Condensation of different pairs of oxidized catechins, one with a dihydroxylated B-ring (catechol-type) and the other with a trihydroxylated B-ring (pyrogallol-type), followed by decarboxylation affords four kinds of theaflavins, theaflavin, **1**, theaflavin 3-*O*-gallate, **2**, theaflavin 3'-*O*-gallate, **3**, and theaflavin 3,3'-*O*-digallate, **4** (Figure 1).[3, 4]

Theaflavins contribute to the astringency of various brewed teas and have various bioactivities, such as antioxidant activity,[5, 6] anticancer activity,[7, 8] antidiabetic effects,[9] and inhibitory activity against bone loss in models of osteoporosis.[10] According to some *in vitro* and *in vivo* studies, **2** specifically decreases the micellar solubility of cholesterol,[11, 12] probably being responsible for the lowering of blood LDL cholesterol by the intake of black tea.[13, 14] In addition, a single oral administration of theaflavins elevates cremasteric blood flow in rats, and the magnitude of this effect is in the order of **3** >> **2** >> **1** = **4**.[15] To elucidate their structure-activity relationships and the mechanisms of their bioactivities, sufficient amounts of theaflavins are required. However, the study and application of theaflavins have been hampered by their fairly low amounts in black tea[16] and their instability in aqueous solutions,

especially under neutral or alkaline conditions.[17-19]

Researchers have investigated the mechanism of theaflavin synthesis using plant homogenates,[4] PPO purified from tea leaves,[1, 20, 21] or tyrosinase (EC 1.14.18.1) from the mushroom *Agaricus bisporus*, a copper-containing oxidase with both monophenol monooxygenase and diphenol oxidase activities, as a commercially available PPO model.[22] Through the prospective mechanism of PPO-catalyzed theaflavin synthesis (Figure 1), the enzymatic and non-enzymatic reactions proceed concurrently: 1) catechol-type catechins are enzymatically oxidized to the quinone form, 2) pyrogallol-type catechins are oxidized either by enzymatic oxidation or a coupling redox reaction with catechol quinones, and 3) theaflavins are produced by the condensation of a pair of catechol- and pyrogallol-type quinones and subsequent decarboxylation. There is another explanation in which the benzotropolone moiety in theaflavins is produced by direct coupling between catechin quinone and pyrogallol.[23, 24] This hypothesis concerning the first step is based on organic reactions in aprotic media. The higher electron-donating property of the pyrogallol group[25, 26] and our previous kinetic analyses of tyrosinase-catalyzed reactions with catechins in aqueous media (i.e., catechol-type catechins decrease very slowly in spite of their higher constant efficiency (k_{cat}/K_m) for tyrosinase until almost all of the combined pyrogallol-type ones are consumed)[22] strongly suggest that coupling redox reactions between catechol quinones and

pyrogallol occur predominantly under aqueous conditions essential for enzymes. The sub-products interfere with the synthesis of theaflavins; in particular, highly reactive quinones attack theaflavins leading to oxidative degradation.[4, 20, 22] Pyrogallol quinones are known to cause self-dimerization,[27-30] restricting the efficiency of the condensation critical for the production of theaflavins as described above.[22] In our previous study, we found that the synthetic yields of galloylated theaflavins, such as **2**, **3**, and **4**, by the tyrosinase-catalyzed reaction are significantly lower compared with that of **1**.[22]

Considering the differences in the partition coefficients of catechins,[31] we conceived a 1-octanol/buffer biphasic system that would allow for mild separation of hydrophobic (-)-epigallocatechin gallate, **8**, from the aqueous buffer phase, where tyrosinase preferentially oxidizes (-)-epicatechin (EC), **5**, a relatively hydrophilic catechol-type catechin (Figure 2).[22] A coupling redox reaction involving EC quinone and **8** then rapidly occurs, and the condensation of EC- and EGCg quinones synthesize **2**. The partition of hydrophobic **2** into the 1-octanol phase may prevent EC quinone-mediated oxidative degradation of **2**. In this study, we investigated the effects of a 1-octanol/buffer biphasic system on the tyrosinase-catalyzed synthesis of **2** and found that this system effectively increased the synthetic yield of **2**.

Materials and methods

Materials

(-)-Epicatechin (EC), **5**, (-)-epicatechin gallate (ECg), **6**, (-)-epigallocatechin (EGC), **7**, and (-)-epigallocatechin gallate (EGCg), **8**, were kindly provided by Mitsui Norin Co., Ltd. (Shizuoka, Japan). Tyrosinase from *A. bisporus* was obtained from Sigma-Aldrich Co. (St. Louis, MO). 1-Octanol was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). *o*-Phenylenediamine was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other reagents used were of analytical grade.

Measurement of partition coefficients of catechins and theaflavins

The respective four catechins were dissolved in pure water at 1 mM. An aliquot (200 μL) of aqueous catechin or theaflavin solution was mixed with an equal volume of 1-octanol. After the mixture was vigorously shaken, it was centrifuged at approx. 2046 × *g* for 3 min at room temperature (25 °C). Polyphenols in both the upper layer (1-octanol phase) and the lower one (aqueous buffer phase) were analyzed by RP-HPLC. RP-HPLC was carried out as described previously.[22] Briefly, 30 μL of sample were injected to a 150 mm × 4.6 mm i.d., 4 μm, Synergi Polar-RP 80Å column (Phenomenex, Torrance, CA). Catechins and theaflavins were eluted with aqueous 20% and 32% MeCN solutions containing 0.05% phosphoric acid at flow rate of 1 mL/min, respectively, and were detected at 280 nm. Chromatogram analysis

was performed with the data processing software Chromato-PRO (Run Time Corp., Tokyo, Japan). Partition coefficients (log P_{ow} values) were determined as follows:

$$\log P_{ow} = \log_{10} [\text{polyphenol in 1-octanol phase}]/[\text{polyphenol in aqueous phase}]$$

Tyrosinase-catalyzed synthesis of 2 in the absence or presence of 1-octanol

A series of reaction mixtures containing **5**, **8**, and tyrosinase were constructed as shown in Table 1. Final concentrations of respective catechins and tyrosinase were 2.5 mM and 0.025 mg/mL in 50 mM sodium phosphate buffer, pH 6.0. The enzymatic reaction was conducted at 25 °C. At intervals of 15 min for up to 60 min, the reaction mixture was shaken at 2800 rpm/min for 1 min with a vortex mixer, centrifuged at approximately 2046 × *g* for 3 min, and then each aliquot (30 μL) of the 1-octanol and buffer fractions was taken and added into 180 μL of the stop solution (a mixture of equal volumes of 25 mM citric acid and ethanol). The stopped samples were analyzed by RP-HPLC using a 150 mm × 4.6 mm i.d., 4 μm, Synergi Polar-RP 80Å column (Phenomenex, Torrance, CA). The mobile phase consisted of 0.05% phosphoric acid in water (A) and 0.05% phosphoric acid in 80% MeCN (B). Polyphenols were eluted by gradient elution according to the following program: 0-10-30 min/20-25-50% B at flow rate of 1 mL/min. They were detected at 280 nm to identify the peaks of catechins and theaflavins.

Analysis of phenazine derivatives

Each aliquot (30 μL) of the 1-octanol and buffer fractions was taken and added into 180 μL of 0.5 mg/mL *o*-phenylenediamine in 0.5% acetic acid/ethanol.[29] Phenazine derivatives were analyzed by RP-HPLC as described above. Some peak compounds were isolated by RP-HPLC with similar eluting conditions, except that 0.2% acetic acid was substituted for 0.05% phosphoric acid. They were concentrated by a centrifugal evaporator CC-105 (Tomy Seiko, Tokyo, Japan) for mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses. High resolution electrospray ionization time-of-flight (HR-ESI-TOF) MS spectra were measured with a JMS-T100LP AccuTOF LC-plus (JEOL, Tokyo, Japan) operated in positive ion mode. Monoisotopic masses were calculated by a Molecular Mass Calculator.[32] MS spectra of **P1** and **P2** showed $[M+Na]^+$ peaks at m/z 1007.1744 and m/z 1007.1738, respectively. Thus, both molecular formulae of **P1** and **P2** were identical, corresponding to $C_{50}H_{36}N_2O_{20}$ (calcd for $C_{50}H_{36}N_2NaO_{20}$, 1007.1759). NMR spectra were recorded on an ECP-600 FT-NMR (JEOL, Tokyo, Japan) spectrometer operating at ^1H and ^{13}C resonance frequencies of 600.18 MHz and 150.93 MHz, respectively. A field-gradient system was used for 2D-NMR measurements. Chemical shift values were referenced with respect to tetramethylsilane (TMS) at 0 ppm.

Results and Discussion

Efficient synthesis of 2 by a tyrosinase-catalyzed reaction with 5 and 8 in the presence of

1-octanol

The log P_{ow} values of four catechins (Table 2) indicated that the order of their hydrophobicity is **6**>**8**>**5**>**7**. While **5** was partitioned almost evenly into both 1-octanol and water fractions, the concentrations of **6** and **8** in 1-octanol were about 40- and 10-fold of those in the water phase, respectively. The log P_{ow} values of theaflavins (Table 2) also indicated that galloylated theaflavins are more hydrophobic in the order of **4**>**3**>**2**>**1**. As expected, **2** was preferentially partitioned into the 1-octanol phase. After a small volume (10 μL) of tyrosinase solution (1 mg/mL in 50 mM sodium phosphate buffer, pH 6.0) was added into the 1-octanol phase (400 μL) containing 2 mM of **5** or **8**, these two catechins hardly decreased (data not shown), suggesting that the enzyme was denatured and inactivated by 1-octanol. Therefore, we examined the tyrosinase-catalyzed reaction with **5** and **8** in the 1-octanol/buffer biphasic system.

In a simple buffer solution, tyrosinase oxidized **5** (Figure 3A) and **8** individually (Figure 3B). As previously reported,[22] the tyrosinase-catalyzed reaction with a mixture of **5** and **8** produced **2**; however, the yield of **2** was very low: 0.20 mM of **2** at 60 min corresponding to 32% and 9% based on consumed **5** and **8**, respectively (Figure 3C). On the other hand, in the presence of 1-octanol, tyrosinase decreased **5** by itself in both 1-octanol and buffer phases, keeping its distribution pattern between the two phases (Figure 4A). The decrease of **5**

accompanied an orange colorization of the buffer phase (data not shown). This is probably due to oxidative polymerization of **5** and hydrophilicity of the resulting products. Although the generated EC quinone is considered to be more hydrophobic than **5**, it may be so highly reactive as to induce the polymerization of **5** before its distribution into the 1-octanol phase. In the case of the tyrosinase-catalyzed reaction with **8** by itself, a slightly pale-yellow colorization in the buffer phase and a moderate decrease of **8** in the 1-octanol phase were observed (Figure 4B). The latter might be due to compensation for the enzymatically consumed **8** in the buffer phase, because the enzymatic oxidation of **8** did not occur in 1-octanol as described above. The tyrosinase-catalyzed reaction with a mixture of **5** and **8** in the 1-octanol/buffer biphasic system showed efficient synthesis of **2** with a higher yield in the 1-octanol fraction: 0.46 mM of **2** at 60 min corresponding to 91% and 34% based on consumed **5** and **8**, respectively (Figure 4C). Preferential localization of **2** into the 1-octanol phase (Figure 4C and D) was visually confirmed. Although it is still unclear in which of the two phases the redox reaction between EC quinone and **8** occurs, the resulting product, EGCg quinone, might be effectively utilized for synthesis of **2** through condensation with EC quinone. The increase of **2** as well as the consumption of catechins reached a plateau after a 45-min reaction (Figure 4C and 4D) despite **5** remaining in both phases at higher concentrations than with treatment of **5** alone (Figure 4A). A higher concentration of

tyrosinase produced more **2**: 0.70 mM of **2** at 60 min with 88% and 47% yields based on consumed **5** and **8**, respectively (Figure 5A), though the reaction rate also decreased after 45 min (Figure 5). These results imply that enzyme inactivation during the reaction restricts the yield of **2**. Thus, in order to improve such a biphasic reaction system, further examinations are necessary into the adequate stirring conditions and technologies for enzyme immobilization required to prevent the enzyme from being denatured by 1-octanol.

Suppression of self-dimerization of EGCg quinone in the 1-octanol/buffer biphasic system

To investigate effects of the 1-octanol/buffer biphasic system on quinone production and oxidative self-dimerization of **8**,[28, 29] we compared the formation of phenazine derivatives between the reaction mixtures in the absence and presence of 1-octanol. In RP-HPLC chromatograms of samples in the absence of 1-octanol, compared with the slight peaks detected in the reaction of **5** alone with retention times of 9.4 min and 19.4 min (Figure 6A), two more distinguishable peaks, **P1** and **P2**, possibly corresponding to phenazine derivatives were detected in both the individual **8** reaction (Figure 6B) and the mixture of **5** and **8** (Figure 6C). Although **2**, **P1**, and **P2** were also detected in the 1-octanol phase (Figure 7), the peak area of **2** was remarkably larger than those of **P1** and **P2**, the latter being smaller than those from samples without 1-octanol (Figure 6C). The HR-ESI-TOF MS spectra of **P1** and **P2**

showed $[M+Na]^+$ peaks at *m/z* 1007.1744 and *m/z* 1007.1738, respectively. These data indicated that both molecular formulae of **P1** and **P2** were identical, corresponding to $C_{50}H_{36}N_2O_{20}$. The ^1H NMR spectrum of compound **P2** showed signals for eight protons derived from two C-rings (δ_H 2.34–5.59), two pairs of meta coupled benzene protons (δ_H 5.90 (d, J = 2.2 Hz), and δ_H 5.95 (d, J = 2.2 Hz), δ_H 6.08 (d, J = 2.2 Hz), and δ_H 6.12 (d, J = 2.2 Hz)), two singlet benzene protons (δ_H 7.12 and δ_H 8.22, each 1H), two galloyl protons (δ_H 6.98 and δ_H 7.09, each 2H), and protons of the phenazine moiety (δ_H 7.91–8.26) in the ^1H NMR spectrum (Table 3). In the ^{13}C NMR spectrum, 45 signals including two signals for 2 carbons and one signal for 4 carbons were observed as shown in Table 3. These spectra were analyzed by the aid of COSY, HMQC, and HMBC experiments, and the signals were assigned. Based on the chemical and spectroscopic data, **P2** was determined to be the phenazine derivative as shown in Figure 8, which is identical to a compound prepared by treatment of an ethanol extract of fermented tea leaves with *o*-phenylenediamine.[27] Comparison of the ^{13}C NMR data of **P2** with those reported confirmed the identity.[27] Similarly, the ^1H NMR spectrum of **P1** revealed two C-rings (δ_H 1.93–3.02), two pairs of doublets (δ_H 5.88 (d, J = 2.2 Hz) and δ_H 5.92 (d, J = 2.6 Hz), δ_H 6.05 (d, J = 2.2 Hz), and δ_H 6.06 (d, J = 2.2 Hz)), two galloyl moieties (δ_H 7.11 and δ_H 7.12, each 2H), and a phenazine moiety (δ_H 7.89–8.22). In the ^1H NMR of **P1**, one singlet benzene signal was observed at δ_H

7.15 as the corresponding signal (δ_H 7.12) of **P2**. However, another singlet benzene signal was significantly shifted to a higher magnetic field (δ_H 7.78) in comparison with the signal (δ_H 8.22) of **P2** (Table 3). These observations indicated that **P1** is a structural isomer of **P2** in which the phenazine moiety is bound to a different position of the B-ring of the original EGCg quinone dimer (Figure 8). Compounds **P1** and **P2** should be produced from quinone equivalents (**9a** and **9b**) of dehydrotheasinensin A,[28, 29] respectively (Figure 8). These results indicate that oxidative dimerization of EGCg quinone rapidly occurs in an aqueous buffer solution, but not in the 1-octanol phase. This is one of the reasons for differences in yield of **2** between reaction systems in the absence and presence of 1-octanol. In the present study, the phenazine derivative of EGCg quinone could not be detected; however, it is so hydrophobic compared to **8** as to be preferentially distributed into the 1-octanol phase. Such a water-immiscible solvent might lower the polarization of **8** and EGCg quinones, inhibiting the Michael addition reaction required for their dimerization. On the other hand, catechol quinones in aprotic solvents are known to be relatively stable but not completely stop the quinone-induced oxidation.[23, 24] Therefore, this suggests that the highly reactive EC quinone enables its condensation with EGCg quinone either in the buffer or 1-octanol phase, resulting in synthesis of **2**.

Preventive effect of the 1-octanol/buffer biphasic system against EC quinone-induced

oxidative degradation of 2

To investigate whether the preferential partition of **2** into the 1-octanol phase prevents its oxidative degradation caused by the attack of EC quinone, tyrosinase was added into the mixture of **2** and **5** in the absence or presence of 1-octanol. As a result, both **2** and **5** more rapidly diminished in the absence of 1-octanol than in the 1-octanol/buffer biphasic system (Figure 9), whereas without tyrosinase these two polyphenols were stable under the experimental conditions used (data not shown). It is known that tyrosinase does not directly use **2** as a substrate.[22] Therefore, it is clear that EC quinone, the product of enzymatic oxidation of **5**, is involved in the degradation of **2**.[4, 22] Considering that the decreases of **2** and **5** were not completely inhibited even in the 1-octanol phase (Figure 9), the degraded **2** in the buffer phase might have been compensated for by **2** from the other phase to keep its partition equilibrium. Although it is unclear why degradation of **2** was not noticeable in the 1-octanol/buffer biphasic system containing **8**, the latter may be more susceptible to EC quinone-induced oxidation than **2**.

Using a 1-octanol/buffer biphasic system is a promising approach to synthesize **2** in good yield by the tyrosinase-catalyzed reaction with **5** and **8**. Water-immiscible solvent-water biphasic systems are attractive for other enzymatic condensation reactions, giving products

with no dissociating groups.[33-38] The expected effects of organic solvents on product yields are dependent on the partition coefficients of substrates and products. Furthermore, these solvents can play a role in limiting the reverse hydrolysis of products by lowering water activity.[36-38] The present study for the first time elucidated the utility of a 1-octanol/buffer biphasic system for the tyrosinase-catalyzed synthesis of **2**, suggesting that the solvent restricts undesirable non-enzymatic reactions and enhances product yield. On the other hand, this system is not applicable to synthesis of the other theaflavins, because the distribution of combined **5** and **7** into the aqueous phase is not effective for **1**, and the excessive localization of **6** into the 1-octanol phase prevents it from enzymatic oxidation, leading to very slow synthesis of **3** and **4** (data not shown). Therefore, further study is needed to find other methods for enzymatic syntheses of **1**, **3**, and **4**. Screening other highly volatile solvents that not only have similar effects of 1-octanol on the synthesis of **2**, but are also available in the food industry is the next step for applying synthesized theaflavins into health foods, pharmaceuticals, and to study their various bioactivities.

Abbreviations used

EC: (-)-epicatechin; EGC: (-)-epigallocatechin; ECg: (-)-epicatechin gallate; EGCg: (-)-epigallocatechin gallate; log P_{ow}: 1-octanol–water partition coefficient

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Supporting Information

The HR-ESI-TOF MS spectra of **P1** and **P2** (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure captions

Figure 1 Mechanism of tyrosinase-catalyzed synthesis of theaflavins (**1**-**4**) from catechins (**5**-**8**) in aqueous conditions. R_1 and R_2 are H or a galloyl group (G).

Figure 2 Expected reactions in tyrosinase-catalyzed synthesis of **2** in the 1-octanol/buffer biphasic system. Additional Q means the quinone form of catechins.

Figure 3 Tyrosinase-catalyzed reactions with (A) **5** only; (B) **8** only; and (C) a mixture of **5** and **8** in buffer solution without 1-octanol. The concentrations of **2** (□), **5** (○), and **8** (△) are indicated. Data are means ± SD (n=3).

Figure 4 Tyrosinase-catalyzed reactions with (A) single **5** only; (B) single **8** only; and (C) 1-octanol; (D) buffer phase of a mixture of **5** and **8**; in a 1-octanol/buffer biphasic system containing tyrosinase at 0.025 mg/mL. For **2** (□, ■), **5** (○, ●) and **8** (△, ▲), closed and open symbols indicate the concentrations in 1-octanol and buffer phases, respectively. Data are means ± SD (n=3).

Figure 5 Tyrosinase-catalyzed reactions with a mixture of **5** and **8**; (A) 1-octanol; (B) buffer phase; in a 1-octanol/buffer biphasic system containing tyrosinase at 0.05 mg/mL. For **2** (□, ■), **5** (○, ●) and **8** (△, ▲), closed and open symbols indicate the concentrations in 1-octanol and buffer phases, respectively. Data are means ± SD (n=3).

Figure 6 HPLC chromatograms of *o*-phenylenediamine-treated samples after an enzymatic reaction for 30 min in buffer solution without 1-octanol. Each aliquot of (A) **5** only; (B) **8** only; and (C) a mixture of **5** and **8** was treated with *o*-phenylenediamine and analyzed (signal output = 1.0 AU/1000 mV).

Figure 7 HPLC chromatograms of *o*-phenylenediamine-treated samples. After an enzymatic reaction for 30 min in the 1-octanol/buffer biphasic system, each aliquot of 1-octanol phases of (A) **5** only; (B) **8** only; and (C) a mixture of **5** and **8** was treated with *o*-phenylenediamine and analyzed (signal output = 1.0 AU/1000 mV).

Figure 8 Derivatization scheme of EGCg quinone dimers by *o*-phenylenediamine,[27,28] and chemical structures of **P1** and **P2**.

Figure 9 Tyrosinase-catalyzed reactions with a mixture of **2** and **5** in the absence (dotted lines) or presence (solid lines) of 1-octanol containing tyrosinase at 0.025 mg/mL. For **5** (○ and ● in A) and **2** (□ and ■ in B), closed and open symbols indicate the concentrations in 1-octanol and buffer phases, respectively. Data are means ± SD (n=3).

Table 1 Solution Composition (mL) for Reaction Systems.

	5 only		**8** only		Mixture of **5** and **8**	
1-Octanol	(–)	(+)	(–)	(+)	(–)	(+)
5 mM of **5** solution[a]	0.2	0.2	-	-	0.2	0.2
5 mM of **8** solution[a]	-	-	0.2	0.2	0.2	0.2
50 mM phosphate buffer, pH 6.0	0.2	0.2	0.2	0.2	-	-
1-Octanol	-	0.4	-	0.4	-	0.4
1 mg/mL Tyrosinase[b]	0.01	0.01	0.01	0.01	0.01	0.01

a) Catechins are dissolved in 50 mM sodium phosphate buffer, pH 6.0.

b) Final concentration of tyrosinase in buffer is 0.025 mg/mL.

Table 2 log P_{ow} Values of Catechins and Theaflavins.

Catechins	log P_{ow}	Theaflavins	log P_{ow}
5	0.15 ± 0.03	**1**	0.09 ± 0.01
6	1.66 ± 0.04	**2**	0.90 ± 0.01
7	−0.43 ± 0.02	**3**	1.31 ± 0.05
8	1.04 ± 0.05	**4**	2.29 ± 0.09

Initial concentrations of catechins and theaflavins in water were 1 mM and 0.5 mM, respectively. Data are means ± SD (n=3).

Table 3. NMR Spectroscopic Data of **P1** and **P2** in Acetone-d_6

	P1	**P2**	
position	^{1}H	^{1}H	^{13}C
2	5.30 (1H, s)	5.22 (1H, s)	76.6
3	5.73 (1H, s, *J* = 4.0 Hz)	5.59 (1H, br d, *J* = 4.0 Hz)	67.8
4	2.69 (1H, dd, *J* = 4.4 Hz, 17.6 Hz) 3.02 (1H, d, *J* = 17.6 Hz)	2.63 (1H, dd, *J* = 4.6 Hz, 17.4 Hz) 3.04 (1H, d, *J* = 17.2 Hz)	27.1
5			157.3 [b)]
6	6.06 (1H, d, *J* = 2.2 Hz)	6.12 (1H, s, *J* = 2.2 Hz)	96.1
7			157.9
8	6.05 (1H, d, *J* = 2.2 Hz)	6.08 (1H, s, *J* = 2.2 Hz)	95.9
4a			98.6
8a			157.1
1'			143.2
2'			116.5
3'			144.3 [c)]
4'			135.7
5'			152.1
6'	7.78 (1H, s)	8.22 (1H, d, *J* = 0.8 Hz)	119.5
2"	4.71 (1H. s)	4.79 (1H, s)	76.4
3"	5.33 (1H, br d, *J* = 2.6 Hz)	5.44 (1H, ddd, *J* = 0.9 Hz, 1.8 Hz, 4.4 Hz)	68.6
4"	1.93 (1H, dd, *J* = 4.9 Hz, 17.6 Hz) 2.45 (1H, d, *J* = 17.2 Hz)	2.34 (1H, dd, *J* = 4.4 Hz, 17.6 Hz) 2.77 (1H, d, *J* = 17.2 Hz)	27.1
5"			157.5 [b)]
6"	5.92 (1H, d, *J* = 2.6 Hz)	5.95 (1H, d, *J* = 2.2 Hz)	96.9
7"			157.6
8"	5.88 (1H, d, *J* = 2.2 Hz)	5.90 (1H, d, *J* = 2.2 Hz)	96.4
4a"			98.7
8a"			157.3
1'"			112.4
2'"			128.5
3'"			144.0 [c)]
4'"			134.0
5'"			146.5
6'"	7.15 (1H, s)	7.12 (1H, s)	108.5
1""			121.4
2"", 6""	7.12 (2H, s) [a)]	6.98 (2H, s)	109.8
3"", 5""			145.9
4""			138.9
CO			166.4
1""'			122.0
2""', 6""'	7.11 (2H, s) [a)]	7.09 (2H, s)	110.0
3""', 5""'			145.9
4""'			138.7
CO'			166.1
phenazine	7.89–7.95 (2H, m) 8.18–8.22 (2H, m)	7.91–7.94 (2H, m) 8.17–8.20 (1H, m) 8.23–8.26 (1H. m)	131.4, 131.8 130.3 130.2 142.3, 144.9

[a–c)] Assignments may be interchanged

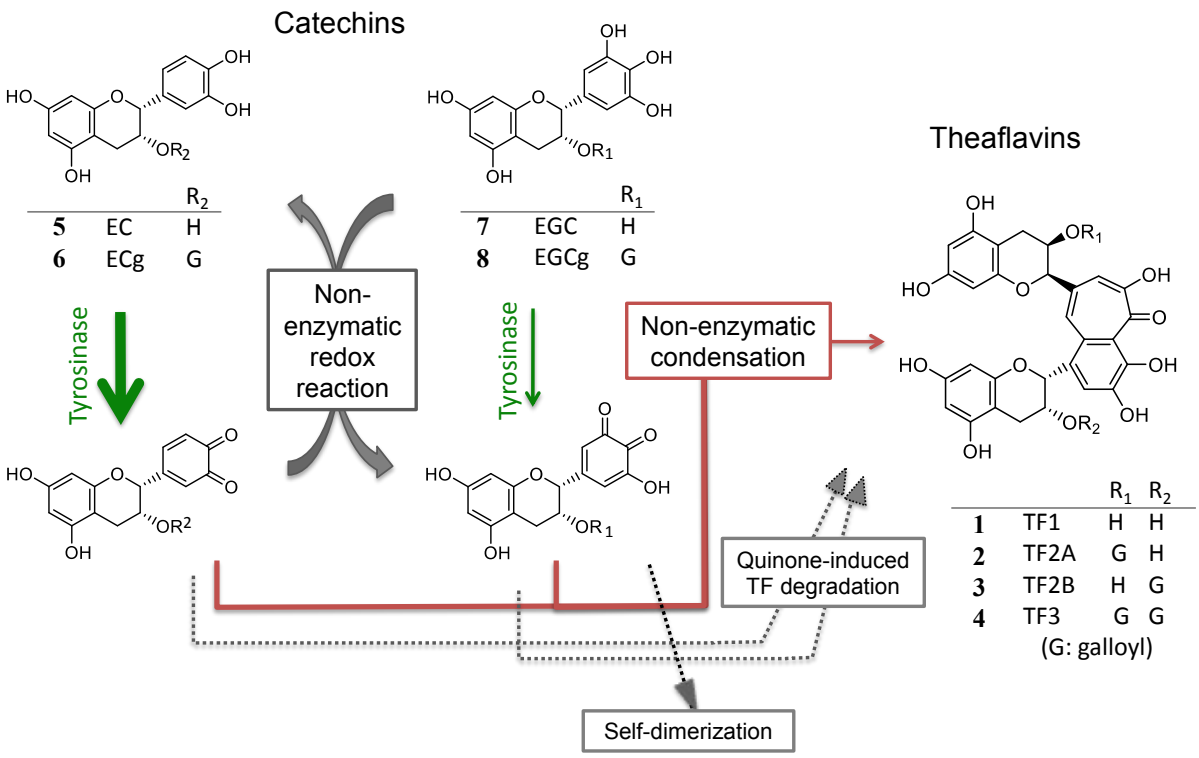

Catechins
Theaflavins
R2
5 EC H
6 ECg G
R1
7 EGC H
8 EGCg G
Tyrosinase
Tyrosinase
Non-enzymatic redox reaction
Non-enzymatic condensation
Quinone-induced TF degradation
Self-dimerization
R1 R2
1 TF1 H H
2 TF2A G H
3 TF2B H G
4 TF3 G G
(G: galloyl)

Figure 1

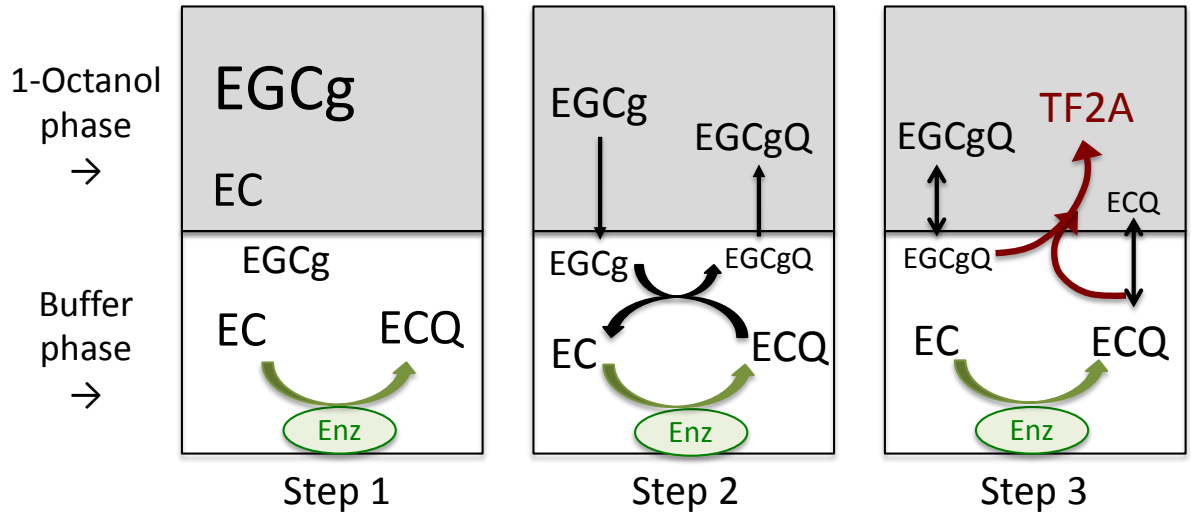
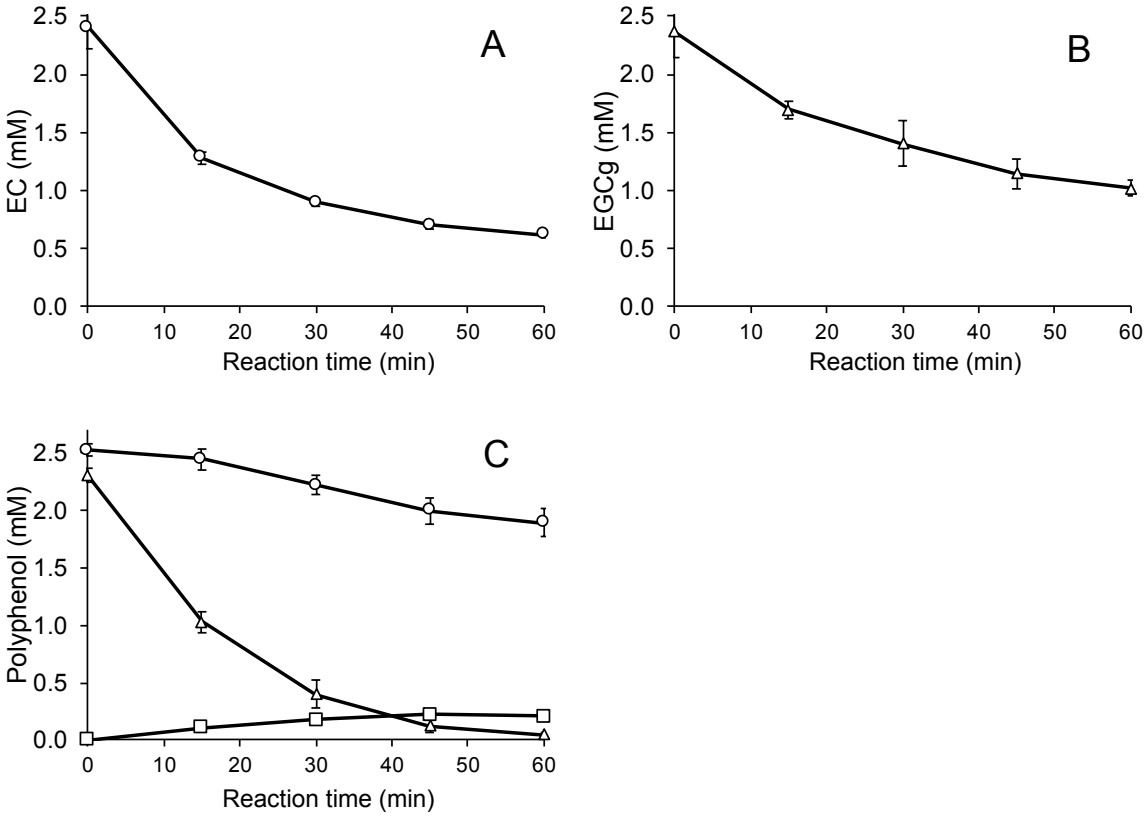

1-Octanol phase
→
Buffer phase
→
EGCg
EC
EGCg
EC
ECQ
Enz
Step 1
EGCg
EGCgQ
EGCg
EGCgQ
EC
ECQ
Enz
Step 2
EGCgQ
TF2A
ECQ
EGCgQ
EC
ECQ
Enz
Step 3

Figure 2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EC (mM)
Reaction time (min)
B
EGCg (mM)
Reaction time (min)
C
Polyphenol (mM)
Reaction time (min)

Figure 3

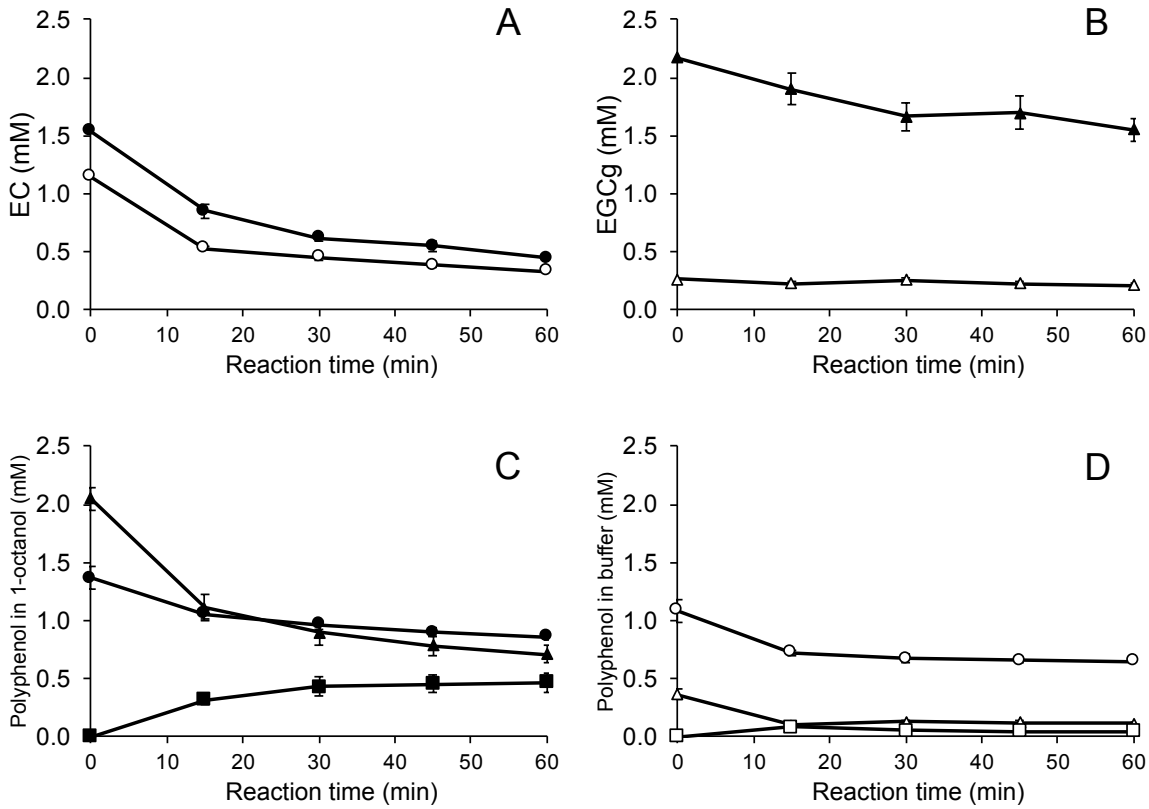
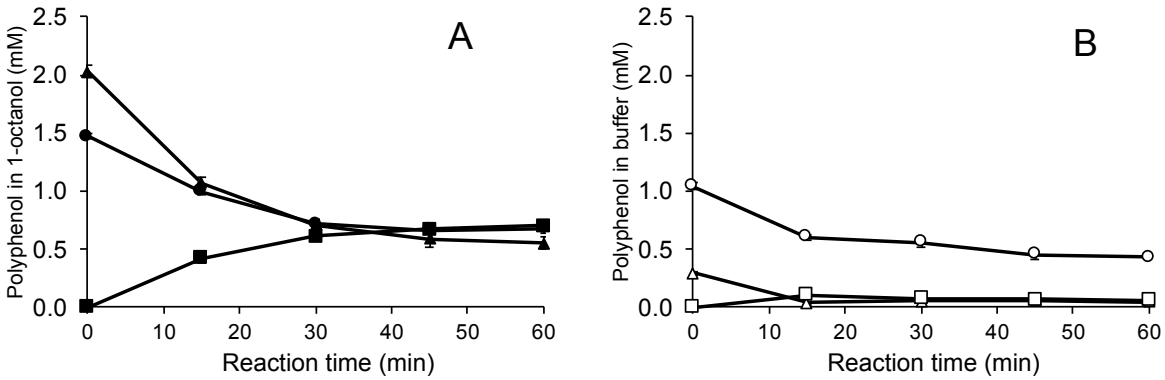

A
EC (mM)
Reaction time (min)
B
EGCg (mM)
Reaction time (min)
C
Polyphenol in 1-octanol (mM)
Reaction time (min)
D
Polyphenol in buffer (mM)
Reaction time (min)

Figure 4


A
B
Polyphenol in 1-octanol (mM)
Polyphenol in buffer (mM)
Reaction time (min)
Reaction time (min)
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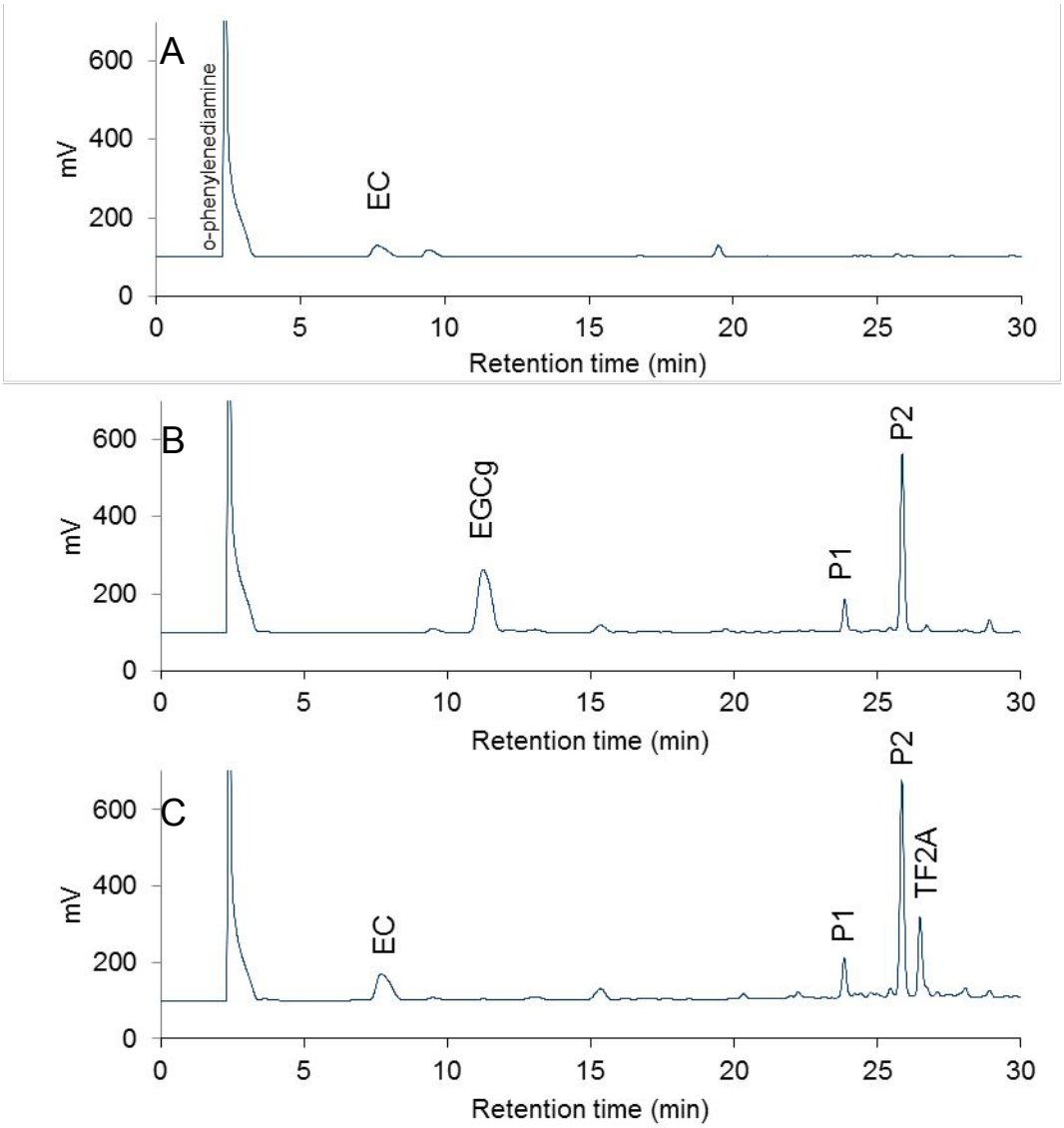
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
50
60

Figure 5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o-phenylenediamine
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mV
600
400
200
0
0
5
10
15
20
25
30
Retention time (min)
B
EGCg
P1
P2
mV
600
400
200
0
0
5
10
15
20
25
30
Retention time (min)
C
EC
P1
P2
TF2A
mV
600
400
200
0
0
5
10
15
20
25
30
Retention time (min)

Figure 6

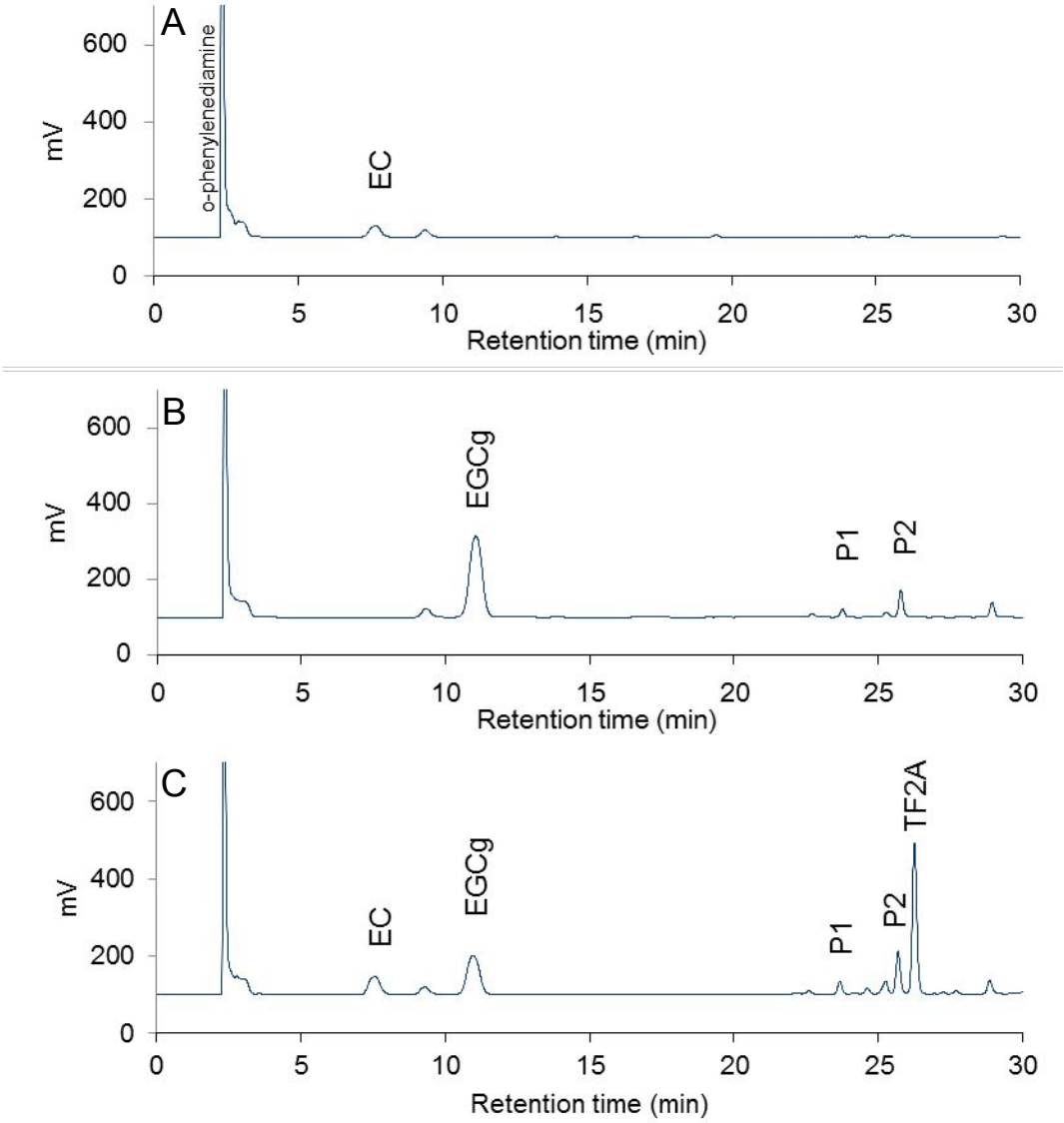

A
o-phenylenediamine
EC
mV
600
400
200
0
0
5
10
15
20
25
30
Retention time (min)
B
EGCg
P1
P2
mV
600
400
200
0
0
5
10
15
20
25
30
Retention time (min)
C
EC
EGCg
P1
P2
TF2A
mV
600
400
200
0
0
5
10
15
20
25
30
Retention time (min)

Figure 7

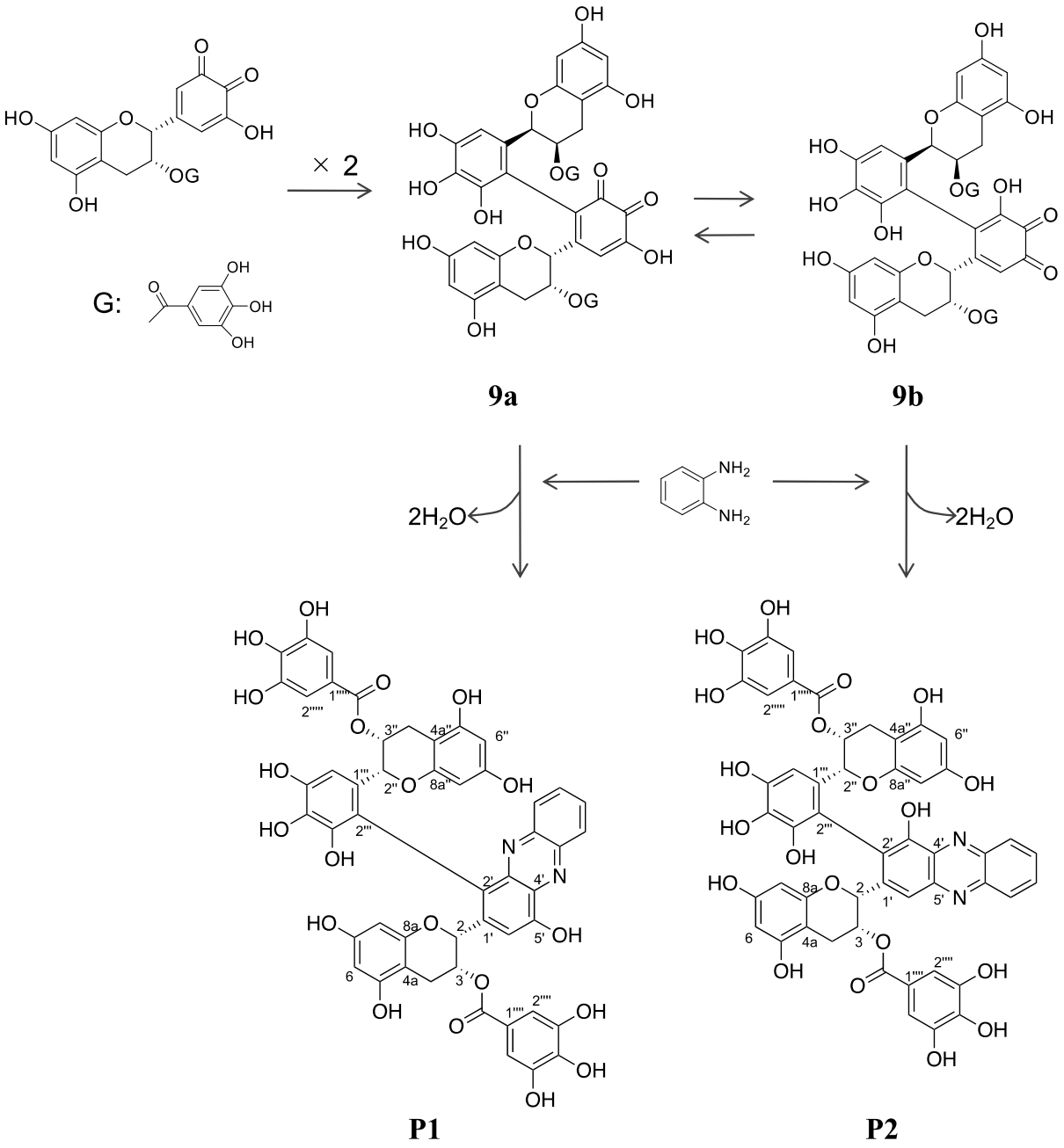


Figure 8

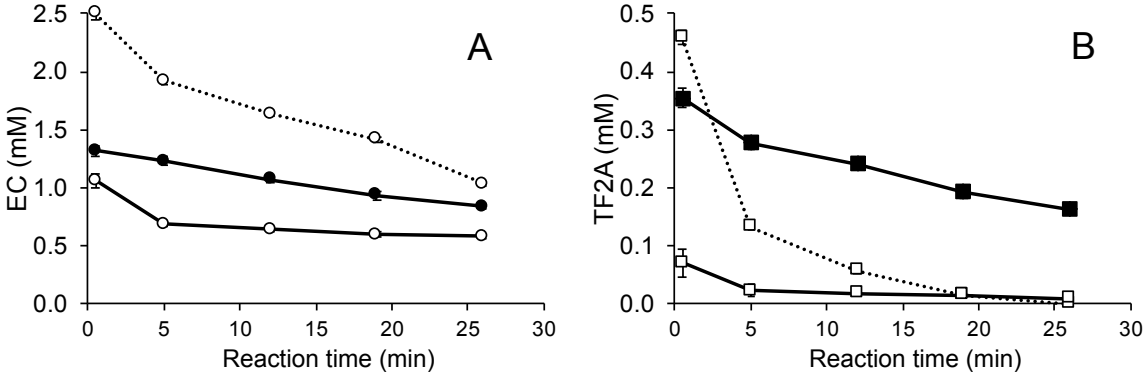
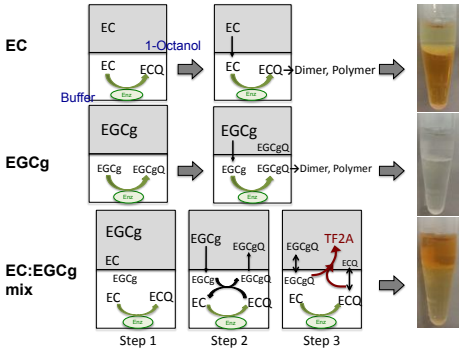

A
EC (mM)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
Reaction time (min)
B
TF2A (mM)
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
30
Reaction time (min)

Figure 9

Table of Contents Graphic



Efficient synthesis of theaflavin 3-gallate by a tyrosinase-catalyzed reaction with (-)-epicatechin and (-)-epigallocatechin gallate in a 1-octanol/buffer biphasic system. by A. Narai-Kanayama et al.