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FRACTIONATION AND CHEMISTRY OF ETHYL ACETATE–SOLUBLE THEARUBIGINS FROM BLACK TEA

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Abstract—Sephadex LH-20 chromatography was used to fractionate purified ethyl acetate-soluble thearubigins, prepared from an aqueous ethanolic extract of black tea. Three subfractions were so produced, each having a MW of about 1500 and each being degradable into cyanidin, delphinidin, gallic acid, the same two flavan-3-ols, and the same two flavan-3-ol gallates, though in different yield. Some evidence for the presence of benzotropolone moieties in at least one of the subfractions was obtained. Overall the ethyl acetate-soluble thearubigins are viewed as pentameric flavan-3-ols/flavan-3-ol gallates, containing both hydrolysable and non-hydrolysable interflavanoid links, as well as benzotropolone units, rather than as polymeric proanthocyanidins, a term previously used for all thearubigin subgroups.

INTRODUCTION

The thearubigins are a heterogeneous group of orange–brown, weakly acidic pigments formed by enzymic oxidation of a number of flavan-3-ols and flavan-3-ol gallates during the manufacture of black tea [1,2]. Despite their relative abundance (7–18%) and important contribution to the colour and taste of black tea brews, only recently has any real progress towards an understanding of their structural features been made [1–5].

Roberts *et al.* [6] sub-divided thearubigins according to whether they could be extracted from aqueous infusions with ethyl acetate (20% of total [7]) or whether they required 1-butanol. We prepared an extract containing ethyl acetate-soluble thearubigins by a combination of the methods of Roberts [6] and Vuataz and Brandenberger [8]. This purified extract, T1, contained only traces of flavonoid material and had a nitrogen content of 1.3%, which was shown to be predominantly due to adsorbed caffeine with only small amounts of amino acid nitrogen (mainly theanine) present. T1 was not homogeneous, but consisted of thearubigins of similar solubilities and chromatographic behaviour. Several unsuccessful attempts [9] to further fractionate it were made, e.g. by means of column chromatography on a variety of support media, countercurrent distribution, differential dialysis, and extraction at different pH values, but only chromatography on Sephadex LH-20 offered a satisfactory means of separation, as detailed below.

RESULTS

T1 was chromatographed on a large column of Sephadex LH-20 and was eluted as one broad peak with a maximum at 3.8 V_0. The eluent was collected in three fractions, A, B, and C, with elution limits of 1.6–3.4 V_0, 3.4–4.3 V_0, and 4.3–7.0 V_0, respectively. Each of these, when evaporated and reapplied to the same column, chromatographed in a manner consistent with its previous behaviour and led to subfractions T1A, T1B, and T1C. The tests described below were then applied to each of these.

Two-dimensional ascending paper chromatography revealed that the thearubigins in each of the T1 subfractions produced streaks between R_f 0.2–0.7 in the first direction, a slightly different pattern of trailing in the second direction being the only discernible difference. The spectra of T1A and T1B between 240–625 nm closely resembled one another and also that for Roberts' S_{ly} thearubigins [2]. The spectrum of T1C, however, exhibited a small peak at 380 nm and a broad shoulder at around 455 nm and so resembled that of the theaflavins, which contain a peak around 380 nm and a small peak around 460 nm. Moreover, the absorbance (1% solution) of T1C at 460 nm was only about 30% lower than that of theaflavin digallate and substantially higher than that of T1A and T1B (Table 1).

Table 1. Spectral characteristics of thearubigins

	λ_{max} (A^{1cm}_{max} of 1% solution)						A^{1cm}_{460} of 1% solution
T1A	271	(171);	—	;	—		6.9
T1B	277	(212);	—	;	—		16.7
T1C	279	(254);	380	(59.9);	—		28.6
Theaflavin*	268	(330);	378	(191);	461	(72.0)	—
Theaflavin digallate*	278	(419);	378	(117);	455	(40.8)	—

* Spectral data according to Collier *et al.* [27].

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Table 2. Degradation products and molecular weights of thearubigins

Determination	Subfraction T1A	T1B	T1C
Formation of anthocyanidins			
Overall % yield of anthocyanidins (as cyanidin chloride)	1·26	2·28	2·01
% Yield of free anthocyanidins (as cyanidin chloride)	0·53	1·35	1·19
Hydrolysis with sulphurous acid			
Relative yields of:			
epicatechin gallate	× ×	× × × × ×	× × × × ×
epigallocatechin gallate	×	× ×	× × ×
epicatechin	×	× ×	× ×
catechin	trace	× ×	× ×
Determination of gallate ester residues			
Yields of gallic acid (as % of thearubigin weight)	1·5	3·5	3·4
Molecular weights of methylated subfractions			
methoxyl content (% OMe)	27·2	31·6	30·6
acetone as solvent	1710	1735	1825
benzene as solvent	—	1770	*ca* 1800

Each of the T1 subfractions was hydrolysed with 0.5 M propanolic HCl, yielding cyanidin and delphinidin (plus small amounts of their isopropyl ethers), which were identified by R_f comparison with authentic anthocyanidins. Visual assessment indicated that the proportion of cyanidin to delphinidin was about 3:1 in each subfraction. The yields of the purified anthocyanidins ranged from 0.5–1.4%, and were approximately half the overall yields (Table 2), which indicates that some flavylium units remain linked to the fragments of the thearubigin molecules under these conditions of hydrolysis.

Sulphurous acid hydrolysis of each of the T1 subfractions, followed by paper chromatographic analysis of ether extracts, demonstrated the presence of epicatechin gallate, epicatechin, catechin, epigallocatechin gallate, and two additional compounds, which appeared from their chromatographic position to be similar to Quesnel's sulphur-containing spots A and B derived from the seeds of *Theobroma cacao* L. [10]. Visual comparison of the spots developed by the ferric chloride/potassium ferricyanide reagent indicated that epicatechin gallate constituted about 50% of all the flavan-3-ols plus gallates released and that the proportion of 3′,4′-dihydroxylated flavan-3-ols to that of 3′,4′,5′-trihydroxylated flavan-3-ols was about 3:1 for each of the subfractions (Table 2).

Brown *et al.* [3] found that their thearubigins contained only relatively small amounts of gallate residues, an unexpected finding since the majority of the flavanol precursors are known to be gallates. Their method of acid methanolysis was adopted for the T1 sub-fractions, the equivalent yields of gallic acid obtained ranging from 1.5–3.5% (Table 2). Application of vapour phase osmometry to repeatedly methylated derivatives of T1A, T1B, and T1C (methoxyl contents all about 30%, which is equivalent to about 3.5 methoxyl groups per epigallocatechin unit) dissolved in acetone showed each of the unmethylated subfractions to have a MW of about 1500. The use of benzene as solvent gave similar results for T1B and T1C, but T1A was not sufficiently soluble in benzene.

DISCUSSION

Our results (Table 2) are compatible with those of Brown *et al.* [3], where comparison is possible, in all but one respect, namely, the overall yield of anthocyanidins, which was found to be about 2% for the T1 subfractions in contrast to 24% reported by the Sheffield workers [3] for equivalent fractions. Our yield of 2% anthocyanidins, together with evidence for other forms of bonding, suggests that, whilst the ethyl acetate-soluble thearubigins contain some proanthocyanidin linkages, other forms of inter-flavanoid bonds predominate, since yields in excess of 5% would be anticipated from oligomers of a MW of 1500 (the value determined for the thearubigins), totally comprised of proanthocyanidin links [11]. Accordingly the description of the ethyl acetate-soluble thearubigins as polymeric proanthocyanidins is inappropriate since the term has been reserved for polymers predominantly, and often exclusively, comprised of linkages which on hydrolysis release anthocyanidins [12]. Other thearubigins, however, may fit this description.

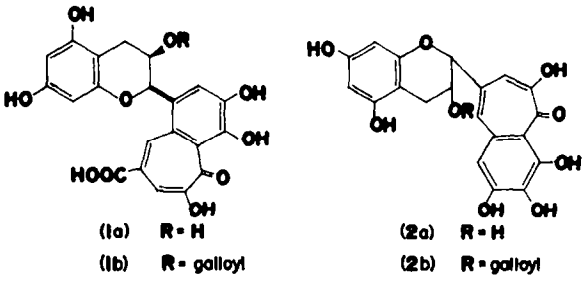
The present study, like that of Brown *et al.* [3], has demonstrated that, whereas the proportion of 3′,4′-dihydroxy- to 3′,4′,5′-trihydroxyflavan-3-ols present in fresh tea shoots is about 1:3, the proportion of cyanidin to delphinidin produced by propanolic HCl hydrolysis of each of the T1 subfractions is approximately 3:1. Similarly, the proportion of 3′,4′-dihydroxy- to 3′,4′,5′-trihydroxyflavan-3-ols released by the action of sulphurous acid on each of the subfractions was found to be 3:1. Thus this ratio-reversal is consistent and suggests a basic structural feature common to all ethyl acetate-soluble thearubigins.

Brown *et al.* [3] put forward two alternative hypotheses to explain the apparent anomaly. In one of these, the 3′,4′,5′-trihydroxyflavan-3-ols are oxidised enzymically to α-hydroxy-*o*-quinones, which survive through to the thearubigins. Although accounting for the colour and weak acidity of the generally unreactive thearubigins, this hypothesis is not in accord with the high reactivity of *o*-quinones, e.g. with proteins present in fermenting tea juice [13,14]. The alternative hypothesis, that the greater reactivity of 3′,4′,5′-trihydroxyflavan-3-ols at position C_2 causes them to form the non-hydrolysable C_2–$C_{2'}$, and possibly $C_{6'}$–C_2 bonds as well, seems more attractive. On this view it would be predicted that end groups attached via a hydrolysable bond, presumably C_4–$C_{6\text{ or }8}$, would be more likely to be a 3′,4′-dihydroxy than a 3′,4′,5′-trihyhydroxy derivative. Thus, where the end group is attached through its position C_4, alcoholic acid hydrolysis will release greater proportions of cyanidin than delphinidin [15], and, where it is connected via its $C_{6\text{ or }8}$, then sulphurous acid hydrolysis will release greater proportions of catechins than gallocatechins [10,16,17]. Implicit in this model of the thearubigin molecule is that hydrolysable bonds not linked to end groups exist. On treatment with alcoholic acid these would create anthocyanidin-like moieties attached to fragments of the thearubigins molecule. The fact that the streaks of oligomeric material on the paper chromatograms of the hydrolysate are red is consistent with this view.

From our results, it appears that the T1 thearubigins are approximately pentameric, which corresponds to a molecular weight of 1500, with, at most, two of their four interflavanoid bonds hydrolysable. The remainder must be non-hydrolysable, but cannot be only C_2–$C_{2'}$, bonds, since the colour and acidity of the thearubigins need to be explained.

Subfraction T1C was found to have a spectrum similar

to that of the theaflavins in exhibiting a small peak at about 380 nm and having a comparable absorbance in 1% solution at 460 nm (Table 1). This similarity suggests that these thearubigins contain a benzotropolone system. The absorption spectra of the other subfractions show less similarity to that of the theaflavins in that they lack the peak at 380 nm. However, the general similarity between the theaflavins and thearubigins makes it likely that the latter contain benzotropolone units, since this grouping dominates the properties of the theaflavins. The finding of Berkowitz *et al.* [4], that mixtures of epitheaflavic acid (**1a**) or its gallate (**1b**) with epicatechin or its gallate are rapidly converted into thearubigins in model tea fermentations, supports the general contention that at least some thearubigins contain benzotropolone systems. The retention of the benzotropolone system of epitheaflavic acid with its carboxy group in the thearubigins would explain not only the colour but also the acidity of the latter. Alternative pathways for thearubigin production involve theaflavins or the flavanotropolones (**2a**, **2b**), which could be formed by the reaction of gallic acid with epigallocatechin and its gallate, respectively [18]. Roberts [2] suggested that they corresponded to his substance R and a component of his substance Q, though more recently Sanderson *et al.* [19] have reported that substance R may be prepared by enzymic oxidation of epigallocatechin alone.



Another aspect of the work of Berkowitz *et al.* [4] is that they found epicatechin gallate alone could be converted into thearubigins in model tea fermentations. In addition to the thearubigins, they also detected gallic acid, epicatechin, epitheaflavic acid and its gallate, and some other unidentified products. They accounted for these by assuming that initially the epicatechin gallate is deesterified, the epitheaflavic acid resulting from a combination of the epicatechin and gallic acid so produced and its gallate from the combination of unhydrolysed epicatechin gallate and gallic acid. Both epitheaflavic acid and its gallate are then converted into thearubigins. Coggon *et al.* [20] have more recently shown that degallation does take place during tea fermentation and that it occurs as a sidereaction to the oxidation of flavanols by the catechol oxidase, no esterase *per se* being involved.

If the thearubigins in black tea fermentations are produced by such processes, initial hydrolysis would again be necessary, as insufficient free gallic acid is present in the fresh tea shoots. Thus even though the thearubigins are produced from substrates 65% esterified, very much lower proportions of gallates would be expected amongst the fermentation products. That this is the case was first reported by Brown *et al.* [1,3], who found that all their thearubigin fractions yielded between 2 and 4% gallic acid, compared with a value of about 25%, which would have been obtained if the gallate precursors had been incorporated into the thearubigins in proportion to their occurrence amongst the flavan-3-ols present in fresh tea shoots. T1 and its subfractions yielded 1.5–3.5% gallic acid, so supporting the results of Brown *et al.* [1,3]. However, these workers explained the unexpectedly low values by suggesting "that either the galloyl residues are oxidized to the corresponding hydroxy-*o*-quinones or that there are inter-aryl bonds involving positions 2″ and/or 6″ of the gallate residues". Whilst the latter proposition is a possibility, the former appears very unlikely for the reasons discussed earlier. Neither suggestion is as attractive as degallation.*

Sanderson *et al.* [19,21] have reported that thearubigins are formed by enzymic oxidation of any of the individual flavanols present in fresh tea shoots (with the possible exception of epigallocatechin gallate) or from combinations of them, though, like all previous workers, they were unable to characterise the different thearubigins formed with any precision. Because of their similar solvent solubilities, the ethyl acetate-soluble thearubigins in T1 have moderately consistent properties, particularly with respect to their molecular weights, but nevertheless they constitute a heterogeneous group of compounds, the complexity of which follows from the involvement of up to six different flavan-3-ol precursors (including their gallates) as well as of some flavan-3,4-diols (also present prior to "fermentation"), from permutation of the sequence of units within the oligomer, from differences in linkages between them, and, to a minor degree, from variation in size of the oligomer. As in *Theobroma cacao* [10], molecular shape may play a role in determining the properties of the thearubigin oligomers and may well account for the lower yields obtained in the degradation reactions of T1A. Clearly the isolation of homogeneous material is essential before structural proposals can be made with any precision. Our experience as well as that of Brown *et al.* [3] of attempting to isolate individual thearubigins from black tea suggests that the alternative approach by Sanderson *et al.* [4,21], using *in vitro* enzymic oxidation, is more likely at this stage to lead to products of a purity sufficient for the determination of their total structure.

EXPERIMENTAL

Isolation of ethyl acetate-soluble thearubigins. 80% aqueous EtOH (4.0 l) at room temp. was added to black tea (1.0 kg) and the mixture infused under N_2 for 2 hr with intermittent stirring. The liquor was filtered, the remaining tea leaf washed with 80% aqueous EtOH (2.0 l) and combined filtrates, reduced to 20% aqueous alcohol, extracted with $CHCl_3$, the extract being discarded. The materials were taken into EtOAc (7 × 2 l) and the solid obtained (106 g) after removal of the EtOAc was repeatedly purified by dissolving in Me_2CO and precipitating successively with $CHCl_3$, Et_2O, H_2O, and Et_2O, the final yield being 15 g (1.5%).

Fractionation of the ethyl acetate-soluble thearubigins. T1 (3.0 g) was chromatographed on a large column (60 × 6.7 cm) of Sephadex LH-20, equilibrated with 60% aqueous acetone, and the eluent collected in three portions with elution limits of 1.6–3.4 V_0 (A), 3.4–4.3 V_0 (B), and 4.3–7.0 V_0 (C). These were bulked with the corresponding portions from two similar fractionations, and each was then evaporated to dryness below 35°. Each of the three residues was re-applied to the same column, in the order A, C, and B, and produced three overlapping fractions A–C, from which the subfractions T1A, T1B, and T1C were selected. The cuts of A and C not appropriate

* See note in proof overleaf.

for T1A and T1C, respectively, were added to B prior to its re-chromatography. The cuts of B not appropriate for T1B were discarded. The subfractions T1A, T1B, and T1C, were each evaporated to dryness, dissolved in Me_2CO (100 ml), and precipitated from $CHCl_3$ (1.0 l), prior to collection.

Formation of anthocyanidins Each of the three T1 subfractions (25 mg) was dissolved in 0.5 M propanolic HCl (25 ml) and $A^{1\,cm}$ at 535 nm measured before 2 × 10 ml aliquots were sealed into ampoules and heated at 100° for 20 min. The yield of anthocyanidins was assessed by diluting 5 ml of each hydrolysate to 25 ml with 0.5 M propanolic HCl and re-measuring $A^{1\,cm}$ at 535 nm. The percentage yield of anthocyanidins (as cyanidin) was calculated by comparison of the increase in colour at 535 nm, using $\epsilon = 29270$ at 545 nm for cyanidin [22]. The yields of free anthocyanidins were determined by evaporating the remaining 5 ml of each hydrolysate to dryness, dissolving it in EtOAc (10 ml), and extracting with 0.1 M HCl (2 × 10 ml). The combined aq layers were washed with EtOAc (10 ml), extracted with 2-BuOH (2 × 10 ml), and the bright red upper layers made up to 25 ml prior to measurement of $A^{1\,cm}$ at 550 nm and subsequent calculation of the percentage yield of free cyanidin equivalent. The purified anthocyanidin extracts were concentrated and then examined by PC in HOAc–conc HCl–H_2O (30:3:10) and in BuOH–HOAc–H_2O (4:1:2.2). The identification of cyanidin and delphinidin and their isopropyl ethers was made from R_f values [23] and by comparative chromatography with authentic anthocyanidins.

Hydrolysis with sulphurous acid. Samples (30 mg) of each of the three T1 subfractions were dissolved in 33% aq EtOH (50 ml) and heated for 2 hr at 80°, whilst SO_2 was bubbled through. The residues were dissolved in 20% EtOH, extracted with Et_2O (4 × 25 ml), and the combined ethereal extracts along with the remaining aq layer examined quantitatively by 2-D PC in BuOH–HOAc–H_2O (4:1:2.2), followed by 2% HOAc. The flavan-3-ols, their gallates, and gallic acid were detected by use of $FeCl_3$–$K_3Fe(CN)_6$ dip and identified by R_f [24,25], whilst the presence of the flavan-3-ols and gallic acid was confirmed by co-chromatography with authentic samples.

Determination of gallate ester residues. The three T1 subfractions (100 mg) were dissolved in MeOH (3.8 ml) and conc HCl (1.2 ml) was added. Aliquots (2 × 2.0 ml) of each were heated in ampoules (capacity about 2.3 ml) at 65° for 24 hr and then evaporated to dryness. The residues were dissolved in Me_2CO (0.5 ml) and applied to two Whatman 3 MM papers, previously washed with 0.1 M HCl These were developed two-dimensionally in BuOH–HOAc–H_2O (4:1:2.2), followed by 2% HOAc, and, after location under UV light, the spots corresponding to methyl gallate and gallic acid (weak) cut from the papers and extracted with MeOH (4 × 15 ml) containing one drop of 2 M HCl. The values of $A^{1\,cm}$ at 275 nm for each and for a blank were recorded and the yield of methyl gallate/gallic acid calculated (methyl gallate: λ_{max} 276 nm, ϵ_{max} 10 000) [26].

Molecular weight determination. The T1 subfractions were methylated with an excess of CH_2N_2 at 0° over 5 days in MeOH with increasing quantities of added Et_2O, and the crude products purified on Sephadex LH-20 (80 × 1.50 cm) with $CHCl_3$ as eluent. The material eluted at or around V_0 was collected in fractions which were examined by TLC on microcellulose in 2% HOAc. Fractions which produced only a brown–yellow fluorescing spot at R_f 0.00 with no trailing were pooled and used for subsequent measurement of MWs, by means of vapour phase osmometry with both Me_2CO and benzene (methylated T1B and T1C only) used as solvents and benzil as standard.

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Note added in proof: Although the thearubigins are a heterogeneous group, undoubtedly derived by a variety of pathways, the points discussed on the previous page do integrate into a self-consistent model on which future work can be based: (a) degallation leads to the low gallate content of thearubigins, (b) the gallic acid produced (together with the small amount originally present) leads to the formation in tea fermentation of epitheaflavic acid and its gallate and, indirectly, of flavanotropolones, (c) the participation of these substances (and possibly of theaflavins) in further reactions leads to some of the non-hydrolysable bonds of the thearubigins and the colour of the thearubigins, including in particular the spectrum of T1C, (d) the participation of epitheaflavic acid and its gallate accounts of the acidity of the thearubigins, (e) the limited number of hydrolysable bonds leads to the relatively low yields of anthocyanidins, (f) the predisposition of the originally prepondering gallocatechins to form non-hydrolysable 2′-2′ bonds leaves the *vic*-dihydroxylated compounds to predominate among the C_{15} hydrolysis products of the ethyl acetate-soluble thearubigins, (g) some higher molecular weight compounds containing flavylium groups linked to other residues by non-hydrolysable bonds result on treatment with propanolic HCl, and (h) the low methoxyl content of the methylated T1 subfractions suggests the presence of some non-hydrolysable ether links.