Pergamon

PII: S0031-9422(98)00153-8

Phytochemistry, Vol. 49, No. 8, pp. 2511–2519, 1998

Printed in Great Britain
0031-9422/98/$—see front matter

THEAFLAVATE B, ISOTHEAFLAVIN-3′-*O*-GALLATE AND NEOTHEAFLAVIN-3-*O*-GALLATE: THREE POLYPHENOLIC PIGMENTS FROM BLACK TEA

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(*Received in revised form* 9 *February* 1998)

Key Word Index—*Camellia sinensis*; black tea; tea polyphenols; catechins; theaflavins; theaflavates; theaflavic acids; NMR.

Abstract—Three new polyphenolic compounds, theaflavate B, isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate, have been characterised in extracts from black tea (the fermented leaves of *Camellia sinensis*). The structures of these compounds were determined using 1D and 2D NMR spectroscopy, mass spectrometry and chemical oxidation of catechin precursors. Theaflavate B contains a benzotropolone moiety produced from oxidation of the galloyl ester group of a flavan-3-*O*-gallate and as such represents a new class of polyphenol pigments obtained from black tea.

INTRODUCTION

Theaflavins are a group of polyphenol pigments found in black tea that are formed at the fermentation stage of black tea manufacture [1]. It is known that theaflavins make important contributions to the properties of black tea such as colour [2], 'mouthfeel' [3] and extent of tea cream formation [4] and therefore studies of the structures of these pigments have long been of interest. Theaflavin itself was first isolated four decades ago [5] and its structure identified ten years later [6]. These and other studies [7–9] have demonstrated that theaflavin and its galloyl esters possess an hydroxy-substituted benzotropolone ring formed from oxidative coupling of the B-rings of a pair of flavan-3-ol (or flavan-3-*O*-gallate) precursors.

Stereoisomers of theaflavin such as iso- and neotheaflavin have been identified [10, 11] and a number of closely related polyphenolic compounds, including theaflavic acids [11, 12] and theaflagallins [13], have also been isolated from black tea. It is speculated that further minor components exist amongst the theaflavin pigments. However, the theaflavin pigments constitute only 2% of the dry weight of black tea (3–5% of the soluble solids) [14] and the minor theaflavin compounds are present at very low levels (less than 0.1% of the dry weight). Therefore, isolation of these minor pigments presents a significant technical challenge.

This paper describes the isolation of three novel polyphenol compounds from black tea: theaflavate B, isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate. These compounds have been characterized using spectroscopic methods, primarily NMR spectroscopy, and the structures confirmed by chemical syntheses from the precursor catechins.

RESULTS AND DISCUSSION

The three new polyphenol dimers, theaflavate B (1), isotheaflavin-3′-*O*-gallate (2) and neotheaflavin-3-*O*-gallate (3), were obtained from black tea using a combination of techniques including column chromatography and reverse-phase preparative and semi-preparative HPLC methods. UV-Vis, MS and HPLC retention time data for selected theaflavins including 1–3 are listed in Table 1. The characterisation of 1–3 is described below and was achieved primarily by NMR spectroscopy, in particular by means of 2D HMQC [15] and HMBC [16] $^{13}C-{}^{1}H$ correlation experiments.

Theaflavate B (1)

This compound was isolated as an orange-red amorphous powder that is soluble in MeOH and

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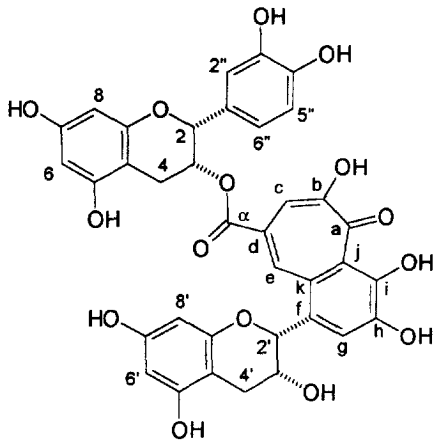
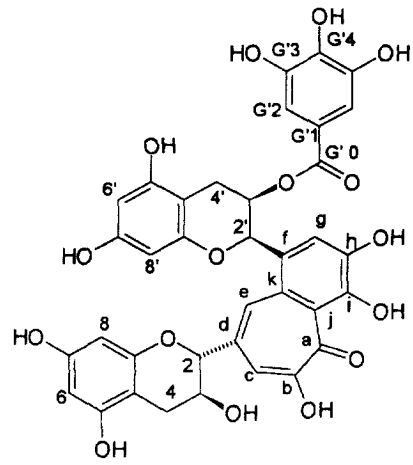
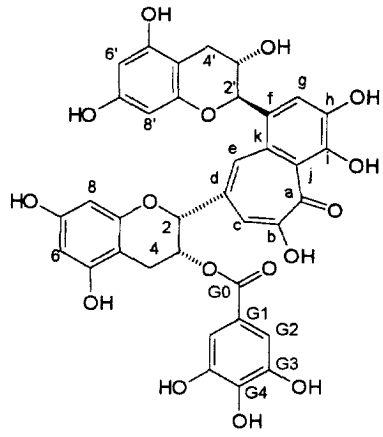


Theaflavate B (1)



Isotheaflavin-3'-O-gallate (2)



Neotheaflavin-3-O-gallate (3)

Me_2CO. Its UV-Vis spectrum (absorption maxima at 284 and 406 nm) is very similar to that of epitheaflavic acid [20] and the previously characterised theaflavate A [21], indicating that it may have a carboxy-substituted benzotropolone group. The M_r of 1 is 700, 152 amu less than that of theaflavate A, suggesting that it differs from the latter by the absence of a galloyl ester group and therefore has an empirical formula of

Table 1. UV-Vis, M_r data and HPLC retention times (analytical conditions) for selected black tea polyphenols

Compound	UV λ_{max} (nm)	M_r*	R_t (min)
Isotheaflavin-3′-*O*-gallate	284, 375, 455	716	36.32
Neotheaflavin-3-*O*-gallate	277, 373, 451	716	36.71
Theaflavate B	284, 406	700	38.99
Theaflavin	273, 375, 455	n.m.†	39.52
Theaflavin 3,3′di-*O*-gallate	273, 375, 455	868	42.99

* Measured by electrospray mass spectrometry.
† n.m = not measured.

Table 2. ^{1}H NMR chemical shift data for theaflavate B, isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate*

H	Theaflavate B	Isotheaflavin-3′-*O*-gallate†	Neotheaflavin-3-*O*-gallate
2	5.19 *br,s*	4.78 *d*	5.32 *br,s*
3	5.69 *m*	4.04 *m*	5.69 *m*
4α	3.04 *dd*	3.08 *dd*	2.96 *d*
4β	3.14 *dd*‡	2.57 *dd*‡	3.05 *dd*‡
6	6.09 *d*§	6.08 *d*§	6.08 *s*
8	6.21 *d*§	5.96 *d*§	6.08 *s*
2′	5.65 *br,s*	6.00 *br,s*	5.67 *br,s*
3′	4.22 *d*	5.70 *d*	4.25 *m*
4′α	2.93 *dd*	2.95 *d*	2.97 *dd*
4′β	3.27 *dd*‡	3.17 *dd*‡	2.70 *dd*‡
6′	6.17 *d*§	6.09 *d*§	6.11 *d*
8′	6.09 *d*§	6.13 *d*§	5.98 *d*
e	8.46 *d*	7.79 *br,s*	8.29 *br,s*
g	8.04 *s*	8.06 *s*	7.73 *s*
c	7.69 *d*	7.39 *d*	7.71 *d*
2″	7.01 *d*	—	—
5″	6.65 *d*	—	—
6″	6.97 *dd*	—	—
G′2	—	6.96 *s*	—
G2	—	—	7.02 *s*
OH-i	14.77 *s*‖	14.88 *s* ‖	—

* Measured in ppm at 303 K in acetone-d_6 plus D_2O and referenced relative to internal TMS or the solvent peak = 2.087 unless otherwise stated. Multiplicities stated are those observable when only mild resolution enhancement is employed.

† Measured at 300 K.

‡ Distinguished from 4α (or 4′α as appropriate) by chemical shift and coupling constant data [17–19].

§ Assignment follows from HMQC and unequivocal data from analogous compounds showing that δC-6 > δC-8 [17, 18].

‖ Measured in acetone-d_6 and referenced relative to internal TMS or the solvent peak = 2.052.

$C_{36}H_{28}O_{15}$. Its ^{1}H and ^{13}C NMR spectra (Tables 2–4) are very similar to those of theaflavate A [21], with the only major differences being the absence of the galloyl ester signals and a large downfield shift of H-3′ in the ^{1}H NMR spectrum of 1; such a change in δH-3′ is consistent with loss of a galloyl group from C-3′ [17, 18].

The use of 2D ^{1}H-^{13}C correlation techniques (HMQC and HMBC; long-range correlations given in Table 5) allows assignment of the ^{1}H and ^{13}C NMR spectra of theaflavate B as previously described [17, 21], confirming the conclusions above. Long-range correlations from H-3, H-e and H-c to the ester carbonyl C-α are observed, linking one of the flavanol groups to the carboxy-substituted benzotropolone ring, whilst correlations from H-2 to C-1″, C-2″ and C-6″ demonstrate that the 1, 3, 4 substituted aromatic ring is attached to this same flavanol group, as expected. The position of the other flavanol group in the molecule is confirmed by the correlations from H-2′ to C-g and C-f. The pattern of long-range correlations within the benzotropolone ring is exactly as observed for theaflavate A and all benzotropolone ^{13}C chemical shifts are consistent with the structure shown (δC-a to δC-k are almost identical in theaflavate B, theaflavate A [21] and epitheaflavic acid [22]).

The C-ring chemical shifts and coupling constants of theaflavate B are very similar to those of theaflavate A, demonstrating that the relative stereochemistry of theaflavate B at C-2 and C-3 (and similarly at C-2′ and C-3′) is a *cis*-2,3 geometry (epicatechin-like), rather than a *trans*-2,3 geometry (catechin-like); of particular diagnostic importance in this respect is the observation that the $J_{2,3}$ and $J_{2',3'}$ coupling constants are small (1.2 Hz or less) [19]. This clearly indicates that theaflavate B has been formed from coupling of epicatechin-3-*O*-gallate and epicatechin with the usual accompanying mass loss of 32 amu (442 + 290 − 32 = 700). A compound with identical spectroscopic (NMR and UV-Vis) and chro-

Table 3. ^{1}H NMR coupling constant data for theaflavate B, isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate*

J	Theaflavate B	Isotheaflavin-3′-*O*-gallate†	Neotheaflavin-3-*O*-gallate
2,3	1.2	8.6	≤2
3,4α	2.2	5.6	n.m.‡
3,4β	4.6	9.6	4.5
4α,4β	17.7	15.8	17.2
6,8	2.3	2.3	n.r.§
2′,3′	n.r.	0.8	8.2
3′,4′α	1.8	1.7	5.4
3′,4′β	4.6	4.6	8.8
4′α,4′β	17.1	17.7	16.1
6′,8′	2.3	2.3	2.4
e,c	1.3	1.1	1.1
2,2″	0.6	—	—
2,6″	0.7	—	—
2″,6″	2.1	—	—
5″,6″	8.2	—	—

* Measured in Hz at 303 K in acetone-d_6 plus D_2O unless otherwise stated.
† Measured at 300 K.
‡ n.m. = not measured.
§ H-6 and H-8 are coincident.

matographic properties was isolated from chemical oxidation of (−)-epicatechin-3-*O*-gallate and (−)-epicatechin, confirming these conclusions. Since (−)-epicatechin-3-*O*-gallate and (−)-epicatechin (and not their enantiomers) are present in tea, the absolute configuration of theaflavate B is concluded to be as shown above.

Mechanism of formation of theaflavate B

Theaflavate B represents a new class of polyphenol compounds isolated from black tea. Previous work has shown that it is possible to prepare theaflavates by chemical oxidation and provided evidence that they are present in tea [8, 11, 21]; the current work has confirmed this. The theaflavate structure (and the results of chemical oxidation studies) strongly suggests that theaflavates are formed during black tea manufacture by oxidative coupling of two flavanols via a galloyl ester group on one of the molecules. This finding is of interest because, prior to the identification of the theaflavates, it had been widely assumed that galloyl esters are chemically inactive during the black tea fermentation process.

It is not known whether direct enzymic oxidation of the galloyl ester group occurs during the formation of theaflavate B; however, this route would be very slow since it is known that polyphenol oxidase (the enzyme responsible for initiating fermentation of black tea) does not readily oxidise gallic acid [23, 24]. This suggests that galloyl ester groups too, would only be very slowly oxidised by this enzyme. A more plausible mechanism for the formation of theaflavate B is analogous to that proposed by Berkowitz *et al.* [25] for the formation of theaflavic acids. In this case, oxidation of the galloyl ester group to a quinone occurs via a chemical redox reaction with a flavanol quinone (formed by the action of polyphenol oxidase on a flavanol) and subsequent coupling of the galloyl ester quinone and a flavanol produces the theaflavate. It is a possibility that interflavanol linkages similarly formed from oxidative coupling of galloyl ester groups and flavanol B-rings may occur in the poorly characterised and heterogeneous thearubigin pigments that are present in black tea [14].

Isotheaflavin-3′-O-gallate (2)

Isotheaflavin-3′-*O*-gallate was isolated as the major component in a mixture containing epitheaflagallin-3-*O*-gallate, in the approximate relative proportions of 2:1 on a molar basis. Further purification was not successful. The UV-Vis spectrum of this compound is very similar to that of the theaflavins (Table 1). Electrospray mass spectrometry indicated that the M_r of the major component in the sample is 716, identical to the theaflavin monogallates. It was therefore concluded that 2 is an isomer of one of these molecules.

Examination of the ^{1}H NMR spectrum confirms that 2 is a theaflavin possessing one galloyl ester group since the expected double intensity singlet is observed at *ca.* 7.0 ppm. One flavanol group has a *cis*-2,3 geometry [epicatechin-like; $J_{2',3'} < 1$ Hz] and the other flavanol unit has a *trans*-2,3 geometry [catechin-like; $J_{2,3} \approx 9$ Hz] [18, 19]. Further structural information is easily obtained by the use of 2D ^{1}H–^{13}C correlation techniques (HMQC and HMBC; long-range correlations are given in Table 5), once again assignments

Table 4. ^{13}C-{^{1}H} NMR data for theaflavate B, isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate*

	Theaflavate B	Isotheaflavin-3′-*O*-gallate†	Neotheaflavin-3-*O*-gallate
2	77.43	85.97	80.29
3	71.65	68.24	70.00
4	26.37	29.87	26.70
4a	98.81	100.79	98.49
5	157.40‡	157.23‡	157.92‡§
6	96.90‡	96.70‡	96.89‡
7	157.95‡	158.03‡	157.84‡§
8	96.27‡	95.54‡	95.62‡
8a	156.77‡	156.44‡	156.23‡
2′	76.67	75.30	79.19
3′	66.02	67.85	68.96
4′	29.81	26.95	29.17
4′a	100.15	99.28	100.49
5′	157.78‡	157.61‡	157.53§
6′	96.90‡	97.02†	96.66
7′	157.62‡	157.93‡	157.34§
8′	96.04‡	96.02‡	95.34
8′a	157.04‡	156.97‡	156.60
α	166.78	—	—
a	185.99	185.39	185.22
b	154.44	155.44	154.60
c	115.76	117.88	117.79
d	124.21	135.19	133.94
e	132.02	128.08	128.40
k	126.39	128.43	129.66
f	134.77	130.28	132.15
g	123.76	123.31	122.90
h	148.97	146.44	146.76
i	151.52	151.13	151.11
j	121.92	121.68	121.84
1″	130.95	—	—
2″	113.97	—	—
3″	145.82	—	—
4″	145.48	—	—
5″	115.86	—	—
6″	118.72	—	—
G0	—	—	166.43
G1	—	—	121.08
G2	—	—	109.98
G3	—	—	145.99
G4	—	—	139.18
G′0	—	165.74	—
G′1	—	121.38	—
G′2	—	109.93	—
G′3	—	145.99	—
G′4	—	138.96	—

* Measured at 303 K in acetone-d_6 (except neotheaflavin-3-*O*-gallate, measured in acetone-d_6 plus D_2O) and referenced relative to internal TMS or the solvent peak = 29.83.

† Measured at 300 K.

‡ Assignment follows from HMBC connectivities *and* unequivocal data from analogous compounds showing that δC-6 > δC-8 [17, 18].

§ Assignment interchangeable.

Table 5. Long-range proton–carbon correlations measured for the novel polyphenol dimers isolated from black tea*

H	Theaflavate B C	Isotheaflavin-3′-*O*-gallate C	Neotheaflavin-3-*O*-gallate† C
e	α, c, d, f, j	2, c, d, f, j	2, c, d, f, j
g	2′, f (w), h (w), i (s), k (s)	2′, f (w), h (w), i (s), k (s)	2′, h (w), i (s), k (s)
c	α, a, b, d, e	2, a, b, d, e	2, a, b, d, e
6″	2, 2″, 4″	—	—
2″	2, 3″, 4″, 6″	—	—
5″	1″, 3″, 4″	—	—
G′2 or G2	—	G′0, G′1, G′3, G′4	G0, G1, G3, G4
8	4a, 6, 7, 8a	4a, 6, 7, 8a	(4a, 6, 7, 8a)
6′	4′a, 5′, 7′, 8′	4′a, 5′, 7′, 8′	4′a, 8′, (5′, 7′)
6	4a, 5, 7, 8	4a, 5, 7, 8	(4a, 5, 7, 8)
8′	4′a, 6′, 7′, 8′a	4′a, 6′, 7′, 8′a	4′a, 6′, 8′a, (7′)
3	4a, α	4a	4a
2′	f, g	3′, f, g, k	3′, 4′, 8′a, f, g
2	3, 1″, 2″, 6″	3, 4, c, d, e	3, c, d, e
3′	4′a	4′a, G′0	n.o.‡
4ß′	4′a, 5′, 8′a	4′a, 5′, 8′a	4′a, 5′, 8′a
4ß	4a, 5, 8a	4a, 5, 8a	4a, 5, 8a
4α	2, 3, 4a, 5, 8a	2, 3, 4a, 5, 8a	(2, 3, 4a, 5, 8a)
4α′	2′, 3′, 4′a, 5′, 8′a	2′, 3′, 4′a, 5′, 8′a	(2′, 3′, 4′a, 5′, 8′a)
OH-i	j, h, i	n.m.§	—

* Measured at 303 K in acetone-d_6. Only those correlations with sufficient signal-to-noise to be fully reliable are listed. Numbers in parentheses represent correlations that cannot be unambiguously assigned due to signal overlap. Where assignments were made on the basis of the intensity of the correlation the following notation is used: (s) = strong, (w) = weak. If no correlation from H-2 to C-8a (or H-2′ to C-8′a) was noted then ambiguitites in A-ring assignments due to the inability to distinguish H-6 from H-8 *etc.* were resolved by ^{13}C shifts (δC-6 > δC-8) [17, 18].

† Measured in acetone-d_6 plus D_2O.

‡ Not observed.

§ Not measured.

were obtained as described previously [17, 21]. The chemical shifts δC-a to δC-k of isotheaflavin-3′-*O*-gallate are very similar to those of the known theaflavins [17], confirming the presence of a benzotropolone ring. The H-2′ resonance [$J_{2',3'} < 1$ Hz] correlates to C-g and C-f, whilst H-2 [$J_{2,3} \approx 9$ Hz] correlates to C-c, C-e and C-d, confirming the positions of the epicatechin- and catechin-like flavanol units on the benzotropolone ring. The position of the galloyl ester group in the molecule is given by the correlation from H-3′ to C-G′0. Therefore, the structure of isotheaflavin-3′-*O*-gallate is as shown above, indicating that it is formed from coupling of (+)-gallocatechin and (−)-epicatechin-3-O-gallate.

A small scale chemical oxidation of (+)-gallocatechin and (−)-epicatechin-3-*O*-gallate produced a reaction mixture containing a compound with an identical HPLC retention time and UV-Vis spectrum to those observed for isotheaflavin-3′-*O*-gallate. This reinforces the belief that structure 2 is correct, with the absolute configuration of the molecule fixed by knowledge of the configurations of the catechin precursors present in tea, as described above.

Neotheaflavin-3-O-*gallate (3)*

The UV-Vis spectrum of neotheaflavin-3-*O*-gallate was also very similar to that of the theaflavins (Table 1). The electrospray mass spectrum gave the M_r of 3 as 716, indicating it is another isomer of the theaflavin monogallates.

Interpretation of the ^1H and ^{13}C-$\{^1H\}$ NMR spectra for this molecule was not as straightforward as in the examples described above, due to the presence of a number of minor impurities. However, reference to the 2D ^1H-^{13}C correlation spectra (long-range correlations given in Table 5) allowed the peaks from the main component to be distinguished from those of the impurities. In the ^1H NMR spectrum, benzotropolone resonances (H-e, H-g and H-c), a double intensity galloyl ester resonance (H-G2), A-ring resonances [H-6, H-8, H-6′ and H-8′ (former pair overlapped)] and C-ring resonances (H-2, H-3, H-4α, H-4β and their dashed counterparts) are observed, indicating that the molecule is indeed a theaflavin monogallate. The resonances of one of the C-rings clearly indicate a *trans*-2,3 geometry (catechin-like) as characterized by the large $J_{2,3}$ coupling constant (as described above), whilst the other flavanol has a *cis*-2,3 geometry. The ^{13}C chemical shifts of the benzotropolone carbons are analogous to those of the other theaflavins (and also show the expected correlations in the HMBC spectrum), confirming the presence of a benzotropolone moiety in this molecule.

The flavanol group with *trans*-2,3 stereochemistry

has long-range correlations from H-2′ to C-g and C-f whilst the other group shows correlations from H-2 to C-c, C-e and C-d. Therefore, the positions of the flavanol groups relative to the benzotropolone ring are fixed as shown above.

No correlation is observed to the galloyl ester carbon in the HMBC spectrum, however, it is clear from data obtained in previous studies that the point of attachment of the galloyl ester group is C-3. It is well known from studies of catechins that the chemical shift of H-3 increases by *ca.* 1.5 ppm on introduction of a galloyl group at C-3 [18]. This trend is also observed in theaflavins [17]. Therefore, the molecule isolated here is the 3-*O*-gallate and not the 3′-*O*-gallate since it has H-3 = *s* 5.69 and H-3′ = *s* 4.25. This conclusion is confirmed by the ^{13}C chemical shift data where C-3 and C-4 = *s* 70.0 and 26.7 respectively, which is in agreement with the shifts obtained for theaflavin-3-*O*-gallate and theaflavin-3,3′-di-*O*-gallate (*ca. s* 69.5 and 26.6) rather than theaflavin and its 3′-*O*-gallate (*ca. s* 66.5 and 29.5) [17, 22]. The expected trends are also observed on comparison of the shifts of C-3′ and C-4′ to those of catechin and catechin-3-*O*-gallate [18].

Once again the findings of NMR spectroscopy were confirmed by chemical synthesis: a compound with an identical HPLC retention time and UV-Vis spectrum was observed in the reaction mixture obtained from oxidation of (+)-catechin and (−)-epigallocatechin-3-*O*-gallate. This provides strong evidence that the structure shown above, including its absolute configuration, is correct.

Formation of minor theaflavins

The presence of isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate in black tea has been predicted [26], but these compounds have not been previously isolated, presumably due to the very low levels at which they are present in black tea (<0.01% dry wt). One factor that partly accounts for the scarcity of these compounds in comparison to theaflavin is the low level of catechins with *trans*-2,3 stereochemistry that are present in black tea leaves. Catechins with *cis*-2,3 stereochemistry (the precursors of theaflavin and its gallate esters) are present at levels ranging from twice to ten times the amount of (+)-gallocatechin and (+)-catechin (the *trans*-2,3 precursors of isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate respectively) [2]. However, this is unlikely to be the sole cause of the low levels of the minor theaflavins found in black tea: it is known from both enzymatic and chemical studies that oxidation reactions involving gallated catechins yield theaflavins far less readily than reaction of ungallated precursors [27–29]. Therefore, the minor theaflavins isolated in this work each have one precursor which is not abundant in tea (catechin or gallocatechin) and one precursor which is gallated and does not react readily to give theaflavins. Furthermore, there is evidence that oxidative coupling of *trans*-2,3 catechins to give theaflavins is less favoured than that of *cis*-2,3 catechins [27–29] (though this effect is less marked than the difference in reactivity caused by the presence of galloyl ester groups). Consequently, it is likely that a combination several factors is responsible for the low level of isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate observed in black tea.

CONCLUSIONS

The characterisation of several novel polyphenol dimers, theaflavate B, isotheaflavin-3′-*O*-gallate and neotheaflavin-3-*O*-gallate, has been described. The identification of these molecules in tea has led to a greater understanding of the processes occurring during black tea fermentation and the range of catechin oxidation products that may result from these processes.

EXPERIMENTAL

Isolation of theaflavins, theaflavates and theaflavic acids from black tea

Black tea leaf (1.0 kg; Ruo Mimosa clone, ex. C.T.G.) was extracted with MeOH, the spent leaf discarded and the MeOH extracts combined and concentrated. An equal vol of H_2O was added to this soln and the MeOH removed. The resulting aq. soln was heated to *ca.* 80°C, extracted with $CHCl_3$ to remove caffeine and chlorophylls, and the $CHCl_3$ soluble material discarded. The aq. soln was then repeatedly extracted with EtOAc and the extracts combined and retained. Removal of the solvent under reduced pressure gave a red/purple solid which was dissolved in a minimal amount of H_2O and freeze dried to produce a fine orange/red solid (*ca.* 35 g). The complete process was repeated 2× to yield 105 g of orange/red solid in total.

The orange/red solid (*ca.* 100 g) was dissolved in EtOH and applied to a Sephadex LH-20 column (26 cm × 12 cm i.d.) which was eluted with EtOH (flow rate 3.5 l/h) to give 7 fractions. The progress of the separation was followed by TLC on cellulose plates developed with a *sec*-BuOH–HOAc–H_2O (14:1:5) solvent mixture. The plates were visualised by spraying with a fresh aq. mixture of $FeCl_3$ and K_3FeCN_6 (1% each, 1:1). The bright red fraction identified as being theaflavin-rich (Rf 0.35; fraction 4) was evaporated to dryness under reduced pressure. Chromatography of an aq. Me_2CO sol of this material (30.28 g) on a Sephadex LH-20 column (26 cm × 12 cm i.d.) eluted with 28% aq. Me_2CO (flow rate *ca.* 3 l/h) yielded six fractions (I-VI), which were collected and evaporated to dryness. Progress of the separation was followed by TLC as before.

The fraction of interest (IV or VI; 100 mg) was dissolved in 20% aq. MeCN (4.0 ml) and applied to a prep 40 μm Novapak C_{18} column (300 mm × 47.5 mm

i.d; Waters Ltd) on an HPLC system with a Waters 4000 System Controller and Pump, a Waters Fraction Collector and UV detection at 280 nm. The column was operating at ambient temp and was eluted with MeCN (A) and MeCN–HOAc–H_2O (20.0:0.5:79.5; B) in the following composition: 100% B for 50 min followed by a wash procedure of 50% B for 5 min and then re-equilibration at 100% B for 10 min. The eluent flow rate was 75 ml/min. Multiple injections were made and the required peaks were collected and combined. The MeCN was removed and the remaining aq. fraction was extracted with three equal volumes of EtOAc which were combined and concentrated to dryness.

Purification of fraction VI (2.0 g) on a Sephadex LH-20 column eluted with H_2O (to remove HOAc) and then 50% aq. Me_2CO yielded theaflavate B. Products obtained from fraction IV required further purification by semi-prep HPLC.

The solid (20 mg) from fraction IV was dissolved in 20% aq. MeCN (2.0 ml) and 250 μl injected onto a semi-prep 5 μm Hypersil C_{18} column (300 mm × 10.0 mm i.d; Fisons Ltd) operating at ambient temp on an HPLC system equipped as described above. The eluent consisted of MeCN (A) and MeCN–HOAc–H_2O (18.0:0.5:81.5; B) with the following composition: 100% B for 50 min followed by a wash procedure of 50% B for 1 min and then re-equilibration at 100% B for 10 min. The solvent flow-rate was 4.75 ml/min. Multiple injections were made and fractions corresponding to the major components were collected and combined. Following removal of the MeCN, the aq. fraction remaining was extracted with EtOAc and the extracts combined. Purification on a Sephadex LH-20 column (as above; eluent H_2O then 50% aq. Me_2CO) yielded isotheaflavin-3′-*O*-gallate (25 mg) and neotheaflavin-3-*O*-gallate (30 mg) from fraction 4 (2.25 g).

Synthesis of novel theaflavins/theaflavates

Epicatechin-3-*O*-gallate, epigallocatechin-3-*O*-gallate, epigallocatechin and gallocatechin were prepared as previously described [18]; epicatechin, catechin and $K_3Fe(CN)_6$ were supplied by Sigma–Aldrich; $NaHCO_3$, anhydrous $MgSO_4$, Na_2EDTA and L-ascorbic acid were supplied by Sherman chemicals.

Appropriate pairs of catechins [(−)-epicatechin-3-*O*-gallate (180 mg) and (−)-epicatechin (120 mg) for theaflavate B; (−)-epicatechin-3-*O*-gallate (19 mg) and (+)-gallocatechin (15 mg) for isotheaflavin-3′-*O*-gallate; (−)-epigallocatechin-3-*O*-gallate (190 mg) and (+)-catechin (120 mg) for neotheaflavin-3-*O*-gallate] were oxidised as described in Ref. [21].

The crude oxidation product was dissolved in a small amount of EtOH and applied to a Sephadex LH-20 column (15 cm × 4 cm i.d) which was eluted with EtOH (flow rate *ca.* 0.6 l/h). The progress of the separation was followed by HPLC analysis and fractions containing the dimers of interest were combined and evaporated to dryness *in vacuo*. If necessary, further purification of the products was achieved by repeating the process described above but with the following modification: Sephadex LH-20 column (20 cm × 1 cm i.d) eluted with aq. Me_2CO (flow rate 2 ml/min). Purified fractions were combined and the Me_2CO removed prior to freeze drying.

Analytical HPLC

Analysis of the various fractions/extracts previously described was carried out using a method adapted from those reported by Bailey *et al.* [26]. and Powell *et al.* [4]. Samples were dissolved in aq. stabiliser sol (250 ppm EDTA, 250 ppm ascorbic acid and 20% aq. MeCN) and applied to a 3 μm Nucleosil C_{18} column (150 mm × 4.6 mm i.d; Phenomenex Ltd) operating at 30°. The eluent compositions were MeCN–HOAc (49:1; A) and H_2O–HOAc (49:1; B) with the following gradient: 8% to 40% A over 55 min (linear), followed by a wash procedure of 40% A for 5 min, then 40% to 8% A over 1 min (linear) and 8% A for 15 min to re-equilibrate. The eluent flow rate was 1 ml/min. Photodiode array spectra (range 260 to 500 nm) were obtained using a Shimadzu SPD–M10A detector with Shimadzu CLASS Software version 1.20.

UV-Vis spectroscopy

All UV-Vis spectra were obtained by the diode-array detector of the HPLC system (see above).

Mass spectrometry

Mass spectra were obtained on a VG Biotech Quattro triple quadruple mass spectrometer. Solvent was delivered by an Isco SFC-500 Micro Flow Pump delivering 10 μl/min of a mixture of 49.5 ml MeCN, 49.5 ml H_2O, and 1 ml HOAc. The solvent was microfiltered and He sparged before use. Manual injection of the sample sol was via a Rheodyne injector fitted with a 10 μl loop. Electrospray conditions were as follows: nitrogen bath gas flow 300 l/h and nebulising gas 15 l/h. Source temp 80°C. Capillary potential 3.5 kV, HV lens 0.20 kV. Negative ion mode, MS1, acquiring continuum data, m/z 100–1000, with cone voltage set alternately at 30 V and 70 V. Detector 650 V.

NMR

NMR spectra were measured on a Bruker AMX400 spectrometer operating at a probe temperature of 300 K or 303 K using either a dual $^1H/^{13}C$ 5 mm probe or a multinuclear 5 mm inverse probe as appropriate. The solvent used was acetone-d_6 and spectra were referenced relative to internal TMS or the residual solvent peak [2.087 ppm for ^1H (D_2O exchanged solutions) and 29.83 ppm for ^{13}C]. Sample concentrations were typically *ca.* 2–3 mg per 0.5 ml. HMQC [15] and

HMBC [16] experiments were acquired with the parameters described previously [17, 18, 21]. HMBC spectra were typically acquired in the mixed mode format using TPPI for t_1 amplitude modulation [30].

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