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Theanaphthoquinone, a novel pigment oxidatively derived from theaflavin during tea-fermentation

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Treatment of a mixture of epicatechin and epigallocatechin with extracts of fresh tea leaf or banana fruit generated a new pigment named theanaphthoquinone, which has a 1,2-naphthoquinone moiety oxidatively derived from the benzotropolone unit of theaflavin.

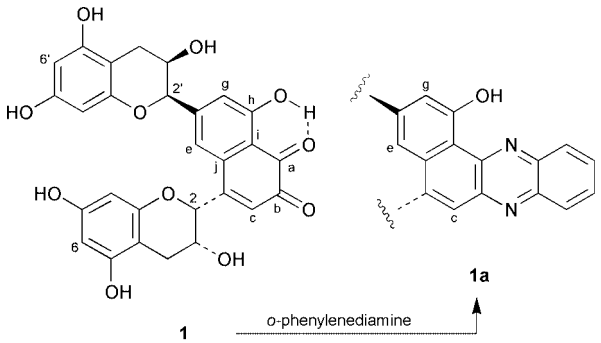
Theaflavins and thearubigins are major pigments of black tea and it is generally accepted that these are formed from flavan-3-ols (catechin) during tea fermentation.[1] Although structures and biogenesis of theaflavins having a benzotropolone unit are well studied, little is known about thearubigins despite many spectroscopic and chemical studies on these heterogeneous polymers.[2] Biochemical studies on tea fermentation indicated that oxidation of theaflavins might participate in thearubigin formation.[1]

For the purpose of clarification of the oxidative metabolism of flavan-3-ols in tea fermentation and characterization of thearubigins, a mixture of (−)-epicatechin (EC) and (−)-epigallocatechin (EGC), the major flavan-3-ols of tea leaf, was treated with aqueous extract of fresh tea leaf (*Camellia sinensis* var. *sinensis*). Prior to the reaction, almost all flavan-3-ols in the tea extract were removed by homogenization with Polyclar AT and subsequent filtration.[3] Analysis of the reaction mixture by HPLC equipped with a photodiode-array detector showed accumulation of theaflavin (**2**) at the initial stage (5 h). Subsequently, a peak due to an unknown product (**1**) having an absorption maximum at 440 nm appeared (10–20 h). Although an attempt to isolate **1** from the reaction mixture failed owing to the presence of many minor products, we found that **1** and **2** were also synthesized in a similar reaction using banana fruit instead of tea leaves.

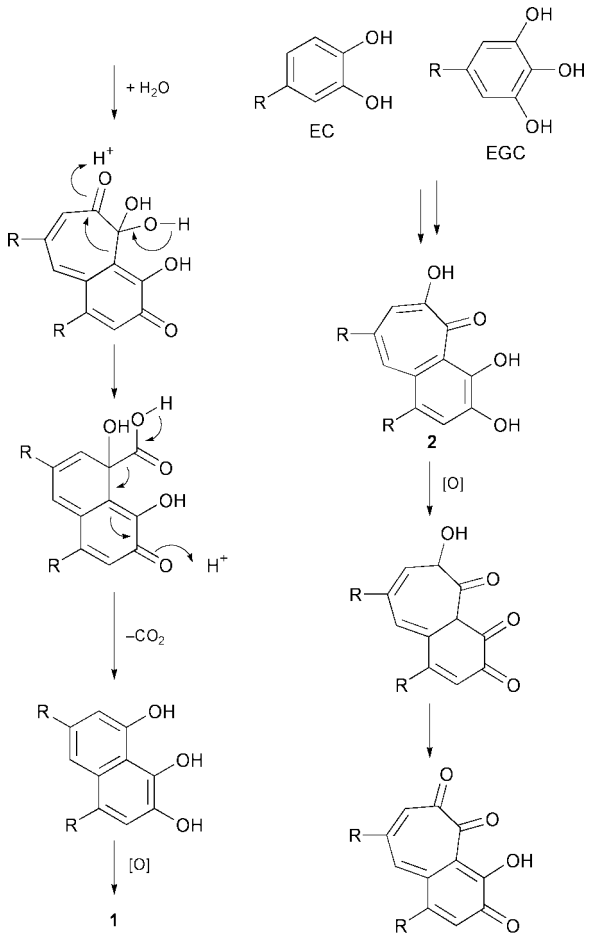
Separation of **1** and **2** from the reaction mixture treated with banana fruit was much easier than that in the tea leaf experiment (isolation yield from EGC: **1**, 16%; **2**, 39%).[4] Extracts of apple, potato, sweet potato, persimmon and black mushroom were also examined and found to be capable of synthesizing **2**; however, **1** was not detected in their reaction mixtures.

Structure elucidation of the dark yellow pigment **1**, named theanaphthoquinone, was as follows: ^{1}H and ^{13}C NMR spectra[5] resembled those of **2** and showed signals arising from two sets of A and C rings of flavan-3-ols. The ^{13}C NMR spectrum indicated the presence of two conjugated carbonyl groups [δ_C 180.62 (C-a) and 183.38(C-b)] and 8 sp^2 carbons besides those of the A and C rings. The HMBC correlation[6] of these carbons with three aromatic protons [δ_H 6.789 (H-c), 7.327 (H-g), 7.441(H-e)] and H-2 (δ_H 5.377) and H-2′ (δ_H 5.163) of the two C-rings revealed the presence of a 1,2-naphthoquinone structure. In addition, the appearance of a signal due to a phenolic hydroxy group at very low field (δ_H 12.28) indicated an intramolecular H bond with one of the C=O groups. The negative FABMS showed a pseudo-molecular ion peak at *m/z* 535, which was two mass units larger than that expected (*m/z* 533 [M − H]$^-$), and probably arose from a reduction product of **1**, because similar phenomena are known for some quinones with low redox potentials.[7] The final confirmation for the structure was made by condensation with *o*-phenylenediamine affording a phenazine derivative **1a** (*m/z* 605 [M − H]$^-$) and its spectral analysis (Scheme 1).[8]

1 and **2** were not detected when EC and EGC were treated separately with banana extract, and it was clear that **2** was



Scheme 1



Scheme 2

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synthesized from a combination of EC and EGC.[1] Therefore, it is suggested that **1** is biosynthesized from **2** with the aid of polyphenol oxidase as shown in Scheme 2. The discovery of **1**, the first pigment generated by oxidation of **2**, is of great interest from the viewpoint of food manufacturing. In addition, a prolonged experiment using tea leaf extract until 30 h showed a decrease of **1** and **2** and an increase of polymeric substances, since only a broad peak was detected on HPLC analysis, suggesting that **1** was further metabolized during tea fermentation and might be related to thearubigin formation.

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Notes and references

1 A. P. Davies, C. Goodsall, Y. Cai, A. L. Davis, J. R. Lewis, J. Wilkins, X. Wan, M. N. Clifford, C. Powell, A. Parry, A. Thiru, R. Safford and H. E. Nursten, in *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*, ed. G. G. Gross, R. W. Hemingway and T. Yoshida, Kluwer Academic/Plenum Publishers, 1999, p. 697.

2 T. Ozawa, M. Kataoka, K. Morikawa and O. Negishi, *Biosci. Biotech. Biochem.*, 1996, **60**, 2023.

3 Fresh tea leaves (30 g) were homogenized with 150 ml of water in the presence of Polyclar AT (10g). After filtration, the filtrate (3 ml) was mixed with an aqueous solution (0.5 ml) of EC (10 mg) and EGC (10 mg) and vigorously stirred at 20 °C. An aliquot of the mixture was extracted with aqueous acetone and analyzed by reverse phase HPLC.

4 Isolation of **1**: Banana fruit flesh (*Musa acuminata* Colla cv. giant cavendish) (200 g) was homogenized with water (600 ml) in a Warring blender and filtered with four layers of muslin. The filtrate (300 ml) was mixed with an aqueous solution (50 ml) of EC (1.0 g, 3.45 mmol) and EGC (1.0 g, 3.27 mmol) and stirred vigorously for 5 h at 20 °C. The mixture was poured into acetone (800 ml) and filtered. The filtrate was concentrated and extracted with AcOEt (300 ml × 4) and the extract was applied to a column of Sephadex LH-20. Elution with EtOH afforded **1** (280 mg, 0.524 mmol) and **2** (728 mg, 1.29 mmol).

5 *Selected data* for **1**: red amorphous powder, $[\alpha]_D$ − 386.9° (*c*. 0.2, MeOH), λ_{max}^{EtOH}(log ε): 249 (4.29), 440 (3.70); δ_H(500 MHz, acetone-d_6) 5.377 (br s, H-2), 4.396 (br d, *J* 2.7, H-3), 2.955 (dd, *J* 4.6, 16.5, H-4), 2.815 (br d, *J* 16.5, H-4), 5.163 (br s, H-2′), 4.429 (ddd, *J* 1.9, 3.7, 4.6, H-3′), 2.713 (dd, *J* 3.7, 16.6, H-4′), 2.899 (dd, *J* 4.6, 16.6, H-4′), 6.062 and 6.065 (each d, *J* 2.3, H-6 and H-6′), 6.005 and 6.006 (each d, *J* 2.3, H-8 and H-8′) and 12.28 (s, OH); δ_C(125 MHz, acetone-d_6 + D_2O) 180.62 (C-a), 183.38 (C-b), 126.97 (C-c), 152.16 (C-d), 118.05 (C-e), 152.88 (C-f), 120.13 (C-g), 166.97 (C-h), 115.27 (C-i), 133.91 (C-j), 75.40 (C-2), 64.84 (C-3), 28.92 (C-4), 99.68 (C-4), 156.15 (C-8a), 78.85 (C-2′), 66.30 (C-3′), 28.78 (C-4′), 99.38 (C-4a′), 156.09 (C-8a′); (calc. for $C_{28}H_{22}O_{11}\cdot 7/4H_2O$: C, 59.42; H, 4.54. Found: C, 59.78; H, 4.98%).

6 *Selected HMBC correlations* for **1**: H-c to C-b, C-d, C-j and C-2; H-e to C-d, C-j, C-i, C-f, C-g and C-2′; H-g to C-e, C-f, C-h, C-i and C-2′; H-2 to C-c, C-d, C-j and C-4; H-2’ to C-e, C-f, C-g, C-4′ and C-8a′.

7 L. D. Detter, O. W. Hand, R. G. Cooks and R. A. Walton, *Mass Spectrom. Rev.*, 1988, **7**, 465.

8 Synthesis of **1a**: A solution of **1** (15mg) and *o*-phenylenediamine (10 mg) in EtOH (3 ml) containing 10% acetic acid was stirred at 45 °C for 2 h. The mixture was separated by Sephadex LH-20 column chromatography using EtOH to yield **1a** (16.0 mg) as a red amorphous powder. $[\alpha]_D$ −361.3° (*c*. 0.1, MeOH), λ_{max}^{EtOH}(log ε): 266 (4.563), 438 (4.03); δ_H (500 MHz, acetone-d_6 + D_2O) 8.243 (m), 7.983 (m), 8.048 (d, *J* 0.9, H-c), 7.756 (d, *J* 1.1, H-e), 7.465 (br s, H-g), 5.757 (br s, H-2), 4.565 (m, H-3), 3.100 (dd, *J* 4.5, 16.5 H-4), 2.927 (br d, *J* 16.5, H-4), 5.373 (br s, H-2′), 4.565 (m, H-3′), 2.996 (dd, *J* 4.5, 16.3, H-4′) and 2.770 (dd, *J* 3.9, 16.3, H-4′). Long-range H–C correlations observed in the HMBC spectrum were entirely consistent with the structure **1a**.