Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 19 February 2009, Revised 31 March 2009, Accepted 4 April 2009 Published online 28 May 2009 in Wiley Interscience

(www.interscience.wiley.com) DOI: 10.1002/jlcr.1605

Deuterium labelling of theaflavin

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An efficient deuterium labelling of theaflavin, aiming at radiolabelling of theaflavin, was accomplished for the first time by using 4-deuterium-labelled *epi*-catechin as the precursor and the resulting D-labelled theaflavin was fully characterized by ^{1}H NMR, ^{13}C NMR, IR spectroscopy and ESI-MS. The synthesis features a novel efficient polyphenol oxidase (PPO) catalyzed benzotropolone formation. PPO was bound onto a selected polymer by a novel efficient method developed in this lab.

Keywords: theaflavin; deuterium; polyphenol oxidase; isotopic labelling

Introduction

Black tea and oolong tea, the fermentation and partial fermentation products of green tea leaves are popular beverages in Western and Asian countries, respectively. Both are unique containing theaflavin and its gallates (Figure 1), which embrace a benzotropolone scaffold and contribute to the characteristic red colour and flavour of the tea product. Daily consumption of black tea results in intake of theaflavins, which has been claimed to deliver a variety of physiological benefits, e.g. reducing the risk of cardiovascular diseases and diabetes,[1,2] prevention of cancers,[3,4] slow-down of the ageing process,[5] anti-inflammation,[6,7] anti-rheumatoid arthritis[8] and others.[9] These beneficial functions are generally anticipated to arise from the radical scavenging and antioxidative properties of theaflavins.[10,11] However, their mechanisms of actions have not been investigated in detail. While pharmacokinetics and ADME (absorption, distribution, metabolism and excretion) investigations on green tea catechins have been extensively carried out,[12,13] the same *in vivo* studies on theaflavins have not been thus far reported, though recent *in vitro* studies reveal that theaflavin could interact with some specific biological targets and pathways.[14–17]

The isotopically modified compounds are versatile and non-invasive tools for elucidating the metabolism and physiological role of a bioactive compound in live subjects, and have minimum structural disturbances to the original compound. The radioactive isotopically labelled compound has particular advantages of better sensitivity to enable easy detection and analysis. Developing an isotopically labelled theaflavin would create future potentials to further and better investigate the biological fate and benefits of its consumption. Therefore, specific opportunity was taken in this study to develop radiolabelled theaflavin. Tritium was identified as most suitable to label this molecule and simplify the complexity of the synthesis. This study, conducted in house, accomplishes the non-radioactive deuterium labelling of theaflavin, and henceforth, defines a procedure for radioactive tritium labelling.

It has been discovered that the construction of benzotropolone, requiring multiple steps by conventional synthetic approaches,[18] can be achieved by polyphenol oxidase (PPO) or peroxidase catalyzed oxidative dimerization of catechol and pyrogallol.[19,20] PPO was successfully loaded onto a selected polymer in our laboratory to simplify and improve this transformation, and high catalytic activity and efficiency were attained in the condensation of *epi*-catechin (EC, as catechol) and *epi*-gallocatechin (EGC, as pyrogallol) for the preparation of theaflavin (Figure 2).[21] This enabled us to perform the isotopic labelling of theaflavin with labelled EC as the precursor.

Tritium labelling of EGC gallate has been previously accomplished by several methods using H-T exchange[22] or halogen-T exchange.[23] With the anticipation that the 4-benzylic position of EC is easily susceptible to halogenation and reductive dehalogenation, a halogen and deuterium or tritium exchange strategy was pursued first.

Results and discussion

The synthesis of labelled EC, the precursor for PPO catalyzing benzotropolone formation, commenced with acetyl protection of all the hydroxyls and 4-position bromination of EC (Scheme 1). The former step proceeded smoothly by gently heating in pyridine, resulting in per-acetyl-EC **7** with a yield of 89%. The subsequent reaction conditions were optimized by maintaining the reaction temperature at 65°C, irradiating the reaction mixture with visible light,[24] and adding excess amount of NBS (2 eq.). This ensured the complete conversion of compound **7** within 3 h and allowed the synthesis with reasonable to good yields of a rather chemically unstable bromide **8,** which decomposed completely after two days storage at room temperature. Owing to the anchimeric assistance of the acetyl moiety, the bromo substituent that was introduced was exclusively *trans* configirated.[25] The simultaneous debromination and incorporation of deuterium was accomplished by treatment of compound **8** with a large excess amount of $NaBD_4$ (37 eq.) in anhydrous methanol at room temperature. However, these conditions were found not

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to remove the acetyl moiety at the 3-aliphatic hydroxyl position, though acetyls affiliated to phenolic hydroxyls were successfully removed. This acetyl was further eliminated by treatment of **9** with $LiAlH_4$ to attain 4-deuterium labelled EC **11**. There was evidence that deuterium was introduced stereospecifically because only one singlet signal for 4-proton could be observed, even though the exact configuration was not determined.

Isotope introduction using a large quantity of $NaBD_4$ was determined not to be the best suited for 4-tritium labelling of EC because the expensive $NaBT_4$ seems to mainly accomplish the removal of acetyls and result in a large amount of radioactive waste during the radioactive run. Hence, an alternative labelling procedure viable for tritium labelling was developed (Scheme 1). Bromide **8** was instead treated with 1.2 equivalent of $(n\text{-}Bu)_3SnD$ under an azoisobutyronitrile (AIBN) initiated radical condition to incorporate the isotope. Combined removal of the acetyls on **10** all at one time by $LiAlH_4$ accomplishes the formation of labelled epicatechin **11** with excellent yield (80% yield for two steps).

The condensation of D-labelled EC **11** (D-EC) with EGC **6** (EGC) proceeded straightforwardly in the presence of the freshly prepared polymer bound PPO (Scheme 2). The low concentration solution of D-EC and EGC (0.3%, w/w) was stirred vigorously with air finely bubbled into the reaction mixture at room temperature.

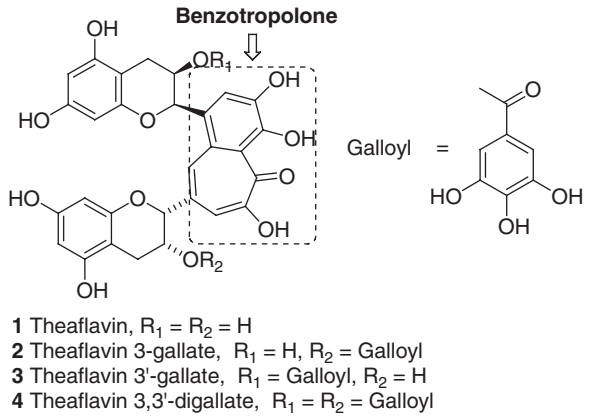


Figure 1. Theaflavin and its gallates.

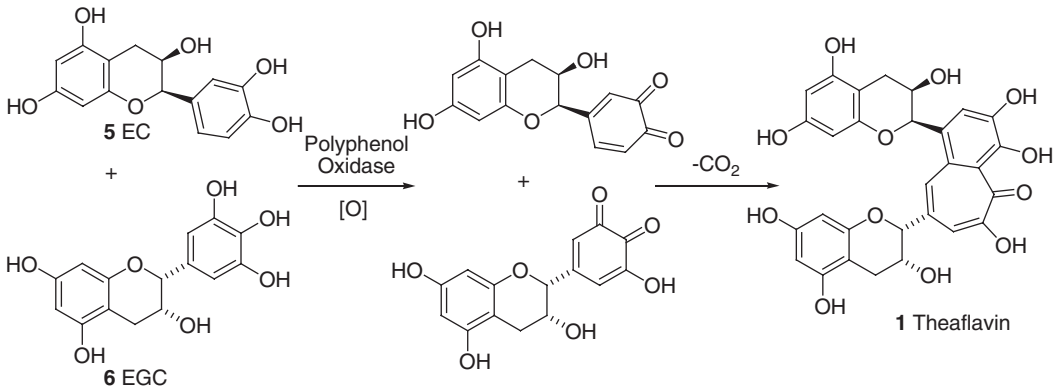
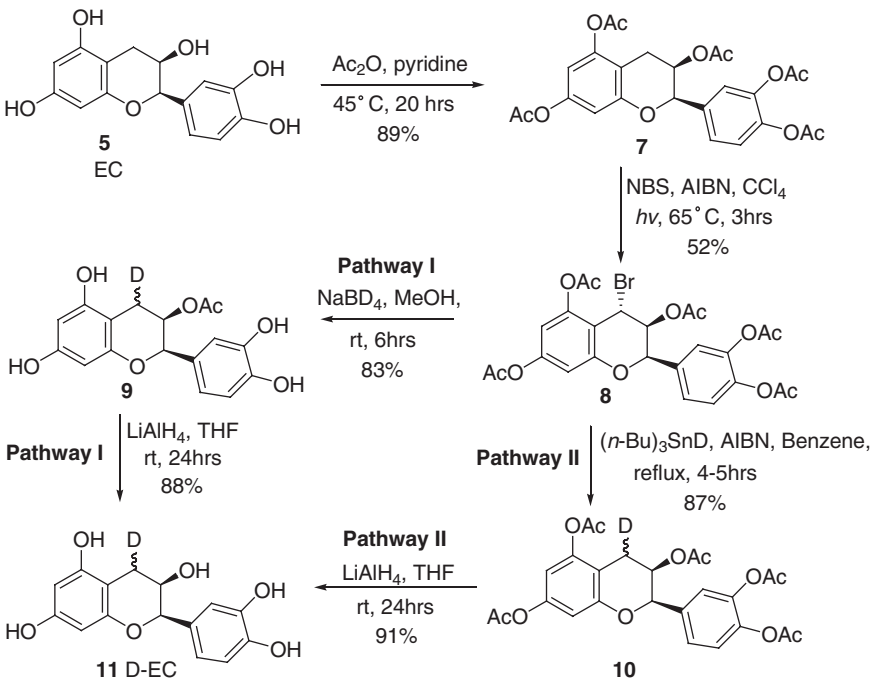
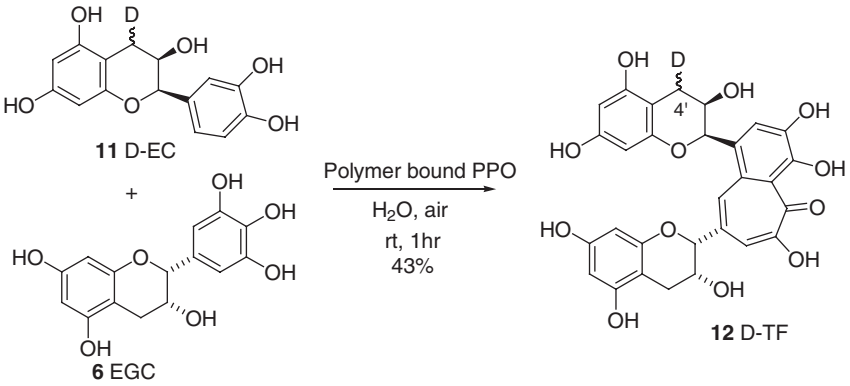


Figure 2. Polyphenol oxidase (PPO) catalyzed formation of theaflavin.



Scheme 1. D-labelling of *epi*-catechin.



Scheme 2. Polymer bound PPO catalyzed formation of D-labelled theaflavin.

D-EC was found to be converted completely in 1 h determined by thin layer chromatography (TLC) monitoring. Routine work-up followed by TLC isolation afforded D-labelled theaflavin **12** in moderate yield (43%). The accomplishment was clearly demonstrated with the AB system signal in the range of 2.8–3.0 ppm that was ascribed to the two protons at the 4′ position of non-labelled theaflavin **1.** This signal indicated a change to a singlet signal at the same chemical shift range with the integration of only one proton instead in the ^{1}H NMR spectrum of **12.** The chemical shifts and splitting pattern for the other ^{1}H NMR signals of **12** were the same as the corresponding ones of non-labelled theaflavin **1**. The similar phenomenon was observed in the ^{13}C NMR spectrum for the singlet signal at 28.2 ppm corresponding to the 4′ carbon of non-labelled theaflavin **1**. This signal became a triplet at the same chemical shift in the spectrum of **12** due to the direct coupling between 4′ carbon and the introduced deuterium, whereas all the other signals remained unchanged due to the distance from the deuterium that was introduced. The IR spectrum of **12** was finely superimposed with that of non-labelled theaflavin **1**. All these spectrum evidences, combined together with the HRMS established $C_{29}H_{24}DO_{12}$ $[M+1]^{+}$ molecular formula, fully suggest the successful formation of mono-deuterated theaflavin **12**. By utilizing $NaBD_4$ containing 98% deuterium as an isotope introducing agent, the deuterium content at the 4′ position of **12** was determined to be 94% by ESI-MS analysis.

In conclusion, D-labelled theaflavin **12** was synthesized in five straightforward steps from EC employing two alternative deuterated reagents, and a procedure for achieving tritium labelling is now established. Tritium labelling of theaflavin following this procedure is currently in progress in the radiosynthesis lab of our collaborator, using tri(n-butyl)tin tritide as tritium source to provide 1 mCi theaflavin with a specific activity of 11 Ci/mmol.

Experimental

Materials

PPO, THF, lithium aluminium hydride, pyridine, methanol, acetic anhydride and sodiumborodeuteride were purchased from Aldrich. EC and EGC were isolated from green tea extract in this lab. All other chemicals were of reagent grade and used without further purification.

General methods

NMR spectrum was recorded on a Bruker B-ACS 60 (400 MHz) spectrometer with $CDCl_3$ or CD_3OD as solvent. IR spectrum was obtained with a FTS185 FT-IR spectrometer using KBr pellets. Electrospray ionization (ESI) mass spectroscopy was obtained with a LCMS-2010 mass spectrometer.

*Procedure for the synthesis of **7***

EC **5** (858 mg, 3.0 mmol) was added to a mixture of pyridine (20 mL) and acetic anhydride (25 mL), and the mixture was maintained for 20 h at 45°C with stirring in an oil bath. The reaction mixture was then poured into ice-cold water (500 mL) with vigorous stirring, and left to stand for 1 h to form white precipitate. The resulting precipitate was collected by filtration with a sintered glass funnel, washed with 500 mL of chilled water, and dried under reduced pressure. The resulting product was chromatographed on a silica gel column with *n*-hexane/ethyl acetate (2:1 followed by 1:1; *v/v*) as the eluent to give the compound **7** (296 mg, 89% yield) as a while solid.

^{1}H NMR ($CDCl_3$, 400 MHz):δ2.27 (s, 12H); 2.89 (dd, 1H, $J = 1.8$, 14.3 Hz); 3.04 (dd, 1H, $J = 1.8$, 14.3 Hz); 5.10 (m, 1H); 5.30 (m, 1H); 6.51 (s, 1H); 6.64 (s, 1H); 7.21–7.25 (m, 2H); 7.42 (s, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz):δ21.2–22.1 ($CH_3CO \times 5$), 26.0, 67.2, 76.6, 108.0, 108.7, 109.6, 122.0, 123.2, 124.4, 135.8, 141.9, 142.0, 149.6, 149.7, 154.9, 168.1, 168.1, 168.1, 169.0, 170.0.

*Procedure for the synthesis of **8***

7 (296 mg, 0.59 mmol) was dissolved in carbon tetrachloride (60 mL) by heating at 65°C. *N*-Bromosuccinimide (210 mg, 1.2 mmol) and AIBN (18 mg, 0.11 mmol) were added to the solution. The reaction mixture was refluxed for 3 h under visible light irradiation. It was then cooled to room temperature and evaporated to dryness under reduced pressure. The resulting product was chromatographed on a silica gel column with *n*-hexane/ethyl acetate (2:1 followed by 1:1; *v/v*) as eluent to give the product **8** (180 mg, 52% yield).

^{1}H NMR ($CDCl_3$, 400 MHz):δ1.95 (s, 3H); 2.30 (s, 9H); 2.37 (s, 3H); 5.23 (m, 1H); 5.42 (m, 1H); 5.82 (s, 1H); 6.70 (s, 2H); 7.23–7.27 (m, 1H); 7.32–7.34 (m, 1H); 7.38 (s, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz):δ20.5–21.1($CH_3CO \times 5$), 21.2, 37.3, 71.2, 72.7, 108.2, 110.1, 110.2, 122.2, 123.4, 124.5, 134.8, 142.2, 150.1, 151.8, 154.6, 168.0, 168.1, 168.2, 168.7, 169.9.

*The first procedure for the synthesis of deuterium-labelled EC **11** (D-EC)*

8 (180 mg, 0.31 mmol) was dissolved in 36 mL of anhydrous methanol, and then $NaBD_4$ (435 mg) was added. Then the reaction mixture was quenched by addition of 5% phosphoric

acid (110 mL) after it was stirred for 6 h at room temperature. The resulting mixture was concentrated to about half of its original volume *in vacuo* and extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed three times with distilled water (50 mL), dried (Na_2SO_4) and evaporated to dryness. The residue was purified by silica gel column chromatography with CH_2Cl_2/ethyl acetate (3:1 followed by 1.5:1; v/v) as eluent to give **9** (92 mg, 83% yield). Consequently, into the solution of **9** in THF (15 mL), $LiAlH_4$ (107 mg, 2.8 mmol) was added. The reaction mixture was poured into ice-cold water (100 mL) after it was stirred under room temperature for 24 h, and then the pH value was adjusted to 1.0 by addition of 5% phosphoric acid. The mixture was then extracted with ethyl acetate (3 × 50 mL). The combined extracts were dried (Na_2SO_4) and concentrated. The residue was chromatographed on a column of silica gel with CH_2Cl_2/AcOEt/MeOH (10:6:3; v/v/v) as eluent to give product **11** (D-EC, 71 mg, 88% yield).

^{1}H NMR (CD_3OD, 400 MHz)δ2.19 (s, 1H); 4.17 (s, 1H); 4.81 (s, 1H); 5.91 (s, 1H); 5.93 (s, 1H); 6.74–6.81 (m, 2H); 6.97 (s, 1H);

^{13}C NMR (CD_3OD, 100 MHz)δ27.9, 66.0, 78.5, 94.5, 95.0, 98.6, 113.9, 114.5, 118.0, 130.9, 144.4, 144.6, 156.0, 156.3, 156.6.

The second procedure for the synthesis of deuterium-labelled EC ***11*** *(D-EC)*

Into a solution of bromide **8** (180 mg, 0.31 mmol) in anhydrous benzene (30 mL), *tri-n*-butyltin deuteride (109 mg, 0.37 mmol) and AIBN (1 mg) were added. The mixture was heated at 90°C for 5 h and concentrated. The residue was purified by silica gel column chromatography with CH_2Cl_2/AcOEt (3:1 followed by 1:1; v/v) as the eluent to give compound **10** (136 mg, 87% yield). Consequently, into the solution of **10** in THF (15 mL), $LiAlH_4$ (104 mg, 2.7 mmol) was added. The mixture was stirred under room temperature for 24 h. The reaction mixture was poured into ice-cold water (100 mL) after it was stirred under room temperature for 24 h, and then the pH value was adjusted to 1.0 by the addition of 5% phosphoric acid. The mixture was then extracted with ethyl acetate (3 × 50 mL). The combined extracts were dried (Na_2SO_4) and concentrated. The residue was chromatographed on a column of silica gel with CH_2Cl_2/AcOEt/MeOH (10:6:3; v/v/v) as eluent to give product **11** (D-EC, 71 mg, 91% yield). NMR spectroscopy characterization confirmed it was the same compound obtained by the first procedure.

Procedure for the synthesis of ***12***

D-EC **11** (71 mg, 0.24 mmol) and EGC **6** (149 mg, 0.45 mmol) were dissolved in 85 mL deionized water, and then 1.421 g of PPO loaded polymer was added. Air was pumped into the vigorously stirred reaction mixture as fine bubbles at room temperature. TLC monitoring showed that D-EC was consumed after 1 h, then the reaction mixture was filtered and the polymer was washed with ethanol (10 mL × 4). Ethanol was evaporated out under vacuum before extraction with ethyl acetate (50 mL × 4). The extracts were dried (Na_2SO_4) and concentrated. The resulted residue was purified by preparative silica gel thin layer chromatography with CH_2Cl_2/AcOEt/MeOH (10:6:3, *v/v/v*) to afford **12** (60 mg, 43% yield) as a brown solid.

^{1}H NMR (CD_3OD, 400 MHz)δ2.79 (s, 1H); 2.89 (AB-d system, 2H, J = 3.8, 1.0 Hz); 4.31 (s, 1H); 4.44 (s, 1H); 4.87 (s, 1H); 5.61 (s, 1H); 5.96 (m, 2H); 5.98 (s, 1H); 6.01 (s, 1H); 7.34 (s, 1H); 7.81 (s, 1H); 7.95 (s, 1H);

^{13}C NMR (CD_3OD, 100 MHz)δ28.0, 28.1 (t, J = 35.3 Hz); 64.2, 65.2, 75.8, 79.9, 94.2, 94.6, 95.3, 98.3, 98.7, 117.0, 120.8, 122.5, 125.1, 127.6, 130.2, 133.2, 145.1, 149.7, 154.1, 155.3, 156.0, 156.3, 156.5, 156.7, 156.8, 184.4;

IR (KBr) 3387, 2921, 2850, 1693, 1630, 1604, 1516, 1469, 1421, 1329, 1260, 1230, 1143, 1100, 1062, 808 cm^{-1};

LRMS (ESI) 564 $[M-1]^-$; HRMS (ESI) Found 566.1403 for $[M+1]^+$, Calculated for $C_{29}H_{24}DO_{12}$ 566.1406.

Acknowledgement

The author thanks Dr Ya Cai, former director of Unilever Research China, for his general advice and Dr Hong-Qiang Wang, current director of Unilever Research China, for his generous support during the progress of this study. We are also grateful to natural product team of URC for supply of catechins and analytic team for their analytic support.

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