Production and degradation mechanism of theacitrin C, a black tea pigment derived from epigallocatechin-3-O-gallate via a bicyclo[3.2.1]octane-type intermediate

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Graphical Abstract

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Yosuke Matsuo, Yan Li, Sayaka Watarumi, Takashi Tanaka,* Isao Kouno
Production and degradation mechanism of theacitrin C, a black tea pigment derived from epigallocatechin-3-\(O\)-gallate via a bicyclo[3.2.1]octane-type intermediate

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Abstract

Black tea is rich in polyphenols and has been shown to have various health benefits; however, its components have not yet been clarified in detail. Enzymatic oxidation of epigallocatechin-3-\(\text{O}-\)gallate, the most abundant polyphenol in tea, is thought to contribute significantly to the production of black tea polyphenols. We identified theacitrin C, an unstable black tea pigment, as an enzymatic oxidation product of epigallocatechin-3-\(\text{O}-\)gallate. Degradation of theacitrin C afforded theacitrinin A and 2,3,5,7-tetrahydroxychroman-3-\(\text{O}-\)gallate. Furthermore, theacitrinin B, which was isolated from black tea, is deduced to be a degradation product of theacitrin A, the desgalloyl analogue of theacitrin C. The structures of theacitrinins A and B were elucidated based on spectroscopic data. This is the first time that a degradation product of theacitrin has been isolated from black tea. We also examined the influence of esterification of the epigallocatechin C-3 hydroxyl group on the decomposition of bicyclo[3.2.1]octane-type intermediates.

Keywords: Black tea; Epigallocatechin-3-\(\text{O}-\)gallate; Theacitrin; Theacitrinin; Bicyclo[3.2.1]octane; Proepitheafagallin
1. Introduction

Plant polyphenols have recently attracted much attention because of their health-promoting effects. Black tea, which is one of the most popular beverages worldwide, contains abundant polyphenols, has various health benefits, and is an important source of plant polyphenols for humans. Black tea is manufactured by the fermentation of fresh tea leaves (Camellia sinensis). During this process, green tea catechins, (–)-epicatechin (17), (–)-epigallocatechin (15), (–)-epicatechin-3-O-gallate (16), and (–)-epigallocatechin-3-O-gallate (1), are enzymatically oxidized to afford complex mixtures of products.

(–)-Epigallocatechin-3-O-gallate (1), which is a catechin present in abundance in tea, is known to have a wide range of biological activities. Enzymatic oxidation of 1 is thought to contribute significantly to the production of black tea polyphenols. Our previous results revealed the structures of various enzymatic oxidation products of 1. However, some of the products were found to be unstable and were insufficiently characterized. In this paper, we report the isolation and identification of theacitrin C (5), an unstable black tea pigment, as a major enzymatic oxidation product of 1. We then examined the degradation of 5. We also describe the isolation of theacitrinin B (9), which is likely produced by degradation of theacitrin A (8), found in black tea. Furthermore, we examined the enzymatic oxidation products of (–)-epigallocatechin-3-O-acetate (10), to clarify the influence of the hydroxyl
group at the C-3 position of the C-ring on the oxidation of epigallocatechin derivatives.

2. Results and discussion

2.1. Production of theacitrin C by enzymatic oxidation of epigallocatechin-3-O-gallate

An HPLC profile of the enzymatic oxidation reaction mixture of (−)-epigallocatechin-3-O-gallate (1) is shown in Fig. 1. Previously, we characterized the structures of the three major products, corresponding to peaks 2–4, as dehydrotheasinensin A (2), EGCG quinone dimer A (3), and EGCG quinone dimer B (4), respectively (Scheme 1). However, until now, the product corresponding to peak 5 could not be isolated due to its instability. Here we report our successful efforts to isolate this unstable product.

![HPLC profile of enzymatic oxidation products](image)

**Fig. 1.** HPLC profile of the enzymatic oxidation products of epigallocatechin-3-O-gallate (1).
Scheme 1. Enzymatic oxidation of epigallocatechin-3-O-gallate (1).

An aqueous solution of 1 was stirred vigorously with Japanese pear fruit homogenate, which has a high polyphenol oxidase activity. After the reaction was observed to reach equilibrium, the mixture was acidified with trifluoroacetic acid (TFA) to inactivate enzyme activity and to suppress the degradation of 2. After filtration, the filtrate was applied to MCI-gel CHP20P and eluted to afford five fractions. Fraction 3 containing product 5 was further purified using Cosmosil 40C_{18}-PREP and YMC-Pack ODS-AQ.

The molecular formula of 5 was determined to be C_{44}H_{32}O_{22} by HRFABMS (m/z 935.1302 [M + Na]^+, +1.9 mmu). The $^1$H and $^{13}$C NMR spectra indicated the presence of two sets of flavan A- and C-rings, along with two galloyl groups. The remaining 12 carbon signals in the $^{13}$C NMR spectrum were attributable to the B-rings and consisted of three carbonyl
carbons ($\delta_{C} 200.2, 194.5, 177.8$), four $sp^2$ quaternary carbons ($\delta_{C} 173.3, 150.8, 149.5, 118.4$),
two $sp^2$ methines ($\delta_{C} 130.0, 118.4$), two $sp^3$ quaternary carbons ($\delta_{C} 87.2, 50.3$), and one $sp^3$
methine ($\delta_{C} 57.6$). $^1H$ and $^{13}C$ NMR data of the B-ring moiety of 5 indicated its similarity to
the B-ring of theacitrin A (8). The molecular formula of 5 (C_{44}H_{32}O_{22}) corresponded to that
of mono-galloyl substituted theacitrin A. The structure of 5 was therefore presumed to be
theacitrin A 3-O-gallate, i.e., theacitrin C, as shown in Scheme 1. The structure of 5 was confirmed using an HMBC experiment, which showed correlations similar to those observed
in 8.

Theacitrins A (8), B, and C (5) were previously isolated from black tea as yellow
pigments; however, 5 has only been characterized using MS and UV spectroscopic data. In
this paper, the structure of 5 has been characterized for the first time by 1D and 2D-NMR
spectroscopic analysis. This is also the first report of the isolation of theacitrins from the
enzymatic oxidation of epigallocatechins.

A production mechanism of 5 is proposed, as shown in Scheme 2. The B-ring of 1 is
oxidized to its $o$-quinone form (1a), and 1,4-addition between 1 and 1a then occurs to
generate a C–C bond. Successive oxidation and intramolecular 1,2-addition produces a
bicyclo[3.2.1]octane-type intermediate, which subsequently rearranges to afford 5.
2.2. Degradation of theacitrin C

During the final step of black tea manufacture, tea leaves are heated to deactivate enzymes and to dry the tea. On heating, theacitrin C (5) present in the tea leaves is likely to decompose because of its instability. We therefore examined the degradation products of 5. An aqueous solution of 5 was heated at 80 °C for 60 min and then analyzed by HPLC. Photodiode array detection revealed a decrease in levels of 5, and the decomposition products were detected as a broad hump on the HPLC baseline. Reaction in McIlvaine buffer at pH 5.0 also led to a similar result. However, heating in aqueous solution containing 0.1% TFA (ca. pH 1.9) gave products that could be detected as sharp peaks by HPLC analysis. The products were purified using Cosmosil 40C18-PREP to afford 6 and 7 (Scheme 3). Based on spectroscopic comparison, compound 7 was identified as
2,3,5,7-tetrahydroxychroman-3-O-gallate,\textsuperscript{4a} previously isolated from black tea.

**Scheme 3.** Degradation of theacitrin C (5) to afford theacitrinin A (6) and 2,3,5,7-tetrahydroxychroman-3-O-gallate (7).

Compound 6 exhibited a [M + Na]\textsuperscript{+} peak at \textit{m/z} 603 in MALDI-TOFMS. The \textsuperscript{1}H and \textsuperscript{13}C NMR spectra (Table 1) were similar to those of 5, except for the absence of signals arising from one set of flavan A- and C-rings, and a galloyl group. Comparison of the \textsuperscript{13}C NMR and HSQC spectra of 6 with those of 5 showed the presence of a 4,5-di-C-substituted pyrogallol ring (δ\textsubscript{C} 156.0, 146.7, 143.2, 133.1, 114.1, 106.8) instead of the 2,5-cyclohexadien-1-one moiety found in 5. The structure of the B-ring was also confirmed by the appearance of HMBC correlations of H-f (δ\textsubscript{H} 6.94) with C-e (δ\textsubscript{C} 55.7), C-g (δ\textsubscript{C} 156.0), C-h (δ\textsubscript{C} 133.1), C-j (δ\textsubscript{C} 114.1), and C-i (δ\textsubscript{C} 146.7; \textsuperscript{4}J), and the correlations of H-e (δ\textsubscript{H} 4.53) with C-f (δ\textsubscript{C} 106.8), C-j, and C-k (δ\textsubscript{C} 143.2) (Table 1). These observations indicated that one set of galloylated flavan A- and C-rings, originally attached at C-k of 5, had been eliminated.
Thus, the structure of 6 was determined as shown in Scheme 3 and named theacitrinin A. The production of 6 and 7 by elimination of the flavan A- and C-rings is thought to be promoted by aromatization of 2,5-cyclohexadien-1-one, yielding the more stable pyrogallol ring (Scheme 3).

Table 1. $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR data for theacitrinins A (6) and B (9) (in acetone-$d_6$).

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* Multipliclty and coupling constants (Hz) were shown in parentheses.
The degradation products of theacitrins such as \( \text{6} \) are expected to be contained in black tea, and we therefore examined the constituents of black tea in detail. Black tea was extracted with boiling water, and the extract was successively partitioned with \( \text{CHCl}_3 \), \( \text{EtOAc} \), and \( \text{1-BuOH} \). The \( \text{1-BuOH} \) fraction was separated using a combination of column chromatography on Sephadex LH-20, MCI-gel CHP20P, Chromatorex ODS and Toyopearl HW-40F, to afford \( \text{9} \), although compound \( \text{6} \) has not been isolated at the present stage.

\( ^1 \text{H} \) and \( ^{13} \text{C} \) NMR spectra of \( \text{9} \) were closely related to those of \( \text{6} \) (Table 1), except for the appearance of signals attributable to one set of flavan A- and C-rings and one galloyl group. The presence of two sets of galloylated flavan A- and C-rings was supported by the \([ \text{M} + \text{H}]^+\) peak at \( m/z 761 \) in the FABMS spectrum. The additional flavan-3-ol moiety in \( \text{9} \) was concluded to be attached at C-f, based on the absence of an aromatic singlet signal assigned to H-f in \( \text{6} \) (\( \delta_{\text{H}} 6.94 \)) in \( ^1 \text{H} \) NMR and the down-field shift of C-f observed (\( \text{9}: \delta_{\text{C}} 113.9; \text{6}: \delta_{\text{C}} 106.8 \)) in \( ^{13} \text{C} \) NMR. The HMBC correlations between H-2' (\( \delta_{\text{H}} 6.12 \)) with C-f, C-g (\( \delta_{\text{C}} 154.0 \)), and C-k (\( \delta_{\text{C}} 140.1 \)) also supported the proposed location of the flavan A- and C-rings in \( \text{9} \). Accordingly, the structure of \( \text{9} \) was determined as shown in Scheme 4 and named theacitrinin B.
Scheme 4. Proposed mechanism of theacitrinin B (9) production from theacitrin A (8).

It is likely that compound 9 was produced by dienone-phenol rearrangement\textsuperscript{8} of theacitrin A (8) (Scheme 4), while compound 6 was produced by the elimination of the flavan-3-ol moiety from theacitrin C (5). The degradation mechanisms of theacitrins are similar to those of proepitheaflagallin (20), which is produced by the enzymatic oxidation of epigallocatechin (15).\textsuperscript{9} Heating of an aqueous solution of 20 afforded hydroxytheaflavin by dienone-phenol rearrangement, and the black tea pigment epitheaflagallin, by elimination of the flavan-3-ol moiety.\textsuperscript{9a} It is anticipated that 6 is also contained in black tea.

This is the first report of the isolation of theacitrin degradation products from black tea. These results show that the production and degradation reactions of theacitrins may be responsible for the complex mixture of black tea polyphenols.

2.3. Enzymatic oxidation of epigallocatechin-3-\textit{O}-acetate

Enzymatic oxidation of (–)-epigallocatechin (15) and
(−)-epigallocatechin-3-\textit{O}-gallate (1) affords dehydroasinsensins \textit{C}°\textit{a} and \textit{A} (2), respectively, as major products (Scheme 1). However, enzymatic oxidation of 1 did not afford the proepitheaflagallin-type product corresponding to 20, which is one of the oxidation products of 15. Conversely, enzymatic oxidation of 15 did not afford the theacitrin-type product corresponding to 5. Because proepitheaflagallin (20) and theacitrin C (5) are both thought to be produced from epigallocatechins via bicyclo\{3.2.1\}octane-type intermediates, we postulated that the presence of a hydroxyl group at C-3 may determine the decomposition routes of bicyclo\{3.2.1\}octane-type intermediates. The presence of galloyl esters may also be important because the aromatic ester can interact with the adjacent aromatic ring by \textit{π}-\textit{π} interactions. We therefore synthesized (−)-epigallocatechin-3-\textit{O}-acetate (10), which has neither a hydroxyl group nor an aromatic ester at C-3, and examined the enzymatic oxidation products of 10 and the decomposition of bicyclo\{3.2.1\}octane-type intermediates.

(−)-Epigallocatechin-3-\textit{O}-acetate (10) was synthesized by acetylation of (−)-epigallocatechin (15) and subsequent selective hydrolysis of the phenolic acetyl groups. Enzymatic oxidation of 10 using Japanese pear fruit homogenate afforded three main products, 11 ($t_R = 22.1$ min), 12 ($t_R = 29.5$ min) and 13 ($t_R = 31.2$ min), as shown by HPLC (Fig. 2). These products were purified by column chromatography to give 11 (44%), 12 (2.3%) and 13a (2.9%). Product 13 was unstable, and gradually degraded to 13a ($t_R = 26.7$ min) during purification process and only 13a could be isolated.
Fig. 2. HPLC profile of the enzymatic oxidation products of epigallocatechin-3-O-acetate (10).

The molecular formula of 11 was determined to be C_{34}H_{36}O_{17} by HRFABMS (m/z 711.1558 [M + H]^+, –0.3 mmu). The \textsuperscript{1}H and \textsuperscript{13}C-NMR spectra of 11 were very similar to those of dehydrotheasinensin A (2),\textsuperscript{4a} except for the appearance of signals due to two acetyl groups instead of two galloyl groups. Therefore, 11 was proposed to be dehydrotheasinensin C 3,3′′-di-O-acetate (Scheme 5), and the structure was confirmed using detailed analysis of 2D-NMR spectra. The structure was further evidenced by the reduction of 11 with dithiothreitol to yield 11a (Fig. 3).\textsuperscript{4a} The absolute configuration at C-2′ of 11 was determined to be S because acetylation of 11a gave theasinensin C dodecaacetate (11b) (Fig. 3).
Scheme 5. Enzymatic oxidation of epigallocatechin-3-O-acetate (10).

Fig. 3. Structures of 11a, 11b, and 14.

The molecular formula of 12 was determined to be C_{34}H_{30}O_{17} by HRFABMS (m/z 711.1570 [M + H]^+, +0.9 mmu). The $^1$H and $^{13}$C-NMR spectra of 12 were very similar to those of 3, except for the appearance of signals derived from two acetyl groups instead of
two galloyl groups. Based on a detailed analysis of 2D-NMR spectra, the structure of 12 was determined as shown in Scheme 5.

The molecular formula of 13a was determined to be C_{23}H_{18}O_{11} by HRFABMS (m/z 471.0930 [M + H]^+, +0.3 mmu). The ^1H and ^13C NMR spectra of 13a resembled those of theacitrinin A (6), except for the replacement of a galloyl group by an acetyl group. Therefore, the structure of 13a was concluded to be that shown in Scheme 5 and confirmed by 2D-NMR spectroscopic analysis.

HPLC analysis with photodiode array detection showed that the UV-vis absorption of 13 (λ_{max}: 203, 223 sh, 307 nm, and gradual-slope from ca. 350 to 430 nm) was similar to that of theacitrin C (5). Furthermore, HRFABMS spectrum of the mixture of 13 and 13a obtained in the process of purification confirmed a molecular formula of C_{34}H_{28}O_{16} for 13 [m/z 693.1422 (calcd for C_{34}H_{29}O_{16}, 693.1456)]. These results implied that 13 is a theacitrin-type structure with two acetyl groups as shown in Scheme 5. It is probable that 13a was produced from 13 by a degradation mechanism similar to the production of theacitrinin A (6) from theacitrin C (5). We could not isolate 13 because of its instability, while 5 was sufficiently stable to be isolated. This implies that the B-ring moiety of 5 could be stabilized by intramolecular stacking with the C-3 galloyl group.

Enzymatic oxidation of 10 afforded the theacitrin-type product 13; however, no proepitheaflagallin-type products were detected. This result confirmed that when the hydroxyl
group at C-3 of epigallocatechin was esterified, theacitrin-type products were produced. On the other hand, when the hydroxyl group at C-3 was not esterified, proepitheaflagallin-type products were produced.\(^9\)

On enzymatic oxidation, a pyrogallol quinone produced from epigallocatechin was coupled with the pyrogallol ring of another epigallocatechin molecule to afford a bicyclo[3.2.1]octane-type intermediate (Scheme 6). When the hydroxyl group at C-3 is esterified, theacitrin-type structures are produced from the intermediates by rearrangement. On the other hand, when the hydroxyl group at C-3 is not esterified, proepitheaflagallin-type structures are produced from the intermediate via hemiacetal formation. Theaflavins are also produced by condensation of catechol quinones produced by enzymatic oxidation of epicatechins with pyrogallol rings of epigallocatechins via bicyclo[3.2.1]octane-type intermediates (Scheme 6).\(^11\)
Scheme 6. Production pathways of proepitheafagallin (20), theacitrin C (5), and theaflavins via bicyclo[3.2.1]octane-type intermediates.

Some decomposition products of bicyclo[3.2.1]octane-type intermediates, theaflavins, theacitrins, and epitheafagallin, are known as black tea pigments. The results described here indicate that the formation of the bicyclo[3.2.1]octane-type skeleton by enzymatic oxidation of tea catechins is a key reaction in the production of black tea pigments. We speculate that the production and decomposition of bicyclo[3.2.1]octane-type intermediates from tea catechins also contribute significantly to the production of structurally unknown black tea pigments, including thearubigins.2a
3. Conclusion

In this study, we identified theacitrin C (5) as an enzymatic oxidation product of epigallocatechin-3-O-gallate (1), and its degradation products were examined. Furthermore, theacitrinin B (9), a degradation product of theacitrin A (8), was isolated from black tea. We also examined the influence of the hydroxyl group at the C-3 position of the C-ring of epigallocatechin on the decomposition reactions of bicyclo[3.2.1]octane-type intermediates. The results indicate that the formation of the bicyclo[3.2.1]octane-type skeleton by enzymatic oxidation of tea catechins is a key reaction in the production of black tea pigments.
4. Experimental

4.1. General

$^1$H, $^{13}$C NMR, $^1$H-$^1$H COSY, HSQC, HMBC spectra were measured on a Varian UNITYplus 500 (500 MHz for $^1$H and 125 MHz for $^{13}$C NMR), and a JEOL JNM-AL400 (400 MHz for $^1$H and 100 MHz for $^{13}$C NMR) spectrometer. Chemical shifts were recorded as parts per million (ppm) with the solvent signal used as a standard (DMSO-$d_6$, $\delta_H$ 2.49, $\delta_C$ 39.5; acetone-$d_6$, $\delta_H$ 2.04, $\delta_C$ 29.8; CDCl$_3$, $\delta_H$ 7.24, $\delta_C$ 77.0). Coupling constants are given in Hz. Melting points were determined on a micro melting point hot stage apparatus (Yanagimoto, Japan) and are uncorrected. FABMS spectra were measured on a JEOL JMS-700N spectrometer, and $m$-nitrobenzyl alcohol or glycerol was used as the matrix. MALDI-TOFMS spectra were measured on an Applied Biosystems Voyager-DE PRO, and 2,5-dihydroxybenzoic acid was used as the matrix. Elemental analyses were measured on a Perkin-Elmer 2400 II analyzer. UV/VIS spectra were measured on a JASCO V-560 spectrophotometer. Optical rotations were measured on a JASCO P-1020 digital polarimeter. IR spectra were measured on a JASCO FT/IR-410 spectrophotometer.

Column chromatography was performed with MCI-gel CHP20P (75–150 μm, Mitsubishi Chemical, Japan), Sephadex LH-20 (25–100 μm, GE Healthcare, Sweden), Chromatorex ODS (Fuji Silysia Chemical, Japan), and Toyopearl HW-40F (Tosoh, Japan). MPLC was performed with Cosmosil 40C$_{18}$-PREP (Nacalai Tesque, Japan) and YMC-Pack.
ODS-AQ (S-50 μm, 12 nm, YMC, Japan). TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm thick, Merck, Germany) with toluene/ethyl formate/formic acid (1/7/1, v/v) and spots were detected using ultraviolet illumination and by spraying with 2% ethanolic FeCl₃ or 5% aq H₂SO₄ reagent followed by heating. Analytical HPLC was performed on a Cosmosil 5C_{18}-ARII (Nacalai Tesque, Japan) column (250 × 4.6 mm i.d.) with gradient elution from 4 to 30% (39 min) and from 30 to 75% (15 min) of CH₃CN in 50 mM H₃PO₄ (flow rate, 0.8 mL/min; temperature: 35 °C; detection: JASCO photodiode array detector MD-2010). (−)-Epigallocatechin-3-O-gallate and (−)-epigallocatechin were isolated from commercial green tea and recrystallized from water. Theasinensin C was prepared as described previously.⁹a

4.2. HPLC analysis of the enzymatic oxidation products of (−)-epigallocatechin-3-O-gallate (1)

Japanese pear fruits (50 g) were homogenized with 50 mL of H₂O and filtered through four layers of gauze. The filtrate (1.0 mL) was mixed with an aqueous solution of (−)-epigallocatechin-3-O-gallate (1) (10 mg/1.0 mL) and vigorously stirred for 60 min at room temperature. After stirring, EtOH (300 μL) was poured into the reaction mixture (100 μL) and the resulting mixture was then filtered through a membrane filter (0.45 μm). The filtered solution (5 μL) was analyzed by analytical HPLC.
4.3. Production of theacitrin C (5) by enzymatic oxidation of
(−)-epigallocatechin-3-O-gallate (1)

Japanese pear (*Pyrus pyrifolia*) fruits (150 g) were homogenized with 150 mL of
H$_2$O and filtered through four layers of gauze. The filtrate (200 mL) was mixed with an
aqueous solution of (−)-epigallocatechin-3-O-gallate (1) (2.0 g/200 mL) and vigorously
stirred for 60 min at room temperature. After stirring, the reaction solution was acidified with
trifluoroacetic acid (TFA) (0.5 mL) and filtered. The filtered solution was then directly
applied to a column of MCI-gel CHP20P (3 × 29 cm) and eluted with 0–100% aq MeOH
containing 0.1% TFA (5% stepwise elution, each 200 mL) to yield five fractions: fr. 1 (430.4
mg), fr. 2 (334.3 mg), fr. 3 (508.1 mg), fr. 4 (463.5 mg), and fr. 5 (228.3 mg). Fr. 1 was
identified as pure dehydrotheasinensin A (2) from spectroscopic data. Other fractions were
analyzed by HPLC-DAD, and the presence of 2, EGCg quinone dimer A (3) and 1 in fr. 2; 1
and 5 in fr. 3; and EGCg quinone dimer B (4) in fr. 4 were detected. Fr. 3 was purified by
MPLC on Cosmosil 40C$_{18}$-PREP (20 × 300 mm, 0–40% aq CH$_3$CN containing 0.05% TFA)
and YMC-Pack ODS-AQ (20 × 300 mm, 0–50% aq MeOH) to give theacitrin C (5) (27.5 mg).
At all stages of purification, obtained fractions were evaporated under reduced pressure below
40 °C, then lyophilized.
4.3.1. Theacitrin C (5)

A yellow amorphous powder; \( [\alpha]^{21}_D \) –34.3 (c 0.12, MeOH); Positive FABMS \( m/z \): 913 [M + H]\(^+\), 935 [M + Na]\(^+\); Negative MALDI-TOFMS \( m/z \): 911 [M – H]\(^-\); HRFABMS \( m/z \): [M + Na]\(^+\) calcd for C\(_{44}H_{32}O_{22}Na\), 935.1283; found: 935.1302; UV (MeOH) \( \lambda_{\text{max}} \) nm (log \( \epsilon \)): 275 (4.45), and gradual-slope from ca. 350 to 450 nm; IR (dry film) \( \nu_{\text{max}} \) cm\(^{-1}\): 3381, 1731, 1696, 1607, 1519; \(^1\)H NMR (DMSO-\( d_6 \)-CF\(_3\)COOD, 9:1, 500 MHz) \( \delta \): 2.45 (1H, br d, \( J = 17.4 \) Hz, H-4'), 2.66 (2H, m, H-4, 4'), 2.80 (1H, dd, \( J = 17.4, 4.1 \) Hz, H-4), 4.30 (1H, s, H-e), 4.40 (1H, s, H-2'), 4.86 (1H, br s, H-2), 5.25 (1H, m, H-3'), 5.72 (1H, br s, H-3), 5.97 (1H, s, H-c), 6.01 (1H, br s, H-6'), 6.05 (1H, br s, H-6), 6.11 (1H, br s, H-8'), 6.15 (1H, br s, H-8), 6.68 (1H, s, H-f), 6.72 (2H, s, galloyl H-2', 6'), 6.79 (2H, s, galloyl H-2, 6); \(^{13}\)C NMR (DMSO-\( d_6 \)-CF\(_3\)COOD, 9:1, 125 MHz) \( \delta \): 25.7 (C-4), 28.3 (C-4'), 50.3 (C-k), 57.6 (C-e), 63.3 (C-3'), 65.3 (C-3), 74.7 (C-2), 81.2 (C-2'), 87.2 (C-a), 95.1, 96.8 (C-6, 8), 95.1, 96.6 (C-6', 8'), 98.0 (C-4a), 98.2 (C-4a'), 109.1 (2C, galloyl C-2, 6), 109.5 (2C, galloyl C-2', 6'), 118.4 (2C, C-f, j), 119.3 (galloyl C-1), 119.6 (galloyl C-1'), 130.0 (C-c), 139.0 (galloyl C-4'), 139.3 (galloyl C-4), 145.5 (2C, galloyl C-3, 5), 145.9 (2C, galloyl C-3', 5'), 149.5 (C-g), 150.8 (C-i), 155.0, 156.3, 157.1 (4C) (C-5, 7, 8a, 5', 7', 8a'), 165.4 (galloyl C-7'), 165.7 (galloyl C-7), 173.3 (C-d), 177.8 (C-h), 194.5 (C-l), 200.2 (C-b); HMBC correlations (H to C): H-2/C-c, d; H-4/C-2, 4a; H-6/C-4a, 5, 7, 8; H-8/C-4a, 6, 7, 8a; H-2'/C-3', 4', e, f, j, k; H-3'/C-4a', galloyl C-7'; H-4'/C-2', 4a'; H-6'/C-4a', 5', 7', 8'; H-8'/C-4a', 6', 7', 8a'; H-c/C-2, a, b, d, e; H-e/C-2', a,
4.4. HPLC analysis of the degradation products of theacitrin C (5)

An aqueous solution of theacitrin C (5) (0.5 mg / 0.2 mL) was heated at 80 °C for 60 min, and then 5 μL of the solution was analyzed by HPLC-DAD. The same procedure was also carried out using McIlvaine buffer at pH 5.0 and an aqueous solution containing 0.1% TFA (ca. pH 1.9).

4.5. Isolation of the degradation products of theacitrin C (5) in acidic conditions

An aqueous acidic solution of theacitrin C (5) containing 0.1% TFA (25.5 mg/5.1 mL) was heated at 80 °C for 60 min. After cooling, the solvent was removed under reduced pressure, and the residue was purified by MPLC on Cosmosil 40C18-PREP (20 × 300 mm, 0–30% aq CH3CN containing 0.1% TFA) to afford 6 (11.0 mg, 68%) and 7 (8.4 mg, 86%).

4.5.1 Theacitrinin A (6)

A pale yellow powder; mp 198–201 °C; [α]_{26}^{D} +31.3 (c 0.05, MeOH); MALDI-TOFMS m/z: 603 [M + Na]^+; Anal. Calcd for C_{28}H_{20}O_{14}·2H_{2}O: C, 54.55; H, 3.92. Found: C, 54.50; H, 3.75; UV λ_{max} (MeOH) nm (log ε): 277 (4.10), 293 sh (4.04), 345 sh (3.39); IR (dry film) ν_{max} cm^{-1}:
4.6. Isolation of theacitrinin B (9) from black tea

Black tea (600 g), a blend of tea leaves produced in India and Sri Lanka, was extracted with boiling water (5 L × 3), and the extract was concentrated. The extract was successively partitioned with CHCl₃, EtOAc and 1-BuOH. The 1-BuOH fraction (63.4 g) was separated into ten fractions (fr. 1–10) by Sephadex LH-20 column chromatography (5.0 cm × 35 cm, H₂O–MeOH–50% aq acetone). Fr. 6 was further separated using a combination of column chromatography with MCI-gel CHP20P (H₂O–MeOH), Chromatorex ODS (0–50% aq MeOH), Toyopearl HW-40F (40–100% aq MeOH), and Sephadex LH-20 (EtOH), to give theacitrinin B (9) (57.3 mg).

4.6.1 Theacitrinin B (9)

A reddish brown amorphous powder, [α]²⁺D –377.9 (c 0.1, MeOH); FABMS m/z: 761 [M + H]⁺, 783 [M + Na]⁺, 745 [M – H₂O + H]⁺; Anal. Calcd for C₃₇H₂₈O₁₈·1½H₂O: C, 51.69; H, 4.57. Found: C, 51.58; H, 4.60; UV (MeOH) λ max nm (log ε): 278 (4.21), 290 sh (4.15), 350 sh (3.45); IR (dry film) ν max cm⁻¹: 3378, 1719, 1632, 1517, 1467; ¹H and ¹³C NMR data: see Table 1.
4.7. Synthesis of (–)-epigallocatechin-3-O-acetate (10)

A solution of (–)-epigallocatechin (15) (4.01 g, 13.1 mmol) in a mixture of acetic anhydride (7.5 mL) and pyridine (15 mL) was stirred at room temperature for 8 h. The solution was then poured into ice-cold water and filtered. The filtered residue was purified by column chromatography on silica gel (CHCl₃–MeOH, 100:0–99:1) to give (–)-epigallocatechin pentaacetate (1b) (5.30 g, 72%) as a white powder. All physical and spectroscopic data of 1b were identical to those reported previously.¹² A solution of 1b (4.01 g, 7.17 mmol) in a mixture of McIlvaine buffer at pH 4.0 (30 mL) and dioxane (30 mL) was heated at reflux for 76 h. After cooling, the solvent was removed under reduced pressure. The residue was purified by column chromatography using MCI-gel CHP20P (0–80% aq MeOH) and Sephadex LH-20 (0–100% aq MeOH), to give (–)-epigallocatechin-3-O-acetate (10) (1.49 g, 60%).

4.7.1. (–)-epigallocatechin-3-O-acetate (10)

A white amorphous powder, [α]¹⁹/D –62.0 (c 0.11, MeOH); HRFABMS m/z: [M + H]⁺ calcd for C₁₇H₁₇O₈, 349.0923; found, 349.0910; UV (MeOH) λmax nm (log ε): 270 (3.32); IR (dry film) νmax cm⁻¹: 3340, 1711, 1605, 1513, 1461, 1377; ¹H NMR (acetone-d₆, 400 MHz) δ: 1.86 (3H, s, Ac), 2.76 (1H, dd, J = 17.6, 2.4 Hz, H-4), 2.95 (1H, dd, J = 17.6, 4.9 Hz, H-4), 4.94 (1H, s, H-2), 5.36 (1H, m, H-3), 5.94, 6.04 (each 1H, d, J = 2.4 Hz, H-6, 8), 6.54 (2H, s, H-2'...
6'), 7.30 (1H, br s, OH at C-4'), 7.87 (2H, s, OH at C-3', 5'), 8.13, 8.34 (each 1H, s, OH at C-5, 7); \(^{13}\)C NMR (acetone-\(d_6\), 100 MHz) \(\delta\): 20.9 (Ac(CH\(_3\))), 26.3 (C-4), 68.9 (C-3), 77.7 (C-2), 95.8, 96.4 (C-6 and C-8), 98.9 (C-4a), 106.7 (2C, C-2', 6'), 130.5 (C-1'), 133.2 (C-4'), 146.2 (2C, C-3', 5'), 156,9, 157.4, 157.8 (C-5, 7, 8a), 170.3 (Ac(CO)).

4.8. Enzymatic oxidation of (–)-epigallocatechin-3-O-acetate (10)

Japanese pear fruits (100 g) were homogenized with 100 mL of H\(_2\)O and filtered through four layers of gauze. The filtrate (93 mL) was mixed with an aqueous solution of (–)-epigallocatechin-3-O-acetate (10) (930 mg / 93 mL) and vigorously stirred for 60 min at room temperature. After stirring, the reaction solution was acidified with TFA (0.5 mL) and insoluble materials were removed by filtration. The filtrate was directly applied to a column of MCI-gel CHP-20P (3 × 29 cm) and eluted with 0–100% aq MeOH containing 0.1% TFA (5% stepwise elution, each 200 mL) to yield five fractions (fr. 1–5). Each fraction was concentrated in vacuo below 40 °C and lyophilized. Fr. 1 (420.1 mg) was pure compound 11.

Fr. 2 (57.2 mg) was subjected to Chromatorex ODS (2 × 27 cm, 0–60% aq MeOH containing 0.1% TFA) column chromatography to give 12 (10.8 mg). Fr. 3 (217.4 mg) was separated by Cosmosil 40C\(_{18}\)-PREP (20 × 300 mm, 4–30% aq CH\(_3\)CN) and the same column (4–60% aq MeOH) to afford 13a (18.5 mg) and 12 (10.7 mg).
4.8.1. Compound II

A white amorphous powder; \([\alpha]_{D}^{30} -132.4\) (c 0.095, MeOH); FABMS \(m/z\): 711 [M + H]⁺, 733 [M + Na]⁺, 749 [M + K]⁺, 693 [M – H₂O + H]⁺; HRFABMS \(m/z\): [M + H]⁺ calcd for C₃₄H₃₁O₁₇, 711.1561; found, 711.1558; UV (MeOH) \(\lambda_{max}\) nm (log \(\varepsilon\)): 269 (3.56), 305 (3.30); IR (dry film) \(\nu_{max}\) cm⁻¹: 3407, 1724, 1633, 1519, 1469, 1379; \(^1\)H NMR (500 MHz, acetone-\(d_6\)) \(\delta\): 1.85 (3H, s, 3''-OAc), 1.88 (3H, s, 3-OAc), 2.73 (1H, br d, \(J = 16.9\) Hz, H-4), 2.83 (1H, dd, \(J = 16.9, 4.6\) Hz, H-4), 2.86 (1H, br d, \(J = 17.0\) Hz, H-4''), 3.02 (1H, dd, \(J = 17.0, 4.5\) Hz, H-4''), 4.37 (1H, s, H-2'), 4.87 (1H, br s, H-2), 5.29 (1H, m, H-3), 5.42 (1H, m, H-3''), 5.71 (1H, br s, H-2''), 5.93, 5.96, 5.98, 6.00 (each 1H, d, \(J = 1.6\) Hz, H-6, 8, 6'', 8''), 6.20 (1H, s, H-6'), 6.85 (1H, s, H-6'''); \(^13\)C NMR (125 MHz, acetone-\(d_6\)) \(\delta\): 20.8, 20.9 (3-, 3''-OAc(CH₃)), 26.4 (C-4''), 26.6 (C-4), 45.5 (C-2'), 67.8 (C-3), 68.6 (C-3''), 74.3 (C-2''), 76.9 (C-2), 92.0 (C-3'), 95.7, 95.8, 96.3, 96.8 (C-6, 8, 6'', 8''), 96.6 (C-4'), 98.5 (C-4a''), 98.6 (C-4a), 109.2 (C-6''), 111.9 (C-2''), 122.9 (C-6'), 127.4 (C-1'''), 133.4 (C-4''), 142.8 (C-3'''), 146.0 (C-5'''), 155.4, 156.7, 157.1 (2C) (C-5, 8a, 5'', 8a''), 157.6 (2C, C-7, 7''), 162.0 (C-1'), 169.9 (3-OAc(CO)), 170.6 (3''-OAc(CO)), 191.5 (C-5'); HMBC correlations (H to C): H-2/C-3, 4, 1', 2', 6'; H-3/C-4a, 3-OAc(CO); H-4/C-2, 3, 4a, 5, 8a; H-2'/C-2, 1', 3', 4', 5' (\(^4J\), 6', 1'', 2'', 3'', 4'' (\(^4J\)); H-6'/C-2, 1', 2', 3' (\(^4J\), 4', 2'' (\(^4J\)); H-2''/C-3'', 4'', 1'', 2'', 6''); H-3''/C-4a'', 3''-OAc(CO); H-4''/C-2'', 3'', 4a'', 5'', 8a''; H-6''/C-2' (\(^4J\), 2'', 1'', 2'', 3'' (\(^4J\), 4'', 5''); H-3-OAc/C-3 (\(^4J\), 3-OAc(CO); H-3''-OAc/C-3'' (\(^4J\), 3''-OAc(CO)).

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4.8.1.1. Reduction of 11 with dithiothreitol

To a solution of 11 (55.5 mg, 78 μmol) in H2O (20 mL) was added dithiothreitol (50 mg) and the mixture was stirred at room temperature for 2 h. The solution was directly applied to MCI-gel CHP20P (2 × 23 cm) and eluted with 0–70% aq MeOH to give 11a (31.0 mg, 57%). A pale brown amorphous powder; [α]29D −76.4 (c 0.10, MeOH); FABMS m/z: 695 [M + H]+; HRFABMS m/z: [M + H]+ calcd for C34H31O16, 695.1612; found: 695.1628; UV (MeOH) λmax nm (log ε): 209 (4.95), 236 sh (4.35), 274 sh (3.49); IR (dry film) νmax cm−1: 3403, 1717, 1631, 1518, 1467, 1378; 1H NMR (400 MHz, acetone-d6) δ: 1.88 (6H, s, Ac), 2.52 (2H, dd, J = 17.6, 4.9 Hz, H-4), 2.73 (2H, br d, J = 17.6 Hz, H-4), 4.69 (2H, s, H-2), 5.13 (2H, m, H-3), 5.86, 5.98 (each 2H, d, J = 2.2 Hz, H-6, 8), 6.78 (2H, s, H-6′), 7.03, 7.53, 7.90, 8.09, 8.26 (each 2H, br s, OH at C-5, 7, 3′, 4′, 5′); 13C NMR (100 MHz, acetone-d6) δ: 21.0 (Ac(CH3)), 26.4 (C-4), 68.3 (C-3), 75.5 (C-2), 95.8, 96.5 (C-6, 8), 98.3 (C-4a), 107.9 (C-6′), 111.5 (C-2′), 129.1 (C-1′), 133.9 (C-4′), 144.8, 146.5 (C-3′, 5′), 157.0, 157.3, 157.8 (C-5, 7, 8a), 171.3 (Ac(CO)).

4.8.1.2. Acetylation of 11a

A solution of 11a (20.3 mg, 29 μmol) in a mixture of acetic anhydride (0.5 mL) and pyridine (0.5 mL) was stirred for 20 h. After stirring, the solution was poured into ice-cold water and filtered. The filtered residue was purified by column chromatography on silica gel.
(CHCl₃–MeOH, 100:0–98:2) to give 11b (20.5 mg, 63%). By the same procedure, acetylation of theasinensin C (14) (26.9 mg, 44 μmol) also afforded 11b (29.8 mg, 61%). A white amorphous powder; [α]₃³⁰D +17.7 (c 0.12, CHCl₃); MALDI-TOFMS m/z: 1137 [M + Na]⁺, 1153 [M + K]⁺; Anal. Caled for C₅₄H₅₀O₂₆·H₂O: C, 57.25; H, 4.63. Found: C, 57.33; H, 4.61; UV (MeOH) λmax nm (log ε): 206 (4.92), 270 (3.38); IR (dry film) νmax cm⁻¹: 2925, 2853, 1771, 1744, 1624, 1593, 1487, 1438, 1370; ¹H NMR (400 MHz, CDCl₃) δ: 1.978, 1.985, 2.228, 2.235, 2.25, 2.27 (each 6H, s, Ac), 2.72 (4H, br s, H-4), 4.61 (2H, s, H-2), 5.40 (2H, br s, H-3), 6.49, 6.53 (each 2H, d, J = 2.4 Hz, H-6, 8), 7.62 (2H, s, H-6'); ¹³C NMR (100 MHz, CDCl₃) δ: 19.7, 20.1, 20.7, 20.7, 20.8, 21.0 (Ac(CH₃)), 26.0 (C-4), 65.0 (C-3), 74.1 (C-2), 108.0, 108.7, 109.7 (C-4a, 6, 8), 121.7, 122.9 (C-2', 6'), 134.7, 135.3 (C-1', 4'), 141.7, 143.6 (C-3', 5'), 149.4, 149.5 (C-5, 7), 155.0 (C-8a), 166.5, 167.3, 167.6, 168.5, 169.0, 170.3 (Ac(CO)).

4.8.2. Compound 12

A tan amorphous powder; [α]₁₆¹D –33.7 (c 0.10, MeOH); FABMS m/z: 711 [M + H]⁺; HRFABMS m/z: [M + H]⁺ caled for C₃₄H₃₁O₁₇, 711.1561; found, 711.1570; UV (MeOH) λmax nm (log ε): 206 (4.72), 228 sh (4.27), 268 sh (3.60); IR (dry film) νmax cm⁻¹: 3390, 1719, 1673, 1629, 1607, 1519, 1469; ¹H NMR (500 MHz, acetone-d₆) δ: 1.87 (6H, s, Ac), 2.83 (2H, dd, J = 17.5, 2.0 Hz, H-4), 2.93 (2H, dd, J = 17.5, 4.7 Hz, H-4), 3.29 (2H, s, H-2'), 4.56 (2H, br s,
H-2), 5.63 (2H, m, H-3), 6.01, 6.07 (each 2H, d, J = 2.3 Hz, H-6, 8), 6.44 (2H, d, J = 1.6 Hz, H-6'); \(^{13}\)C NMR (125 MHz, acetone-\(d_6\)) \(\delta\): 20.7 (Ac(CH\(_3\))), 25.7 (C-4), 59.9 (C-2'), 64.7 (C-3), 76.4 (C-2), 85.4 (C-4'), 95.7, 96.8 (C-6, 8), 98.3 (C-4a), 104.7 (C-3'), 126.9 (C-6'), 155.3, 157.2 (C-5, 8a), 156.6 (C-1'), 157.9 (C-7), 170.3 (Ac(CO)), 196.6 (C-5'); HMBC correlations (H to C): H-2/C-4, 1', 2', 6'; H-3/C-4, 4a, Ac(CO); H-4/C-2, 3, 4a, 5, 6 (\(^4J\), 8 (\(^4J\), 8a; H-6, 8/C-4a, 7, 5 or 8a, 8 or 6; H-2/C-2, 1', 3', 4', 5', 6'; H-6/C-2, 1', 2', 3' (\(^4J\)), 4'; H-Ac/C-3 (\(^4J\), Ac(CO)).

4.8.3. Compound 13a

A pale yellow amorphous powder; \([\alpha]\)\(^{30}\)\(_D\) +61.5 (c 0.12, MeOH); FABMS \(m/z\): 471 [M + H]\(^+\), 493 [M + Na]\(^+\), 941 [2M + H]\(^+\); HRFABMS \(m/z\): [M + H]\(^+\) calcd for C\(_{23}\)H\(_{19}\)O\(_{11}\), 471.0927; found: 471.0930; UV (MeOH) \(\lambda_{max}\) nm (log \(\varepsilon\)): 277 sh (3.79), 300 (3.82), 346 sh (3.45); IR (dry film) \(\nu_{max}\) cm\(^{-1}\): 3346, 1711, 1683, 1606, 1509, 1471, 1339; \(^1\)H NMR (500 MHz, acetone-\(d_6\)) \(\delta\): 1.98 (3H, s, Ac), 2.85 (1H, dd, \(J = 17.5, 2.9\) Hz, H-4), 3.00 (1H, d, \(J = 17.5, 4.9\) Hz, H-4), 4.48 (1H, s, H-e), 5.17 (1H, br s, H-2), 5.70 (1H, m, H-3), 5.93 (1H, br s, H-c), 6.14 (1H, d, \(J = 2.3\) Hz, H-6), 6.18 (1H, d, \(J = 2.3\) Hz, H-8), 6.92 (1H, s, H-f); \(^{13}\)C NMR (125 MHz, acetone-\(d_6\)) \(\delta\): 20.9 (Ac(CH\(_3\))), 25.9 (C-4), 55.7 (C-e), 66.5 (C-3), 74.0 (C-2), 85.4 (C-a), 95.9 (C-8), 97.3 (C-6), 99.2 (C-8a), 106.8 (C-f), 114.2 (C-j), 126.0 (C-e), 133.0 (C-h), 143.2 (C-k), 146.6 (C-g), 155.5 (C-8a), 156.1 (C-i), 157.6 (C-5), 158.1 (C-7), 170.7 (Ac(CO)), 174.9 (C-d), 170.7 (Ac(CO)).
198.7 (C-l), 200.7 (C-b); HMBC correlations (H to C): H-2/C-4, 8a, b \(^{4}J\), c, d; H-3/C-4, 4a, d, Ac(CO); H-4/C-2, 3, 4a, 5, 6 \(^{4}J\), 8 \(^{4}J\), 8a; H-6/C-4a, 5, 7, 8; H-8/C-4a, 6, 7, 8a; H-c/C-2, a, b, d, e, k \(^{4}J\); H-e/C-2, a, c, d, f, j, k, i \(^{4}J\), l; H-f/C-d \(^{4}J\), e, g, h, i \(^{4}J\), j, k, l \(^{4}J\); H-Ac/C-3 \(^{4}J\), Ac(CO).

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**References and notes**


7. Recently, Qi obtained 5 by non-enzymatic oxidation of 1 using potassium hexacyanoferrate (III): Qi, X. *Fitoterapia* 2010, 81, 205–209.


**Fig. 1.** HPLC profile of the enzymatic oxidation products of epigallocatechin-3-O-gallate (1).

**Fig. 2.** HPLC profile of the enzymatic oxidation products of epigallocatechin-3-O-acetate (10).

**Fig. 3.** Structures of 11a, 11b, and 14.

**Scheme 1.** Enzymatic oxidation of epigallocatechin-3-O-gallate (1).

**Scheme 2.** Proposed mechanism of theacitrin C (5) production.

**Scheme 3.** Degradation of theacitrin C (5) to afford theacitrinin A (6) and 2,3,5,7-tetrahydroxychroman-3-O-gallate (7).

**Scheme 4.** Proposed mechanism of theacitrinin B (9) production from theacitrin A (8).

**Scheme 5.** Enzymatic oxidation of epigallocatechin-3-O-acetate (10).

**Scheme 6.** Production pathways of proepitheflagallin (20), theacitrin C (5), and theaflavins via bicyclo[3.2.1]octane-type intermediates.