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# THE THEAFLAVINS OF BLACK TEA

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**Abstract**—The structures, relative configurations and precursors of seven pigments isolated from the theaflavin fraction of black tea have been established by synthesis, and by NMR and mass spectrometry. The four principal pigments are the bis-flavan-substituted 1',2'-dihydroxy-3,4-benzotropolones theaflavin, the corresponding isomeric 3- and 3'-monogallates, and the 3,3'-digallate. Collapsed spin-spin couplings and nuclear Overhauser effects in these and a range of mono-flavan-substituted model compounds are interpreted in terms of steric hindrance to rotation of flavan groups with respect to the benzotropolone ring. As a result of these steric effects, the 3-gallate and 3,3'-digallate exist in rotameric forms which are clearly distinguished in the temperature-dependent CD spectra.

## INTRODUCTION

The theaflavin fraction of black tea makes important contributions to the colour[1] and "mouthfeel" properties of its infusions,[2] and is intimately concerned in the process of decreaming.[3,4] Theaflavin, the first of this group of pigments to be isolated,[5] has been shown to be a bis-flavan-substituted 1',2'-dihydroxy-3,4-benzotropolone (TFla, Fig 1) formed in the fermentation of green tea leaf by enzyme-promoted oxidative coupling of (−) epigallocatechin and (−) epicatechin.[6–10]

Consideration of the mechanism of formation of 1',2'-dihydroxy-3,4-benzotropolones from *p*-substituted pyrogallols and catechols[11–16] suggests that black tea should contain similar benzotropolone pigments formed by oxidative coupling of other pyrogallols (gallocatechins and gallic acid) and catechols (catechins) of green tea leaf. Preliminary reports of the isolation, structures and precursors of seven such theaflavin pigments (Fig 1) have appeared;[17–21] we now wish to amplify these reports, and in particular to discuss the configurations and conformations of these and a number of model compounds (Fig 2) in the light of their NMR and CD spectra.

## RESULTS AND DISCUSSION

### *Yields and precursors of pigments isolated*

Chromatography of the ethyl acetate extractables of black tea solubles on Sephadex LH20 (35% acetone[22]) gave five red fractions P1–P5. P1,2 and 5 were chromatographically identical respectively to the products of oxidative coupling of (−) epicatechin (EC) with gallic acid [(−) TF4,[17] epitheaflavic acid[20]], (−) epicatechin gallate (ECG) with gallic acid (epitheaflavic acid gallate[21]) and (−) epigallocatechin gallate (EGCG) with ECG (TF3[17]). Chromatographic comparison of the acetates (TLC on silica) and pertrimethylsilyl derivatives (GLC[23]) of P4 with those of the products of oxidative coupling of EGCG with EC (TF2A) and of EGC with ECG (TF2B) showed P4 to be a mixture of TF2A and B in the ratio 1·9:1. Chromatography of P3 on cellulose revealed three pigments. The main constituent was chromatographically identical with the oxidative coupling product of EGC and EC (theaflavin, TF1a), the second to that of EGC and (+) catechin (TF1c). The mass spectra (Table 3) and GLC retention times[23] of the pertrimethylsilyl derivatives of the three pigments were identical, thus suggesting that they are isomers, and that the third (TF1b) is Ollis' isotheaflavin.[19]

Fractions similar to P3,4 and 5 were isolated more rapidly and in better yield by chromatography on SilicAR CC7 of the theaflavin fraction obtained from a Sephadex LH20 column (60% acetone[24]). Examination of the trimethylsilylated first fraction by GLC under flavanol[23] and theaflavin[25] analysis conditions showed that in addition to theaflavin isomers, it contained a species (A, Fig 3) with the same retention time as epitheaflavic acid and P1. The mass spectra of all three GLC-trapped pigment derivatives were identical (Table 3), and estimation of A by GLC[25] showed it to account for 2·5% of the first fraction eluted from the silica column. Examination of the other two fractions by GLC failed to reveal the presence of P2, whose pertrimethylsilyl derivative has the same retention time as epitheaflavic acid gallate (Fig 3).

These results confirm the presence in black tea of eight pigments formed by enzyme-promoted oxidative coupling of the known flavanols of green leaf.[26] The amounts estimated[23] and isolated (Table 1) reflect the precursor flavanol composition of

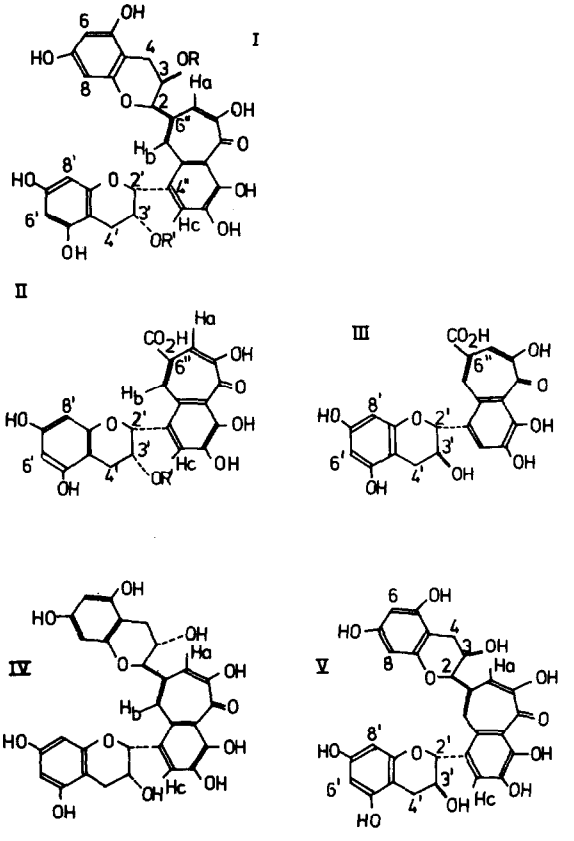


Fig 1. Structures of the Theaflavins of Black Tea

I { Theaflavin, TF1a; R=R'=H
Theaflavin-3-gallate, TF2A; R=galloyl, R'=H
Theaflavin-3'-gallate, TF2B; R=H, R'=galloyl
Theaflavin-3,3'-digallate, TF3; R=R'=galloyl

II { Epitheaflavic acid, (!) TF4; R'=H
Epitheaflavic acid-3'-gallate; R'=galloyl

III Theaflavic acid

IV Isotheaflavin, TF1b

V TF1c.

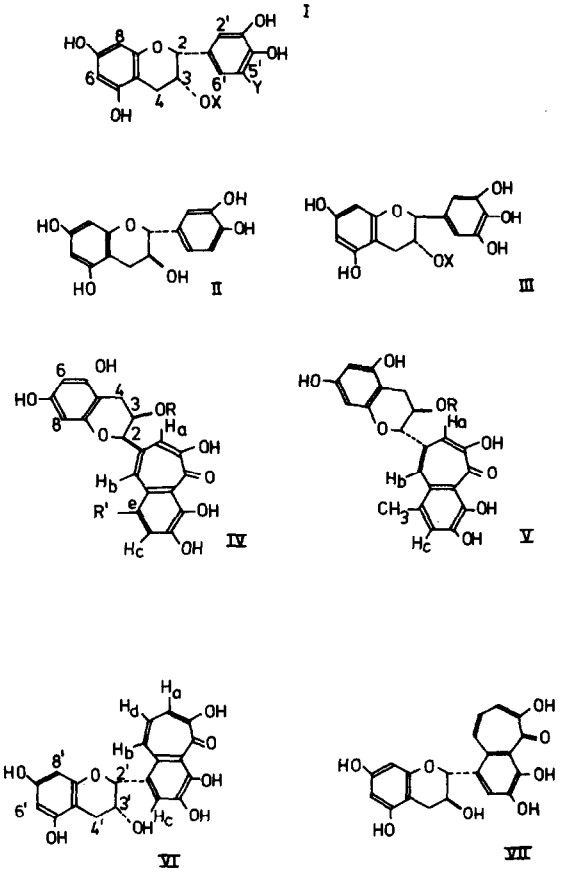


Fig 2. Structures of model Theaflavins and their flavanol precursors

I { (−) Epicatechin, EC; X=Y=H
(−) Epicatechin gallate, ECG; X=galloyl, Y=H
(−) Epitallocatechin, EGC; X=H, Y=OH
(−) Epigallocatechin gallate, EGCG; X=galloyl, Y=OH

II (+) Catechin

III (−) Gallocatechin gallate, GCG; X=galloyl

IV { TF5; R=galloyl, R'=Me
TF7; R=R'=H
TF8; R=galloyl, R'=H

V TF6; R = galloyl

VI TF9

VII TF10

green leaf and the high oxidation-reduction potential of gallic acid relative to those of the flavanols.[27] Similar coupling products from EGCG and (+) catechin, ECG and (+) gallocatechin and gallic acid and (+) catechin [(+) TF4, theaflavic acid] have yet to be isolated from black tea, although theaflavic acid has been synthesised by ferricyanide oxidation of its precursors.[20] It is interesting that epitheaflavic acid is formed as a side product in the ferricyanide oxidation of ECG and gallic acid (Experimental 2). This implies that ECG either undergoes hydrolysis to EC, or yields epitheaflavic acid directly by intramolecular coupling of quinoid groups formed from the B-ring and the gallate group of the same molecule. The first possibility is excluded by the observation that ECG is stable to hydrolysis at pH7 and 0°. The second mechanism (currently under investigation) would involve steric strain in the expected intermediates,[11–16] which might induce the required cleavage of the gallate ester bond. The formation of epitheaflavic acid in enzymic oxidations of ECG[28] probably does not proceed by such intramolecular coupling, since the esterase activity of the enzyme preparation ensures the formation of the direct precursors EC and gallic acid.

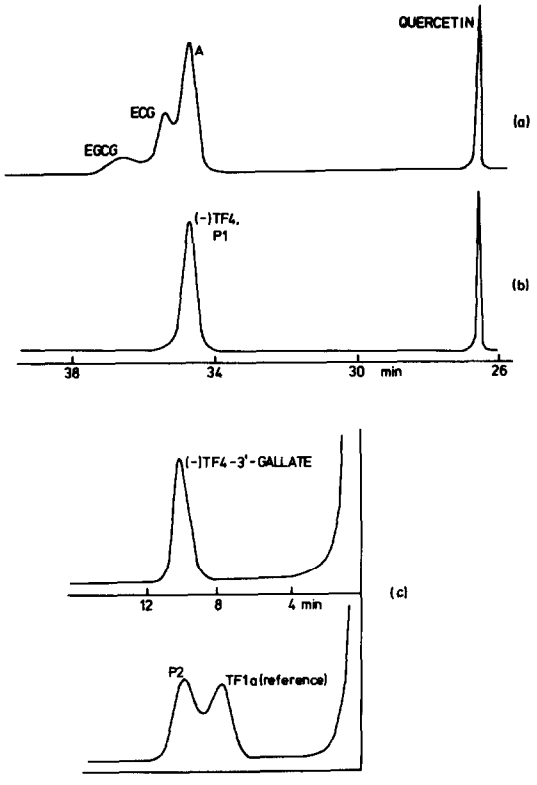



Fig 3. GLC of pertrimethylsilyl derivatives of crude Theaflavin (a), epitheaflavic acid (b) and its gallate (c).

*Structures of the theaflavins*

The natural pigments and their synthetic counterparts were identical in all respects discussed below: the structures given in Fig 1 rest on the following evidence.

Those pigments formed by coupling of flavanols and flavanol gallates (TF1a, b and c; TF2A and B; TF3) possess a common chromophore absorbing in the visible at *ca* 380 and 460 nm (Table 2), a spectral feature characteristic of 1′,2′-dihydroxy-3,4-benzotropolones.[10] Those formed by coupling of gallic acid with flavanols (theaflavic and epitheaflavic acids, epitheaflavic acid gallate) absorb at *ca* 280 and 400 nm, a characteristic of 6″-carboxy-substituted hydroxy-benzotropolones.[6,7,10,29] The IR spectra of all the pigments and their acetates include bands characteristic of tropolone carbonyl groups (1630 $cm^{-1}$) and benzotropolone ring deformations (1595–1600, 1475, 1430, 1230–50 $cm^{-1}$): those of pigments formed from gallic acid or flavanol gallates include in addition bands characteristic of ester or carboxyl carbonyl groups (1698–1700 $cm^{-1}$). Comparison of the intensities of these and the tropolone carbonyl bands indicates, as

Table 2. Absorption spectra of pigments in methanol

| | λ | ε | λ | ε | λ | ε |
|---|---|---|---|---|---|---|
| TF1a | 461 | 4060 | 378 | 10,800 | 268 | 18,620 |
| TF1b | 462 | 2700 | 378 | 7,490 | 270 | 18,760 |
| TF1c | 460 | 2350 | 377 | 5,980 | 272 | 13,350 |
| TF2A | 455 | 3910 | 376 | 10,000 | 272 | 26,440 |
| TF2B | 452 | 4080 | 376 | 10,040 | 278 | 23,670 |
| TF3 | 455 | 3540 | 378 | 10,230 | 278 | 36,350 |
| (−)TF4 | | | 400 | 11,050 | 280 | 23,500 |
| (+)TF4 | | | 398 | 10,620 | 278 | 23,740 |
| (−)TF4 Gallate | | | 398 | 9,090 | 279 | 26,560 |
| TF5 | 460 | 3670 | 383 | 9,520 | 274 | 28,100 |
| TF6 | 456 | 2500 | 383 | 6,650 | 277 | 19,950 |
| TF7 | 454 | 3520 | 374 | 9,480 | 275 | 20,310 |
| TF8 | 454 | 4290 | 375 | 9,650 | 276 | 28,970 |
| TF9 | 460 | 2000 | 375 | 5,000 | 265 | 11,750 |
| TF10 | 456 | 3490 | 372 | 6,280 | 275 | 15,710 |

Table 1. Theaflavin composition of Black Tea solubles

| | Precursors | Estimated Level[a] | Yield isolated A | Yield isolated B |
|---|---|---|---|---|
| TF1a | EGC, EC | | | 0·19 |
| TF1b | (+)GC, EC | | | 0·004 |
| TF1c | EGC, (+)Catechin | | | 0·03 |
| Total TF1 | | 0·38 | 0·29 | 0·23 |
| TF2A | EGCG, EC | 1·17[b] | 0·82[c] | 0·77[c] |
| TF2B | EGC, ECG | 0·58 | 0·44 | 0·43 |
| Total TF2 | | 1·75 | 1·26 | 1·20 |
| TF3 | EGCG, ECG | 1·50 | 0·91 | 0·63 |
| (−)TF4 | Gallic acid, EC | | 0·007[b] | 0·004 |
| (−)TF4 Gallate | Gallic acid, ECG | | | 0·007 |

[a]Expressed as percentage of black tea solubles
[b]Estimated by GLC[23]
[c]Mean of GLC and NMR estimates
A Separated on SilicAR CC7
B Separated on Sephadex LH20

expected,[11–16] that TF2A and B are the isomeric 3- and 3′-monogallates of theaflavin, that TF3 is the corresponding 3,3′-digallate, and that epitheaflavic acid gallate is indeed an ester of epitheaflavic acid.

The mass spectra of the pertrimethylsilyl derivatives (Table 3) support these conclusions. Molecular formulae derived from *m/e* values for the molecular and $(M\text{-}CH_3)^+$ ions accord with those expected. The spectra of all the derivatives reveal retro-Diels-Alder fragments of required composition, which in the cases of TF2A and B, TF3 and epitheaflavic acid gallate are formed from the degallated molecular and $(M\text{-}CH_3)^+$ ions. Fragments derived from the gallate groups appear characteristically at *m/e* 369, 386 and 458.

The 220 MHz NMR spectra of the pigments provide unambiguous support for the proposed structures (Tables 4–7). Comparison of the spectra of theaflavin (TF1a), TF2A, TF2B and TF3 shows that TF3 is theaflavin-3,3′-digallate and that TF2A and B are the isomeric theaflavin-3- and 3′-gallates respectively. Integration of corresponding signals

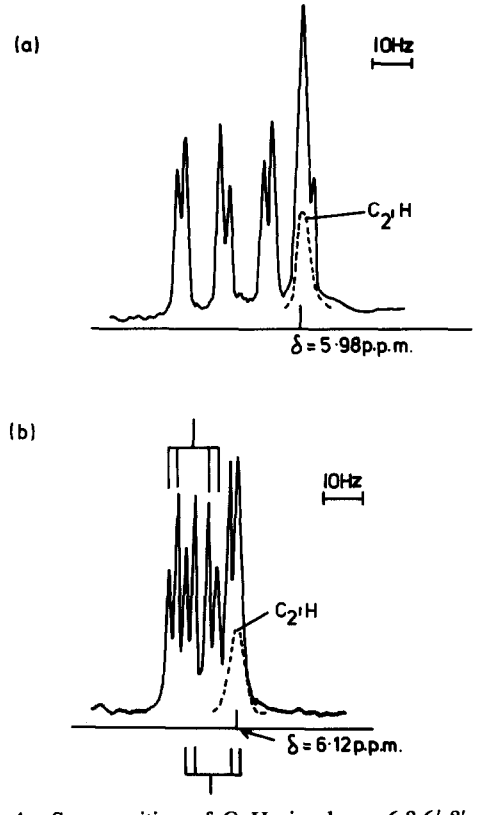



Fig 4. Superposition of $C_{2'}H$ signals on 6,8,6′,8′ AB patterns of Theaflavin-3′-gallate (a) and 3,3′-digallate (b) at 220 MHz.

*Assignments of $C_3H$ and $C_{3'}H$ were made on the basis of decoupling from $C_2H$ and $C_{2'}H$.

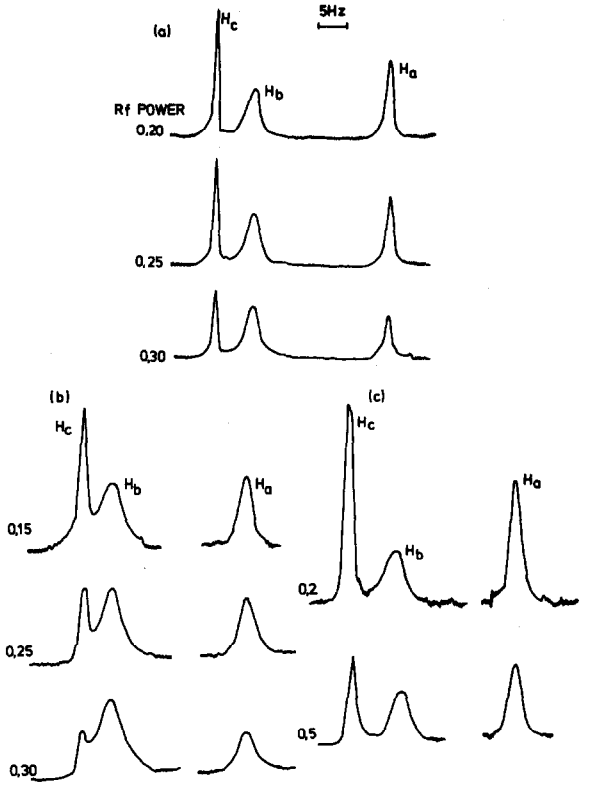



Fig 5. Progressive saturation of $H_a$, $H_b$ and $H_c$ in Theaflavin and its gallates
(a) Theaflavin-3,3′-digallate
(b) Theaflavin-3′-gallate
(c) Theaflavin (TF1a).

in the spectrum of the unresolved mixture of monogallates obtained from tea gives a natural abundance ratio (TF2A:B, 1·75:1) in good agreement with that determined by GLC (1·9:1).[23] Similar comparison of the spectra of epitheaflavic acid and epitheaflavic acid gallate shows that the latter is in fact epitheaflavic acid-3′-gallate. The gallate groups shift the $C_3H$ and $C_{3'}H$ signals downfield by 1·2–1·4 ppm and the $C_2H$ and $C_{2'}H$ signals downfield by 0·25–0·29 ppm from their positions in the spectra of theaflavin* and epitheaflavic acid: shifts of similar magnitudes are observed in the spectra of the precursor flavanols (Table 8). In the 220 MHz spectra of theaflavin-3′-gallate and the 3,3′-digallate, the $C_{2'}H$ signals cannot be precisely located due to the superposition of the AB patterns of the 6,8,6′ and 8′ protons. Integration of these multiplets indicates clearly that they arise from five protons instead of the expected four, and expanded scale spectra show the superposition of a broad $C_{2'}H$ signal on the high-field end of the AB patterns (Fig 4). However, these $C_{2'}H$ signals are resolved from the AB patterns at 100 MHz, probably because of the higher operating temperature of the HA-100.

Table 3. Mass spectra of pertrimethylsilyl derivatives of the theaflavins

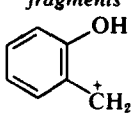
| Molecular formula | TF1a, b & c $C_{29}{\cdot}H_{15}{\cdot}O_3{\cdot}(OTMS)_9$ | TF2A & B $C_{36}{\cdot}H_{17}{\cdot}O_5{\cdot}(OTMS)_{11}$ | TF3 $C_{43}{\cdot}H_{19}{\cdot}O_7{\cdot}(OTMS)_{13}$ | (−)TF4 $C_{21}{\cdot}H_9{\cdot}O_3{\cdot}(OTMS)_7$ | (−)TF4 Gallate $C_{28}{\cdot}H_{11}{\cdot}O_5{\cdot}(OTMS)_9$ |
|---|---|---|---|---|---|
| $M^+$ | 1212 | 1508 | 1804 | 932 | 1228 |
| $(M\text{-}CH_3)^+$ | 1197 | 1493 | 1789 | 917 | 1213 |
| *Gallate fragments* | | | | | |
| $Ph.(OTMS)_3{\cdot}CO_2H^+$; 386 | — | ✓ | ✓ | — | ✓ |
| $Ph.(OTMS)_4{\cdot}CO^{+\cdot}$; 458 | — | ✓ | ✓ | — | ✓ |
| $Ph.(OTMS)_3{\cdot}CO^+$; 369 | — | ✓ | ✓ | — | ✓ |
| *De-gallated fragments* | | | | | |
| M-gallate (385) | | 1123 | 1419 | | 843 |
| $M\text{-}CH_3$-gallate | | 1108 | 1404 | | 828 |
| M-2×gallate | | — | 1034 | | |
| $M\text{-}CH_3$-2×gallate | | — | 1019 | | |
| M-gallate-TMSOH | | 1033 | 1329 | | 753 |
| $M\text{-}CH_3$-gallate-TMSOH | | 1018 | 1314 | | 738 |
| M-2×gallate-TMSOH | | | 944 | | |
| $M\text{-}CH_3$-2×gallate-TMSOH | | | 929 | | |
| *Retro-Diels-Alder fragments* | | | | | |
|  283 | ✓ | ✓ | ✓ | ✓ | ✓ |
| M-282 | 930 | | | 650 | |
| $M\text{-}CH_3$-282 | 915 | | | 635 | |
| M-Gallate-282 | | 841 | | | |
| $M\text{-}CH_3$-Gallate-282 | | 826 | | | 561 |
| M-564 | 648 | | | | 546 |
| $M\text{-}CH_3$-564 | 633 | | | | |
| M-2×gallate-282 | | 752 | | | |
| $M\text{-}CH_3$-2×gallate-282 | | 737 | | | |

Table 4. NMR spectra of theaflavin isomers TFla, isotheaflavin and TFlc

| | TFla | Isotheaflavin[20] | TFlc |
|---|---|---|---|
| 2 | 5·02 | 4·72<br>$J_{2,3} = 8$ | 5·00 |
| 2′ | 5·73 | 5·70 | 5·60<br>$J_{2',3'} = 8·0$ |
| 3 | 4·45 | 4·05 | 4·35 |
| 3′ | 4·55 | 4·42 | 4·12 |
| 4 | 2·86 | 3·09<br>$J_{3,4} = 5·5$<br>$J_{4,4^*} = 16$<br>$J_{3,4^*} = 9$ | 2·85 |
| 4* | 2·96 | 2·56 | 2·90 |
| 4′ | 2·89 | 2·90 (m) | 2·96<br>$J_{3',4'^*} = 9·5$<br>$J_{4',4'^*} = 16·0$<br>$J_{3',4'} = 5·5$ |
| 4′* | 3·02 | | 2·67 |
| 6,6′ | 6·03, 6·10<br>$J_{6,8} = J_{6',8'} = 2·3$ | 5·93 – 6·06 (m) | 5·93, 5·94<br>$J_{6,8} = J_{6',8'} = 2·3$ |
| 8,8′ | 6·07, 6·08 | | 6·02, 6·06 |
| a | 7·54 | 7·40 | 7·62 |
| b | 7·97 | 7·78 | 8·24 |
| c | 8·03 | 8·02 | 7·75 |
| Tropolone OH | 14·93 | 14·83 | 14·98 |

All δ-values quoted (except those for tropolone hydroxyl protons) refer to $D_2O$-exchanged solutions

4* and 4′* denote quasi-axial protons.

Table 5. NMR spectra of the theaflavin gallates

| | Theaflavin-3-gallate | Theaflavin-3′-gallate | Theaflavin 3,3′-digallate |
|---|---|---|---|
| 2 | 5·31 | 5·08 | 5·48 |
| 2′ | 5·76 | 5·98 | 6·12 |
| 3 | 5·70 | 4·40 | 5·76 |
| 3′ | 4·54 | 5·75 | 5·80 |
| 4, 4′ (4H) | 2·8–3·2 (mult) | 2·8–3·2 (mult.) | 3·31, 3·05<br>3·10, 3·01 |
| 6, 8 | 6·03, 6·08 | 5·98, 6·02 | 6·13, 6·16 |
| 6′, 8′ | $J = 2·3$<br>6·09, 6·11 | $J = 2·3$<br>6·15, 6·18 | $J = 2·3$<br>6·15, 6·18 |
| a | 7·62 | 7·58 | 7·78 |
| b | 7·97 | 8·00 | 8·00 |
| c | 8·02 | 8·03 | 8·10 |
| Gallate (2H) | 6·91 | 6·93 | 7·00(2H); 7·04(2H) |
| Tropolone OH | 14·82 | 14·95 | 14·89 |

All δ-values quoted (except those for tropolone hydroxyl protons) refer to $D_2O$-exchanged solutions.

Table 6. NMR spectra of the theaflavic acids

| | Epitheaflavic Acid | Epitheaflavic acid-3′-gallate | Theaflavic Acid |
|---|---|---|---|
| 2′ | 5·69 | 5·98 | 5·49<br>$J_{2',3'} = 8·5$ |
| 3′ | 4·44 | 5·77 | 4·32 |
| 4′* | 3·06<br>$J_{3',4'*} = 4·0$<br>$J_{4',4'*} = 16·5$<br>$J_{3',4'} = 1·5$ | 3·19<br>$J_{3',4'*} = 3·5$<br>$J_{4',4'*} = 17·0$<br>$J_{3',4'} = 1·5$ | 2·71<br>$J_{3',4'*} = 9·0$<br>$J_{4',4'*} = 16·0$<br>$J_{3',4'} = 5·5$ |
| 4′ | 2·89 | 3·08 | 3·09 |
| 6′, 8′ | 6·00, 6·07<br>$J_{6',8'} = 2·0$ | 6·19, 6·14<br>$J_{6',8'} = 2·0$ | 5·96, 6·11<br>$J_{6',8'} = 2·0$ |
| a | 7·83<br>$J_{a,b} = 1·3$ | 7·86 | 7·89<br>$_{a,b} = 1·3$ |
| b | 8·74 | 8·73 | 9·20 |
| c | 8·05 | 8·05 | 7·82 |
| Gallate(2H) | — | 6·96 | — |
| Tropolone OH | 15·0 | 14·85 | 14·90 |

All δ-values quoted (except those for tropolone hydroxyl protons) refer to $D_2O$-exchanged solutions
4′* denotes the quasi-axial proton

As in the flavanols, each pair of methylene protons at $C_4$ and $C_{4'}$ gives the AB pattern of an ABXY system with a geminal coupling of 16–17 Hz. The coupling constants to the adjacent $C_3$ and $C_{3'}H$ protons are clear in the spectra of TF1b, TF1c, the theaflavic acids and model mono-flavan-substituted benzotropolones TF5-10 (Fig 2, Table 7), and are very similar to those observed in the spectra of the corresponding precursor flavanols. The $C_4$ and $C_{4'}$ AB patterns in the spectra of the bis-flavan-substituted theaflavin (TF1a) and its gallate esters are superimposed:† nevertheless, expanded scale spectra show that the downfield complexes include couplings of 4–5 Hz and the upfield complexes couplings of 1–2 Hz with $C_3H$ and $C_{3'}H$, as in the precursor flavanols. $C_2H/C_3H$ and $C_{2'}H/C_{3'}H$ coupling constants also parallel those found in the precursor flavanols. These results confirm that the relative 2,3 configurations and flavan conformations (half chair or sofa[30]) of the precursor flavanols are retained in the derived natural and model theaflavins (Table 9). These relative configurations are consistent with the absolute configurations proposed (Figs 1 and 2, Table 9) on the basis of the known absolute configurations of the precursor flavanols.[31,32]

*NMR evidence for steric interactions in the theaflavins*

The assignments of the benzotropolone aromatic protons $H_a$, $H_b$ and $H_c$ follow from the downfield shifts produced by the 6″-carboxyl substituent in epitheaflavic acid, together with the detection in double resonance experiments of long-range benzylic couplings[33,34] of $C_2H$ with $H_a$ and of $C_{2'}H$ with $H_c$. The $H_b$ signal is in all cases broader than the other two, especially in the spectra of theaflavin and its gallates: for these compounds the expected meta coupling of $H_a$ and $H_b$ and the benzylic coupling of $H_b$ with $C_2H$ cannot be demonstrated, although strong intramolecular nuclear Overhauser effects[35] are found between $H_b$ and $C_{2'}H$. Such effects arise when nuclear dipole interactions between two protons provide more efficient relaxation processes than those available to other protons in the molecule: saturation of either nucleus under double resonance conditions so affects the populations of the spin states of the other as to enhance its absorption relative to those of less efficiently relaxed protons.[35,36] The magnitudes of the $C_{2'}H/H_b$ Overhauser effects for theaflavin and its gallates indicate that in these bis-flavan-substituted pig-

†In these *cis-cis* configuration pigments, the strong deshielding effect of an axial 3 (or 3′) hydroxyl or gallate group on the adjacent 4- (or 4′) axial proton largely overcomes the normal chemical shift difference between axial and equatorial protons.

Table 7. NMR spectra of model theaflavins

| | 2 | 2′ | 3 | 3′ | 4 | 4* | 4′ | 4′* | 6,8 | 6′,8′ | a | b | c | d or e | Gallate (2H) | Tropolone OH | Me (3H) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TF5 | 5·32 | — | 5·78 | — | 2·88 | 3·03 | — | — | 6·13 | — | 7·60 | 8·06 | 7·41 | — | 6·97 | 14·70 | 2·61 |
| | | | | | $J_{4,4*}$ = 17 | | | | (2H) | | | | | | | | |
| | | | | | $J_{3,4}$ = 1·0; $J_{3,4*}$ = 4·0 | | | | | | | | | | | | |
| TF6 | 5·24 | — | 5·50 | — | 3·00 | 2·88 | — | — | 6·10 | — | 7·43 | 7·86 | 7·38 | | 7·03 | 14·70 | 2·48 |
| | $J_{2,3}$ = 6·7 | | $J_{2,3}$ = 6·7 | | $J_{4,4*}$ = 16·5 | | | | 6·14 | | | | | | | | |
| | | | | | $J_{3,4}$ = 5·0; $J_{3,4*}$ = 6·5 | | | | | | | | | | | | |
| TF7 | 4·97 | — | 4·37 | — | 2·86 | 2·93 | — | — | 6·02 | — | 7·64 | 7·77 | 7·49 | 7·49 | — | 14·62 | — |
| | | | | | $J_{4,4*}$ = 17 | | | | 6·09 | | $J_{a,b}$ 1 | | | | | | |
| | | | | | $J_{3,4}$ = 1·5; $J_{3,4*}$ = 4·0 | | | | | | | | | | | | |
| TF8 | 5·22 | — | 5·69 | — | 3·01 | 3·10 | — | — | 6·10 | — | 7·58 | 7·80 | 7·41 | 7·41 | 6·97 | 14·62 | — |
| | | | | | $J_{4,4*}$ = 17·0 | | | | (2H) | | $J_{a,b}$ 1 | | | | | | |
| | | | | | $J_{3,4}$ = 2; $J_{3,4*}$ = 4·3 | | | | | | | | | | | | |
| TF9 | — | 5·70 | — | 4·41 | — | — | 2·91 | 3·06 | | 6·01 | 7·26 | 7·80 | 8·07 | 6·94 | — | 14·90 | — |
| | | | | | | | $J_{4',4'*}$ = 17·0 | | | 6·07 | $J_{a,b}$ 1 | $J_{b,d}$ = 11·5 | | $J_{b,d}$ = 11·5 | | | |
| | | | | | | | $J_{3',4'}$ = 2; $J_{3',4'*}$ = 4·5 | | — | $J_{6',8'}$ = 2·3 | $J_{a,d}$ = 9·5 | | | | | | |
| TF10 | — | 5·48 | — | 4·24 | — | — | 3·00 | 2·69 | — | 5·92 | 7·28 | 8·08 | 7·74 | 6·93 | — | | — |
| | | $J_{2',3'}$ = 8·0 | | | | | $J_{4',4'*}$ = 16·0 | | | 6·08 | $J_{a,b}$ 1 | $J_{b,d}$ = 12·0 | | | | 14·90 | |
| | | | | | | | $J_{3',4'}$ = 5·5; $J_{3',4'*}$ = 9·0 | | | | $J_{a,d}$ = 9·0 | | | | | | |

4* and 4′* denote quasi-axial protons.
All resonances integrate for single protons unless otherwise indicated in parentheses (H)
All δ-values quoted (except those for tropolone hydroxyl protons) refer to $D_2O$-exchanged solutions

Table 8. NMR spectra of the flavanols and flavanol gallates

| | (−) EC | (−) EGC | (+) Catechin | (−) ECG | (−) EGCG | (−) GCG |
|---|---|---|---|---|---|---|
| 2 | 4·84 | 4·82 | 4·60<br>$J_{2,3} = 7$ | 5·13 | 5·04 | 5·09<br>$J_{2,3} = 5·5$ |
| 3 | 4·18 | 4·21 | 4·07<br>$J_{2,3} = 7$<br>$J_{3,4} = 5$<br>$J_{3,4^*} = 8$ | 5·54 | 5·52 | 5·31<br>$J_{2,3} = 5·5$<br>$J_{3,4} = 5·0$<br>$J_{3,4^*} = 5·5$ |
| 4 | 2·71<br>$J_{4,4^*} = 16·5$<br>$J_{3,4} = 3·0$<br>$J_{3,4^*} = 4·5$ | 2·76<br>$J_{4,4^*} = 16·5$<br>$J_{3,4} = 3·0$<br>$J_{3,4^*} = 4·5$ | 2·94<br>$J_{4,4^*} = 16·0$<br>$J_{3,4} = 5$<br>$J_{3,4^*} = 8$ | 2·95<br>$J_{4,4^*} = 17·0$<br>$J_{3,4} = 2·3$<br>$J_{3,4^*} = 4·4$ | 2·91<br>$J_{4,4^*} = 17·0$<br>$J_{3,4} = 2·5$<br>$J_{3,4^*} = 4·5$ | 2·82<br>$J_{4,4^*} = 16·5$<br>$J_{3,4} = 5·0$<br>$J_{3,4^*} = 5·5$ |
| 4* | 2·82 | 2·86 | 2·59 | 3·04 | 3·01 | 2·76 |
| 6,8 | 5·91, 6·02<br>$J_{6,8} = 2·2$ | 5·91, 6·01<br>$J_{6,8} = 2·0$ | 5·90, 6·04<br>$J_{6,8} = 2·3$ | 6·04, 6·07<br>$J_{6,8} = 2·2$ | 6·00, 6·03<br>$J_{6,8} = 2·0$ | 5·95, 6·03<br>$J_{6,8} = 2·3$ |
| 2′ | 7·03 | 6·57 | 6·91 | 7·08<br>$J_{2',6'} = 2·0$ | 6·59<br>$J_{2,2'} = 0·6$ | 6·46<br>$J_{2,2'} = 0·6$ |
| 6′ | 6·80 | 6·57 | 6·77 | 6·89<br>$J_{5',6'} = 8·2$<br>$J_{2',6'} = 2·0$ | 6·59<br>$J_{2,6'} = 0·6$ | 6·46<br>$J_{2,6'} = 0·6$ |
| 5′ | 6·80 | — | 6·77 | 6·77<br>$J_{5',6'} = 8·2$ | — | — |
| Gallate (2H) | | | | 7·04 | 6·99 | 7·02 |

All δ-values refer to $D_2O$-exchanged solutions
4* denotes the quasi-axial proton

Table 9. Configurations of the flavanols and theaflavins

| | Established Relative configuration | | Proposed or established Absolute configuration | | | |
|---|---|---|---|---|---|---|
| | 2,3 | 2′,3′ | 2 | 3 | 2′ | 3′ |
| (−) EC, (−) EGC<br>(−) ECG, (−) EGCG | *cis* | | R | R | | |
| (+) Catechin | *trans* | | R | S | | |
| (−) GCG | *trans* | | S | R | | |
| Theaflavin (TF1a), TF2A and B, TF3 | *cis* | *cis* | R | R | R | R |
| Epitheaflavic acid, its 3′-gallate, and TF9 | | *cis* | | | R | R |
| Theaflavic acid and TF10 | | *trans* | | | R | S |
| TF5, 7 and 8 | *cis* | | R | R | | |
| TF6 | *trans* | | S | R | | |
| Isotheaflavin (TF1b) | *trans* | *cis* | R | S | R | R |
| TF1c | *cis* | *trans* | R | R | R | S |

ments, nuclear dipole interactions between $C_2$·H and $H_b$ provide the major relaxation processes for both protons: saturation of either of two isolated mutually-relaxing protons theoretically leads to a maximum 50% increase in the integrated absorption intensity due to the other,[35,37] and for TF1–TF3, enhancements of 30–40% were obtained (Table 10). In addition, introduction of a gallate group at the 3-position produces a small Overhauser effect between $C_2$H and $H_b$.

There are three direct indications of the distinctively rapid relaxation of $H_b$ in theaflavin and its gallates. First, the $H_b$ signal is much broader than those of $H_a$ and $H_c$: this is not a result of spin-spin coupling, since the meta-related proton ($H_a$) signal is by comparison narrow, and irradiation of the benzylic proton ($C_2$H) and $H_a$ has no sharpening effect on the $H_b$ signal. The large line width therefore indicates $H_b$ to have a shorter relaxation time than $H_a$ and $H_c$.[38] Secondly, progressive saturation experiments (Fig 5) confirm that $H_b$ is less readily saturated than $H_a$ and $H_c$, consistent with its shorter relaxation time. Finally, very rapid relaxation of $H_b$ would be expected to induce collapse of the spin multiplets of protons to which it is nominally coupled.[38] Such collapse occurs when the rate of nuclear spin reorientation

Table 10. Nuclear Overhauser effects observed on $H_b$ in natural and model theaflavins

| Protons irradiated | $C_2H$ | $C_{2'}H$ or 4″-Me |
|---|---|---|
| TF1a | — | A |
| TF1c | — | A |
| Theaflavin-3-gallate | C | A |
| Theaflavin-3′-gallate | — | A |
| Theaflavin-3,3′ digallate | C | A |
| Epitheaflavic acid | — | B |
| Theaflavic acid | — | B |
| Epitheaflavic acid-3′-gallate | — | A |
| TF5 | D | B |
| TF6 | D | B |
| TF7 | — | — |
| TF8 | — | — |
| TF9 | — | B |

The values of the NOE's observed may only be given as ranges, due to the unfavourable signal to noise ratios involved. Four ranges are indicated as
A: 30–40%; B: 15–20%;
C: 10–15%; D: 5–10%.

of the fast-relaxing nucleus is appreciably greater than the coupling constant involved:[36] this explains why in TF1–3 the expected meta coupling of $H_a$ (1·3 Hz) and the benzylic coupling of $C_2H$ with $H_b$ cannot be detected.

These observations, together with the fact that intramolecular relaxation times are proportional to the sixth power of the internuclear distance,[37] clearly show that in theaflavin and its gallates $C_{2'}H$ and $H_b$ are closer (on time average) to each other than to any other protons in the molecule. The small Overhauser effects observed for $C_2H$ and $H_b$ in theaflavin-3-gallate and the 3,3′-digallate (Table 10) show that $C_2H$ lies closer to $H_b$ in these pigments than in theaflavin and the 3′-gallate. Inspection of Dreiding models confirms that of the benzotropolone aromatic protons, $H_b$ can approach closest to $C_{2'}H$, and further suggests that such close approach is a result of the avoidance of interactions between $H_b$ and 3′-substituents on the one hand and between the two bulky flavan groups on the other. Similarly, avoidance of interactions between a 3-gallate group, $H_b$ and/or a 4″-flavan group is responsible for the closer approach of $C_2H$ to $H_b$ in theaflavin-3-gallate and the 3,3′-digallate. These three types of interaction restrict the nominally free rotation of the flavan substituents about the bonds joining them to the benzotropolone ring system, and are especially severe when bulky 3- and 3′-gallate groups are present.

Support for these proposals, and further indications of the effects of these interactions are provided by studies of spin-spin coupling and Overhauser effects in the theaflavic acids and mono-flavan-substituted benzotropolones TF5–10. In the mono-(4″)-flavan-substituted theaflavic acids, TF9 and TF10, the $C_{2'}H/H_b$ Overhauser effects are smaller than in the corresponding bis-flavan-substituted theaflavins TF1a and TF1c (Table 10), and the $H_b$ absorption is less markedly broadened. The meta-coupling of $H_b$ and $H_a$ is clearly seen in the $H_a$ signal, and is revealed in the $H_b$ signal by decoupling of $H_b$ from $C_{2'}H$ (Fig 6). These results show that $H_b$ is more efficiently relaxed in the corresponding 4″,6″-bis-flavan-substituted pigments, and that this is a result of the avoidance of interactions between the two flavan groups forcing $C_{2'}H$ and $H_b$ closer together. Introduction of a 3′-gallate group has a very similar effect, as evidenced in epitheaflavic acid-3′-gallate by the increased $C_{2'}H/H_b$ Overhauser effect and the collapse of the meta coupling of $H_a$ to $H_b$. In this case the effect is clearly a result of the avoidance of interactions between $H_b$ and the 3′-gallate group.

In the mono-(6″)-flavan-substituted benzotropolones TF5–8, the expected meta-coupling of $H_b$ and $H_a$ is clearly seen in the $H_a$ signal unless a 4″-Me substituent is present. In these cases (TF5 and 6), the protons of the methyl group give an Overhauser effect with $H_b$ (Table 10). Further, only 3-gallate-substituted pigments bearing a 4″-methyl substituent give an Overhauser effect between $C_2H$ and $H_b$, thus confirming that avoidance of interactions between the 3-gallate group and the 4″-flavan substituent is principally responsible for the close approach of $C_2H$ to $H_b$ in theaflavin-3-gallate and the 3,3′-digallate. Together with the results for the 4″-flavan-substituted pigments, these observations suggest that $H_b$ is relaxed less efficiently by $C_2H$ than by $C_{2'}H$ (or the equivalent protons of a 4″-Me group) and that a 6″-flavan substituent is freer to rotate about the bond joining it to the benzotropolone ring than is a 4″-flavan substituent.

[Indications that the configuration of a flavan substituent influences the magnetic environments of the adjacent benzotropolone ring protons are provided by comparison of the chemical shifts of $H_a$, $H_b$ and $H_c$ in the pairs of stereoisomers theaflavin and isotheaflavin,[19] theaflavin and TF1c, theaflavic and epitheaflavic acids, TF5 and 6, and TF9 and 10. A change from 2′,3′-*cis*(2′R, 3′R) to 2′,3′-*trans* (2′R, 3′S) configuration moves the $H_b$ signal downfield by 0·27–0·46 ppm. and the $H_c$ signal upfield by 0·23–0·33 ppm. A similar change from 2,3-*cis* (2R, 3R) to 2,3-*trans* configuration (isotheaflavin, 2R, 3S; TF6, 2S, 3R) moves the $H_a$ and $H_b$ signals upfield by 0·14–0·20 ppm. These effects probably arise from shifts of 3- and 3′-substituents from axial (2R, 3R; 2′R, 3′R) to equatorial (2R, 3S; 2′R, 3′S; 2S, 3R[32]) positions: as a result, the time-averaged positions of these deshielding substituents relative to $H_a$, $H_b$ and $H_c$ change. (A less likely alternative is that these changed positions result from a change in the preferred conformation of an entire flavan

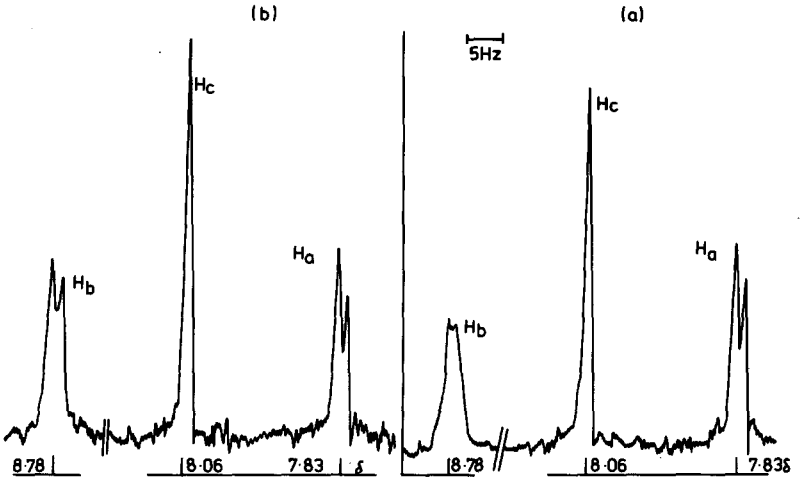



Fig 6. Decoupling of $C_{2'}H$ from $H_b$ and $H_c$ in Epitheaflavic Acid. (a) Normal (b) Irradiated at $C_{2'}H$.

group relative to the benzotropolone ring system. If this were so, the time-averated separations of $C_2H$ and $C_{2'}H$ from $H_b$ would be expected to depend on the configuration of the appropriate flavan group: the associated nuclear Overhauser effects show no such dependence).]

*Conformational mobility in the theaflavins*

Although the NMR studies demonstrate the three types of interaction which determine the preferred rotational conformation(s) of the flavan groups(s) in the theaflavins, they give no indication of the existence of rotamers or rotameric equilibria. The temperature (and solvent) dependencies of the circular dichroism spectra (Tables 11, 12) give better insight into these aspects of theaflavin structure: the UV and visible regions of the spectra are best discussed separately.

*The UV region*

CD bands in the 240–260 nm region give little useful information, since the $\Delta\epsilon/\epsilon$ ratio is very unfavourable and in some cases distinct maxima cannot be discerned. The bands at 280–300 nm are more informative. Three chromophores absorb in this region; the chroman group, the benzotropolone ring, and the gallate group. $\Delta\epsilon$ values for the 280–300 nm CD band in the non-gallate 2R and 2'R configuration theaflavins are negative and larger than those found for the corresponding 2R configuration flavanols.[39] Assuming the conformations of the flavan rings are similar in these two series, the higher $\Delta\epsilon$ values of the theaflavins must arise from negative contributions from the benzotropolone chromophore which are larger than those from the aromatic B-ring in the 2R flavanols. 3R and 3'R configuration pigments (epitheaflavic acid, theaflavin TF1a, TF9) give higher (negative) $\Delta\epsilon$ values than the isomeric 3S and 3'S configuration pigments (theaflavic acid, TF1c, TF10): this parallels the previously observed[39] change in sign (3R, negative; 3S, positive) of the contribution of the $C_3$ chiral centre to the corresponding CD band of the aromatic B ring in the flavanols. Similarly, TF5 (2R, 3R) gives a higher (negative) $\Delta\epsilon$ value than the isomeric TF6 (2S, 3R); again, this parallels the previously observed[39] change in sign (2R, negative; 2S, positive) of the contribution of the $C_2$ chiral centre to the corresponding CD band of the aromatic B ring in the flavanols.

The magnitude of the contribution of the benzotropolone chromophore to $\Delta\epsilon$ depends on the positions of the other groups attached to the chiral centres (2,2', 3,3') relative to the benzotropolone ring, assuming the ring to be planar, non-chiral and conformationally immobile. (There are indications that benzotropone systems may be non-planar,[40,41] but definite evidence is lacking in the case of the theaflavins.) These positions change as the flavan groups rotate about the bonds joining them to the benzotropolone ring. If there are rotamers whose $\Delta\epsilon$ values differ, and whose free energies differ by an amount which is large relative to RT at low temperature and approximates to RT at room temperature, the $\Delta\epsilon$ value actually observed will be temperature dependent. If the free energy difference is larger or smaller than RT at both temperatures, a smaller effect will be observed, since in the first case one rotamer will predominate at both temperatures, and in the second, the position of equilibrium will be relatively insensitive to temperature change. The temperature dependence of $\Delta\epsilon_{280-290}$ in theaflavic and epitheaflavic acid, TF9 and TF10 clearly indicates the co-existence of rotamers in EPA solution at room temperature. (The less pronounced temperature dependence observed in (−) epicatechin and (+) catechin[39] is consistent with

Table 11. CD characteristics of theaflavins in organic solvents

| | | λ | Δε | λ | Δε | λ | Δε | λ | Δε |
|---|---|---|---|---|---|---|---|---|---|
| Theaflavin | MeOH | 254 | −8·85 | 288 | −6·12 | 353 | −0·91 | 505 | +0·09 |
| TF1a | EPA + 25° | 254 | −9·63 | 286 | −7·12 | 375 | −1·80 | 505 | +0·12 |
| | EPA − 185° | 265 | −7·36 | 295 | −8·01 | 380 | −1·88 | 495 | +0·66 |
| TF1c | MeOH | 253 | −3·31 | 277 | −2·58 | 392 | −1·96 | no detect. CD | |
| | EPA + 25° | 255 | −3·52 | 280 | −2·36 | 391 | −2·36 | no detect. CD | |
| | EPA − 185° | | | 300–365 | CD > 0 | 382 | −1·92 | 495 | −0·16 |
| Theaflavin 3-gallate | MeOH | 234 | −11·68 | 300 | −10·28 | 353 | −1·78 | 448 | −0·63 |
| | | 246 | −11·68 | | | 382 | −1·21 | | |
| | EPA + 25° | 247 | −9·97 | 300 | −7·77 | 365 | −1·66 | 455 | −0·74 |
| | EPA − 185° | 250 | −10·27 | 302 | −18·59 | 392 | −2·69 | 500 | +0·31 |
| | | | | | | | | 455 | −0·20 |
| Theaflavin-3′-gallate | MeOH | 226 | −14·33 | 280 | −20·25 | 368 | −1·81 | 490 | +0·40 |
| | EPA + 25° | | | 280 | −23·38 | 380 | −2·91 | 490 | +0·37 |
| | EPA − 185° | 244 | −3·73 | 285 | −23·30 | 385 | −3·09 | 490 | +0·76 |
| Theaflavin-3,3′-digallate | MeOH | 226 | −19·60 | 289 | −24·31 | 368 | −1·96 | 505 | +0·12 |
| | EPA + 25° | 228 | −15·91 | 290 | −19·63 | 368 | −1·78 | 453 | −0·65 |
| | EPA − 185° | | | 298 | −37·64 | 375 | −1·53 | 490 | +0·86 |
| Epitheaflavic acid | MeOH | 252 | −2·63 | 288 | −3·94 | 400 | −0·33 | 480 | +0·49 |
| | EPA + 25° | 253 | −3·93 | 287 | −5·58 | | | 488 | +0·42 |
| | EPA − 185° | 252 | −4·16 | 290 | −8·03 | | | 486 | +1·02 |
| Theaflavic acid | MeOH | 250 | −2·08 | 283 | −2·60 | | | 475 | +0·16 |
| | EPA + 25° | 250 | −1·49 | 286 | −2·19 | | | 465 | +0·04 |
| | EPA − 185° | 248 | −1·91 | 285 | −5·04 | | | 473 | −0·34 |
| Epitheaflavic acid 3′-gallate | MeOH | | | 288 | −18·67 | 390 | −0·79 | 475 | +0·67 |
| | EPA + 25° | | | 282 | −21·93 | 400 | −1·25 | 480 | +0·74 |
| | EPA − 185° | | | 295 | −25·21 | 385 | −0·98 | 485 | +1·29 |
| TF5 | MeOH | 228 | −2·98 | 295 | −11·01 | 380 | −2·29 | neg CD | no extrem. |
| | EPA + 25° | 268 | +0·46 | 298 | −9·75 | 388 | −2·17 | neg CD | no extrem. |
| | EPA − 185° | | | 302 | −14·45 | 385 | −3·37 | 515 | +0·17 |
| TF6 | MeOH | 260 | +0·33 | 292 | −0·39 | | | 482 | −0·072 |
| | EPA + 25° | 260 | +2·91 | 295 | −2·18 | 385 | −0·25 | neg CD | no extrem. |
| | EPA − 185° | 260 | +3·12 | 278 | −2·08 | 385 | −0·21 | neg CD | no extrem. |
| TF7 | MeOH | 252 | −6·23 | CD < 0 | | 363 | −0·98 | 450–500 | CD < 0 |
| | EPA + 25° | 252 | −4·81 | no extrem. | | 355<br>385 | −0·77<br>−0·49 | | no detectable CD |
| | EPA − 185° | 258 | −5·28 | | | 375 | −2·29 | 470 | +0·02 |
| TF8 | MeOH | 265 | +1·63 | 296 | −14·54 | 370 | −1·93 | 450–500 | CD < 0 |
| | EPA + 25° | 265 | +2229 | 296 | −14·14 | 370 | −1·59 | 550 | +0·01 |
| | | | | | | 440 | −0·23 | | |
| | EPA − 185° | 269 | −1·06 | 280 | +0·87 | 378 | −3·06 | 525 | +0·02 |
| | | | | 302 | −12·71 | 440 | −0·22 | | |
| TF9 | MeOH | 240 | +3·19 | 271 | −2·52 | 405 | −0·09 | 495 | +0·37 |
| | | | | 295 | −3·18 | | | | |
| | EPA + 25° | | | 305 | −1·97 | 392 | −0·40 | 507 | +0·24 |
| | EPA − 185° | 240 | +3·19 | 300 | −6·52 | 395 | −0·46 | 528 | +0·31 |
| | | | | 307 | −1·80 | | | | |
| TF10 | MeOH | 240 | +3·49 | 279 | −1·54 | 328 | −0·70 | | no detectable CD |
| | EPA + 25° | | | 280 | −1·08 | 328 | −0·41 | 480 | −0·13 |
| | | | | | | 380 | −0·09 | | |
| | EPA − 185° | | | 290 | −2·77 | 358 | +0·23 | 450 | −0·06 |

their less crowded structures. These permit relatively free rotation of the B ring about its bond to the chroman system, so that the position of rotameric equilibrium is relatively insensitive to temperature.)

Introduction of a gallate group at $C_{3'}$ and/or a non-gallate flavan substituent at $C_{6'}$ virtually eliminates this temperature dependence, thus showing that epitheaflavic acid-3′-gallate, theaflavin and theaflavin-3′-gallate exist predominantly in one preferred conformation over the temperature range 25° to −185°. Evidently in these pigments, all but one of the rotameric possibilities open to epitheaflavic acid and TF9 are destabilised by interactions

Table 12. CD-characteristics of theaflavins in water

| | λ | Δε | λ | Δε | λ | Δε | λ | Δε | λ | Δε | λ | Δε | λ | Δε |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TF1a, Theaflavin | 241<br>257 | −7·92<br>+3·96 | 278 | −29·52 | 312 | +5·04 | 357 | −4·32 | 395 | +4·23 | 472 | −0·58 | 527 | +0·23 |
| TF1c | | | 275 | −6·42 | 311 | +1·10 | 353 | −0·73 | 389 | +1·40 | | | 515 | +0·20 |
| Theaflavin-3-gallate | 230<br>245 | −10·20<br>−12·15 | 274 | −15·90 | 310 | +4·50 | 358 | −3·29 | 398 | +1·74 | 473 | −0·27 | 520 | +0·09 |
| Theaflavin-3′-gallate | | | 278 | −39·52 | 318 | +1·69 | 357 | −3·68 | 395 | +3·48 | 468 | −0·13 | 514 | +0·39 |
| Theaflavin-3,3′-digallate | | | 288 | −46·70 | | | 360 | −5·95 | 400 | +3·57 | 469 | −0·30 | 519 | +0·63 |
| Epitheaflavic acid | 243 | +3·10 | 278 | −6·00 | | | | | 407 | −5·10 | 475 | −1·04 | | |
| Theaflavic acid | | | 284 | −1·20 | | | 364 | +0·36 | 407 | −2·30 | 499 | −0·12 | | |
| (−)TF4 Me-ester | 251 | +1·47 | 286 | −4·05 | | | 376 | +0·99 | 435 | −2·28 | | | | |
| Epitheaflavic acid-3′-gallate | | | 275 | −23·66 | 322 | +0·42 | | | 399 | −2·61 | 455 | −1·01 | | |
| TF5 | | Solubility too small | | | | | | | | | | | | |
| TF6 | | Insoluble | | | | | | | | | | | | |
| TF7 | 250 | −4·60 | | | | | | | 395 | −0·51 | 460 | +0·19 | | |
| TF8 | 266 | +1·03 | 294 | −8·33 | | | | Solubility low, no detectable CD | | | | | | |
| TF9 | 258 | +3·50 | 272<br>295 | +5·17<br>−5·17 | 336 | +0·55 | 387 | −5·37 | | | 442 | +0·10 | 498 | −0·52 |
| TF10 | | | 295 | −0·73 | 348 | +0·06 | 385 | −0·48 | | | 452 | +0·17 | | |

between flavan groups and/or between $H_b$ and a 3'-gallate group. The NMR evidence suggests that both types of interaction constrain $C_2$H to lie closer to $H_b$ so that the 3'-substituent lies on the periphery of the molecule clear of the benzotropolone ring. Hence in theaflavin, gallate can be substituted for hydroxyl at $C_{3'}$ without seriously disturbing the conformation of the molecule. The resulting substantial increase in $\Delta\epsilon$, approximately the same for the 3'-gallates of epitheaflavic acid (16·35–17·18) and theaflavin (15·29–16·26), is due to the contribution of the gallate group. In contrast, introduction of a gallate group at $C_3$ (theaflavin-3-gallate and 3,3'-digallate) results in marked temperature dependence of $\Delta\epsilon$: only at low temperature is the expected contribution of the 3-gallate group to $\Delta\epsilon$ observed. This suggests that in these pigments there are at least two types of conformation of not too disparate free energy open to the 3-gallate group; in its more stable conformations it makes the normal large negative contribution to $\Delta\epsilon$, in the others, its contribution is small or positive. The nominally free rotation of the 3-gallate group about the $C_3$—O and CO—O bonds is in practice limited by its interactions with $H_b$ and the 4''-flavan substituent. These interactions are less serious in the less crowded mono-(6'')-flavan substituted TF5 and 8, so that the free energy difference between the two gallate conformations is smaller, and the position of rotameric equilibrium less sensitive to temperature. This accounts for the smaller temperature dependence of $\Delta\epsilon$ found in TF5 and 8. The temperature dependence is more marked in TF5 (which bears a 4''-Me group) than TF8, which further suggests that interactions between the 3-gallate group and the 4''-flavan substituent are primarily responsible for the temperature dependence of $\Delta\epsilon$ in the theaflavin-3-gallates.

The NMR evidence suggests that these interactions, less serious for the smaller 3-OH group in theaflavin, constrain $C_2$H to lie closer to $H_b$ in the 3-gallates. Thus, besides affecting the preferred positions of the gallate group relative to other groups attached to the chiral centres $C_3$ and $C_2$, these interactions also affect the positions of these centres and groups relative to the benzotropolone chromophore. The expected temperature-dependent contribution of the benzotropolone chromophore to $\Delta\epsilon$ at 280 nm cannot be unambiguously separated from that of the gallate group, which also absorbs at 280 nm. However in the visible region, where only the benzotropolone chromophore absorbs, the CD spectra give ample evidence of temperature-dependent conformations.

*The visible region*

Bands in the visible region arise from the benzotropolone chromorphore only: their signs and intensities may be determined by first, second or higher sphere effects.[42] A first sphere effect demands an inherently chiral (i.e. non-planar) benzotropolone chromophore, which must in addition be conformationally mobile to account for the observed temperature and solvent dependencies. Since firm evidence regarding non-planarity and mobility is lacking, and because the observed CD intensities are rather low for first sphere effects, such effects are neglected in the following discussion, which rests on the assumption that the positions relative to the benzotropolone ring of other groups attached to the chiral centres determine the signs and intensities of the bands.

There are two main points of interest in the CD spectra of theaflavin and its gallates in EPA. First, at room temperature the extremum of the longest wavelength band occurs at 460–470 nm (negative) or 490–505 (positive) according to whether or not a 3-gallate group is present. Secondly, as the temperature is decreased, the intensity of the 460–470 nm band (when seen) diminishes, whilst that of the 500 nm band increases (e.g. Fig. 7). In the absorption spectra, no bands are found at 500 nm irrespective of temperature, which suggests that CD bands at this position are artefacts arising from overlapping negative and positive bands which lie close together. Overlapping bands of opposite sign are often found to produce extrema up to 30 nm apart,[43] but generally these have been found in the UV region. The larger bandwidths in the visible region would be expected to produce a larger separation on a wavelength scale. Close inspection of the room temperature CD spectrum of theaflavin reveals a negative band at *ca* 450 nm (Fig 8) and bands at both 500 nm (positive) and lower wavelength (negative) can be seen in the low temperature spectra of the 3-gallates (Fig 7). The low wavelength negative part of the 460–470 nm band cannot in all cases be seen clearly, because other bands occur around 400 nm. This can be clearly seen in the spectra of aqueous solutions: these spectra (Table 12, Fig 9) show marked and interesting differences when compared with the spectra of solutions in organic solvents. It appears that theaflavin and its gallates adopt very similar conformations in water, since their CD spectra all show six paired bands of alternating sign at 280 and 310 nm, 360 and 395 nm, and 470 and 520 nm. These three pairs of bands almost certainly arise from overlapping bands of different sign, since only three regions of absorption are found between 260 and 600 nm in the absorption spectra.

These results show that in EPA, at least two types of conformation are open to theaflavin and its gallates. In all four pigments, the energetically-favoured conformations are characterised by a resultant positive CD band at 490–505 nm. The others, just detectable in theaflavin at room temperature, are only important in the more crowded theaflavin-3-gallate and 3,3'-digallate, and are characterised by a resultant negative CD band at

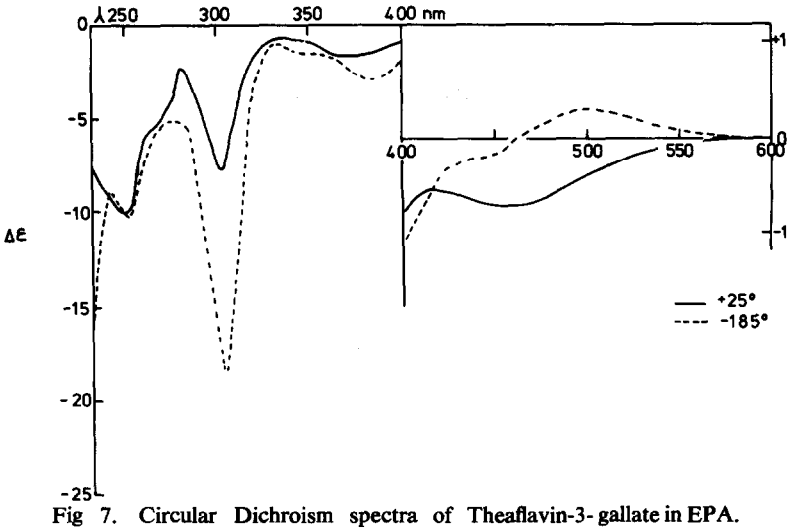



Fig 7. Circular Dichroism spectra of Theaflavin-3-gallate in EPA.

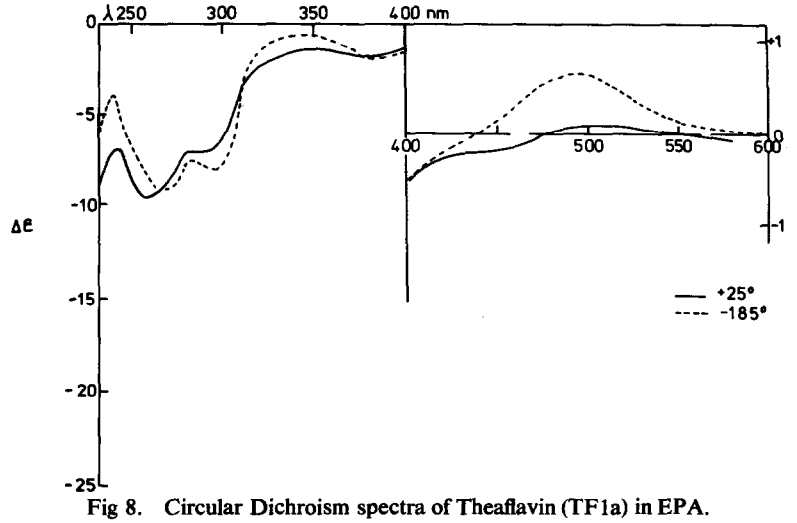



Fig 8. Circular Dichroism spectra of Theaflavin (TF1a) in EPA.

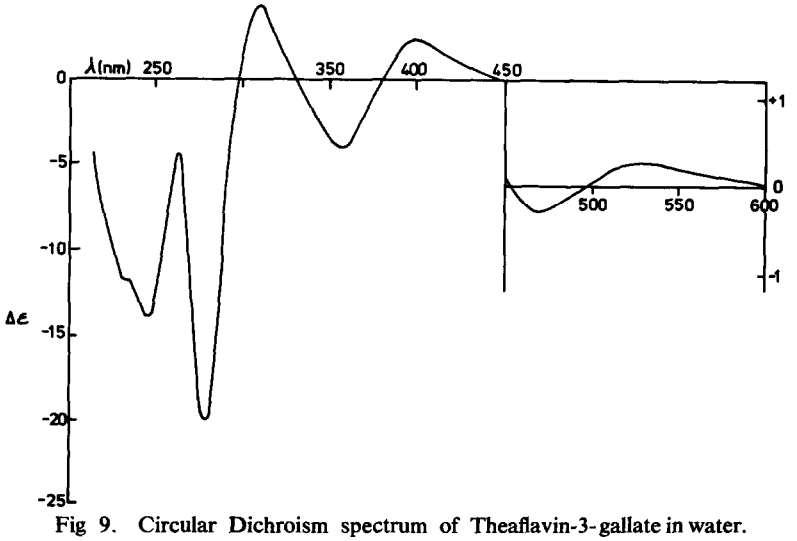



Fig 9. Circular Dichroism spectrum of Theaflavin-3-gallate in water.

450–470 nm. These differing CD characteristics arise from differing positions of groups attached to the chiral centres with respect to the benzotropolone ring. Consistent with this view, the NMR evidence suggests that $C_2H$ is constrained to lie closer to $H_b$ in the 3-gallates than in theaflavin by interactions between the 3-gallate group and the 4″-flavan substituent. These interactions, less serious for the smaller 3-OH group in theaflavin, reduce the free energy difference between the conformations to the extent that in the 3-gallates, conformational equilibrium is detectable even at low temperature. These conformational equilibria almost certainly involve rotation of the 6″-(and possibly also the 4″-) flavan group about the bond joining it to the benzotropolone ring, since changes in the conformations of the flavan rings themselves afford little relief from the above interactions. Similar rotational isomerism has been reported for other polymeric flavanoids.[44–47] Unfortunately examination of aqueous solutions of theaflavins is restricted to a comparatively narrow temperature range: consequently the possibility that the paired CD bands observed at room temperature arise from rotamers has not been confirmed.

## EXPERIMENTAL

1. *Isolation of theaflavins from black tea*

Black tea [2·400 g of a blend of Assam Fred (7), African Standard 2II (2) and Ceylon Poodle (1)] was extracted with hot water (85°, 5 min) to give a filtered soln (24 l) which was freeze-dried to yield black tea solubles (800 g). The solubles were decaffeinated by continuous chloroform extraction of 4% solns in MeOH/water (1:3): MeOH and $CHCl_3$ were distilled from the decaffeinated tea soln (30°, reduced pressure), and the aqueous soln extracted with 5 successive equal volumes of EtOAc. The dried ($MgSO_4$) extract was evaporated to dryness (30°) to yield a red solid (205·5 g). Theaflavins were separated from this material by two methods.

(a) Half of the acetate-soluble solids (5 g per separation) was applied to foil-wrapped columns (80 cm × 4 cm) of Sephadex LH20 preswollen overnight in 60% acetone/water, eluted with 60% acetone (1 ml/min), and the eluate collected in 15 ml fractions. Fractions from 4·5 $V_0$–5·5 $V_0$ were examined spectrophotometrically; those with $E_{380}/E_{460}$ between 2·5 and 3·0 were pooled, the acetone was distilled off (30°), and the pigments extracted into EtOAc. The dried ($MgSO_4$) extract soln was distilled to dryness (30°) to yield a bright scarlet solid (in total, 10·6 g). This theaflavin-rich fraction (1·0 g per separation) was applied in a minimum of acetone-MeOH-water (25:6:2) to foil-wrapped columns (80 cm × 4 cm) of Mallinckrodt's SilicAR CC7 (100–200 mesh) equilibrated overnight with $CHCl_3$-acetone-MeOH-water (40:25:6:2), eluted with the same solvent (0·5 ml/min), and collected in 15 ml fractions. Three clearly-separated red fractions were collected and evaporated to dryness (30°). Yields in order of elution were: fraction 1 (TF1), 1·15 g; fraction 2 (TF2), 5·02 g; fraction 3 (TF3), 3·64 g; recovery from columns, 82%. These three fractions were re-chromatographed once on large (80 cm) SilicAR columns, then twice more (100 mg per separation) on small columns (30 × 2 cm) of SilicAR (first 100–200 mesh, then 200–325 mesh). Recrystallisation of purified TF1 from aqueous MeOH gave crystals of theaflavin (TF1a),[5] m.p. 238–241° (a). Similar recrystallisation of purified TF3 gave theaflavin-3,3′-digallate, m.p. 226–230° (d). TF2, a mixture of theaflavin-3- and 3′-gallates (TF2A and B), could not be recrystallised.

(b) The second half of the acetate-soluble solids (5 g per separation) was applied to foil-wrapped columns of Sephadex LH20 preswollen overnight in 35% acetone/water. The eluted fractions (15 ml, 1 ml/min) were monitored spectrophotometrically; two faint yellow-orange bands B1 and B2 ($\lambda_{max}$ 400 nm) were followed by three more intense orange bands B3, 4 and 5 ($\lambda_{max}$ 380 and 460 nm). Corresponding bands were pooled, acetone removed (30°), and the pigments extracted into EtOAc. The dried ($MgSO_4$) extract solutions were evaporated to dryness to give pigments P1 (15 mg), P2 (29 mg), P3 (890 mg), P4 (4·73 g) and P5 (2·51 g). TLC on polyamide (MeOH), SilicAR TLC-7 ($CHCl_3$-acetone-MeOH-water, 40:25:6:2) and cellulose (Machery & Nagel 300 HR; first dimension, n-BuOH-HOAC-$H_2O$, 4:1:2·2, second dimension, 2% HOAC) showed that P1, 2, 4 and 5 are indistinguishable respectively from synthesised (Exptl. 2) epitheaflavic acid (P1, Fig 1), epitheaflavic acid-3′-gallate (P2, Fig 1), TF2 and TF3. The chromatograms of P3 on cellulose indicated that two trace pigments (TF1b and TF1c, Fig 1 were present in addition to TF1a. Preparative paper chromatography on 3MM paper (n-BuOH-HOAC-$H_2O$, 4:1:2·2) gave TF1b (15 mg) and TF1c (98 mg). TF1c was finally purified by chromatography on LH20 (35% acetone), and obtained in a 48 mg yield. P2 was similarly purified and obtained in a 20 mg yield.

2. *Synthesis of theflavins by ferricyanide oxidation of flavanols*

The required (see below) catechin ($2 \times 10^{-3}$ moles) and gallocatechin or gallic acid ($10^{-3}$ moles) were dissolved in a minimum of water (150–250 ml). The soln was cooled in an ice bath, and a soln of potassium ferricyanide (660 mg) and $NaHCO_3$ (400 mg) in water (20 ml) was run in dropwise with stirring (pH of soln, 6·5–7·5). The soln was allowed to stand for 15 min, was acidified ($10^{-1}$ N HCl) to pH 4·5, and extracted with 5 equal volumes of EtOAc. The dried ($MgSO_4$) extract soln was evaporated to dryness, and the solid so obtained was applied to a Sephadex LH20 column (30 × 3 cm) and eluted with 35% acetone. The orange-red fractions so obtained were finally purified by chromatography on SilicAR CC7 ($CHCl_3$-acetone-MeOH-water, 40:25:6:2): yields are given below.

| Precursors | Products | Yield (mg) |
|---|---|---|
| EGCG + EC | TF2A | 85 |
| EGC + ECG | TF2B | 52 |
| EGCG + ECG | TF3 | 70 |
| Gallic acid + EC | (−) TF4 | 70 |
| Gallic acid + ECG | (−) TF4 | 72 |
| | (−) TF4-3′-gallate | 160 |
| Gallic acid + (+) catechin | (+) TF4 | 110 |
| EGC + EC | TF1a | 106 |
| EGC + (+) catechin | TF1c | 49 |

3. *Synthesis of model theaflavins*

Compounds TF5–10 (Fig 2) were synthesised by ferricyanide (660 mg)/bicarbonate (400 mg) oxidation of solutions containing $2 \times 10^{-3}$ moles of the catechols and $10^{-3}$ moles of the pyrogallols shown below.

| | Catechol | Pyrogallol |
|---|---|---|
| TF5 | 4-methyl catechol | (−) EGCG |
| 6 | 4-methyl catechol | (−) GCG |
| 7 | Catechol | (−) EGC |
| 8 | Catechol | (−) EGCG |
| 9 | (−) EC | Pyrogallol |
| 10 | (+) catechin | Pyrogallol |

In those cases (TF9 & 10) which involve the oxidation of unsubstituted pyrogallol, the oxidant and pyrogallol solns were added separately and simultaneously to the solution of catechol: this procedure minimised the quantity of unwanted purpurogallin formed.

Products were isolated by EtOAc extraction, chromatography on LH20 (35% acetone) and final purification on SilicAR CC7. The (−) gallocatechin gallate (-GCG) required for TF6 was obtained from green tea solubles as previously described:[32] after recrystallisation from water this material gave $[\alpha]_D^{25} = -37°$ (*cf* Bradfield and Penney,[49] −46°) and was probably not completely optically pure. All other optically active precursors were recrystallised to D-line rotations corresponding to literature values.

4. *GLC of theaflavins and model compounds*

(a) All final products (10 mg) were derivatised in dry pyridine (2 ml) with N,O-bis(trimethylsilyl)acetamide (BSA, 2 ml) and examined under flavanol analysis conditions[25] for precursor impurities. P2, TF1c and their synthetic counterparts were also examined under theaflavin analysis conditions.[23]

(b) Fraction TF1 (ex. first SilicAR column, Exptl. 1a; 60 mg) was trimethylsilylated at room temp overnight with BSA (2 ml) in dry pyridine (1 ml). Solvent and excess reagent were removed under vacuum, and the residue taken up in $CCl_4$. (−) TF4 (Exptl 2, 40 mg) was similarly treated and compared with TF1 under flavanol analysis conditions, using (−) EGCG, ECG and quercetin as reference compounds. Trapping experiments were carried out using a 12 in column of 3% OV1 on Chromosorb W (acid washed and silanised with hexamethyl disilazane, 80–100 mesh) attached to a stream splitter (100:1). Materials of interest were trapped in a U-tube capillary cooled in cardice/acetone. For (−) TF4 and TF1, the column was run isothermally at 282° with a carrier gas (argon) flow rate of 75 ml/min. Trapped materials were examined under flavanol analysis conditions and by mass spectrometry.

5. *Theaflavin acetates*

These were prepared from individual theaflavins (25 mg) or theaflavin mixture (TF2, 50 mg) heated (60°, 1hr) in acetic anhydride (5 ml) using a pyridine (10 drops) catalyst. The cooled reaction mixtures were thrown into ice-water, and the precipitated acetates filtered off and washed with water. They were examined by TLC on silica (Merck Kiesilgel G) developed with chloroform-acetone (5:1 and 10:1).

6. *Mass spectrometry*

Theaflavins were trimethylsilylated as described in Exptl. 4b. Pyridine and reagents were distilled off under vacuum, the residue transferred in $CCl_4$ to a small tube, and the solvent removed at room temp in a stream of dry $N_2$. Spectra were obtained at 70 electron volts using an AEI MS 902 spectrometer.

7. *NMR spectra*

These were obtained from degassed solns in deuterioacetone at 220 MHz (Varian HA-220, 13°) with a TMS internal standard. Chemical shifts were obtained correct to ±1 Hz, but concentration effects and addition of $D_2O$ on occasions produced variations greater than this. Coupling constants were measured at this frequency correct to ±0·3 Hz. Decoupling and progressive saturation experiments were carried out at 100 MHz (Varian HA-100, 30°).

8. *Circular dichroism spectra*

These were measured in water, MeOH and EPA (ethanol-isopentane-dimethyl ether, 2:5:5 v/v) using a Jouan Dichrograph 185[43] fitted with the Jouan low temp accessory. Low temp measurements were corrected for solvent shrinkage using reported data.[48] Uvasol (Merck) solvents were used throughout.

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