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FLAVANOL DIGALLATES IN GREEN TEA LEAF

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Abstract The flavanol class which is phytochemically characteristic of green tea leaf has been extended to include the novel natural flavanol-3,5-digallates III and IX in addition to the flavan-3-ols I. VI. VII. and XII and the flavanol-3-monogallates II and VIII.

POLYPHENOLS constitute approximately 30% of the dry weight of green tea leaf[1,2] and they are of considerable economic importance because their oxidative enzymic transformation, into the theaflavin[3–6] and thearubigin[7,8] type pigments characteristic of black tea, is an important part of the fermentation step in black tea manufacture.[1,2,9] The dry weight composition of the polyphenol fraction of green leaf shows considerable local variation, but typical figures[2,10] are as follows: (−)-epigallocatechin gallate (VIII; 9–13%), (−)-epicatechin gallate (II; 3–6%), (−)-epigallocatechin (VII; 3–6%), (−)-epicatechin (I; 1–3%). Other flavanol derivatives present (1–2%) in green leaf include (+)-catechin (VI) and (+)-gallocatechin (XII). Recently the reported isolation[11] of (−)-gallocatechin gallate (XIII) from green tea leaf has been further examined,[12] but it is now suggested[12] that (−)-gallocatechin gallate (XIII) is probably an artefact formed by thermal isomerization of (−)-epigallocatechin gallate (VIII).

Our interest[4,6,13,14] in the structural correlation between the benzotropolone derivatives isolable from black tea and possible polyphenolic precursors present in the green leaf encouraged a detailed examination of green leaf polyphenols. This has resulted in the recognition of flavanol-3,5-digallates as a new class exemplified by the isolation and structural characterization of (−)-epicatechin-3,5-digallate (III) and (−)-epigallocatechin-3,5-digallate (IX). A compound called "Substance H" was previously isolated by Vuataz, Brandenberger, and Egli[10] during a detailed chromatographic fractionation of green tea leaf polyphenols. A constitution for "Substance H" was not proposed,[10] but we now believe that "Substance H" is identical with (−)-epigallocatechin-3,5-digallate (IX).

The flavan-3-ols are unique members of the flavanoid class of natural products in that they sometimes occur as 3-monogallates.[15,16] The recognition of flavanol-3,5-digallates as natural products is an interesting extension of the phytochemistry of flavan-3-ols.[17]

Initially we had some difficulty in successfully repeating the procedure described by Vuataz, Brandenberger, and Egli[10] for the separation of the polyphenol fraction isolated from green tea leaf. However, by slight modification of the original method

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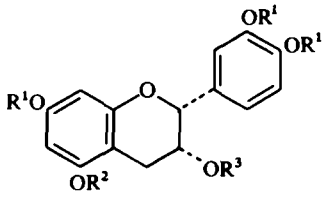
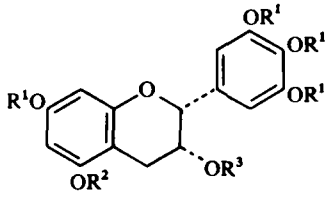
(see Experimental) fractionation was successfully achieved by column chromatography on cellulose powder yielding (−)-epicatechin gallate (II), (−)-epigallocatechin gallate (VIII), and a compound previously described as "Substance H". However, this method for the separation of the flavan-3-ol gallate fraction from green tea leaf was rather tedious and a better method was developed. This involved first a countercurrent distribution[18] of the polyphenol fraction followed by column chromatography using Sephadex LH 20. This yielded (−)-epicatechin gallate (II), (−)-epigallocatechin gallate (VIII), and two new compounds which were subsequently identified as (−)-epicatechin-3,5-digallate (III) and (−)-epigallocatechin-3,5-digallate (IX). "Substance H" and (−)-epigallocatechin-3,5-digallate (IX) were shown to be identical and as this digallate was available in relatively larger amounts its structural examination was carried out first.



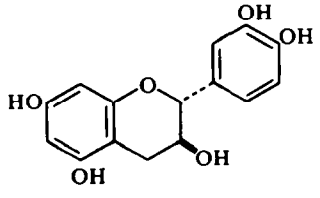
I (−)-Epicatechin
II (−)-Epicatechin-3-gallate
III (−)-Epicatechin-3,5-digallate
IV
V



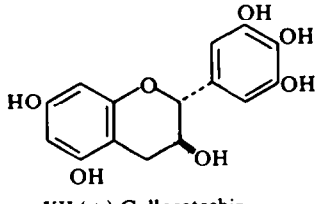
VII (−)-Epigallocatechin
VIII (−)-Epigallocatechin-3-gallate
IX (−)-Epigallocatechin-3,5-digallate
X
XI

	R^1 =	R^2 =	R^3 =
I and VII	H	H	H
II and VIII	H	H	THB
III and IX	H	THB	THB
IV and X	Me	Me	TMB
V and XI	Me	TMB	TMB

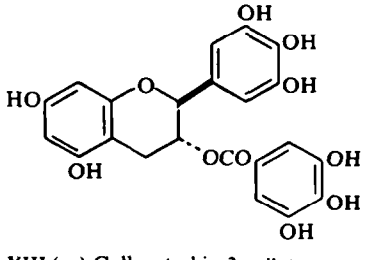
In the above formulae THB = 3,4,5-trihydroxybenzoyl (galloyl) and TMB = 3,4,5-trimethoxybenzoyl.



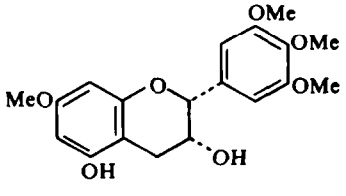
VI (+)-Catechin



XII (+)-Gallocatechin



XIII (−)-Gallocatechin-3-gallate



XIV

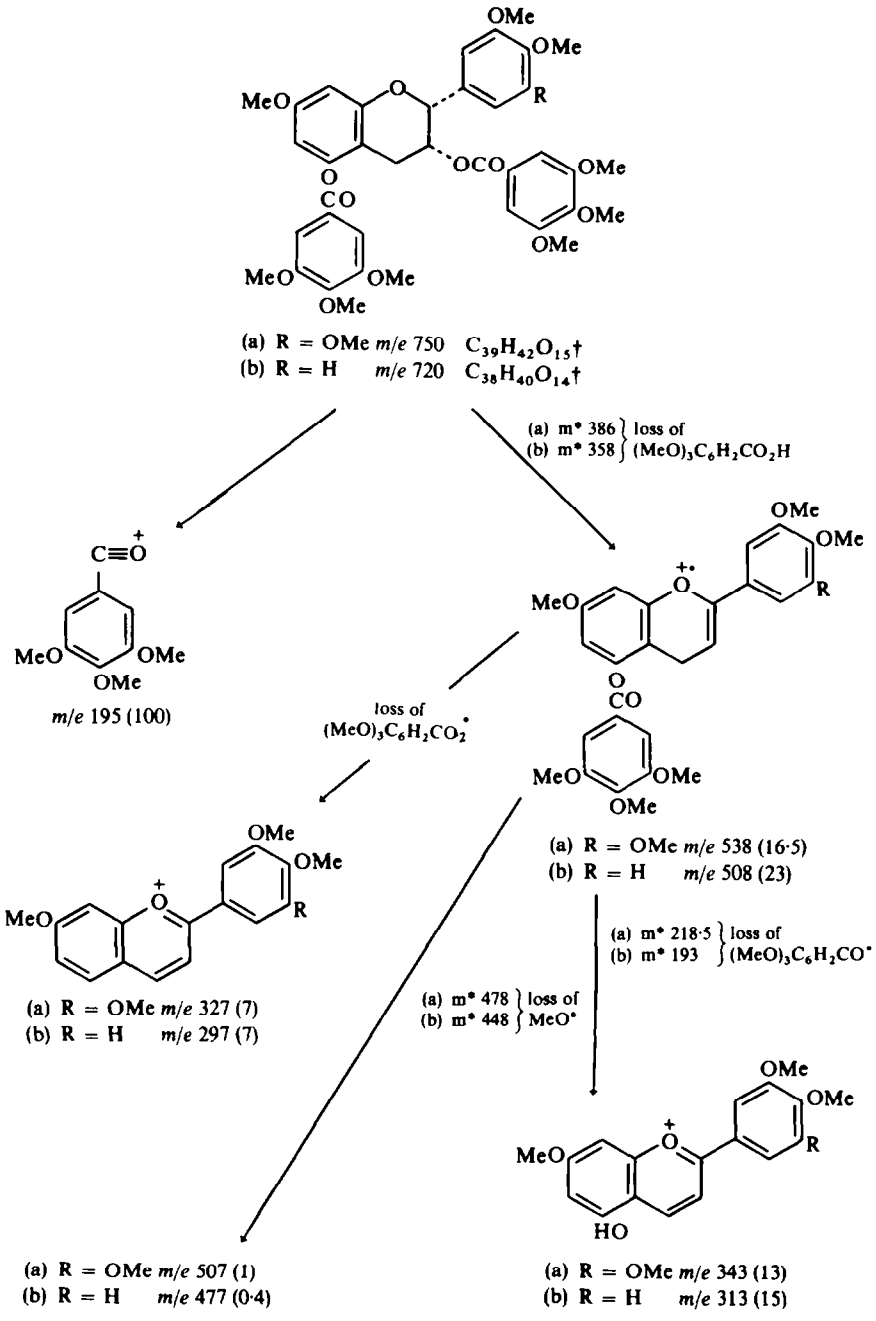


FIG. 1. Mass spectra of the decamethyl ether (XI, see a) and the nonamethyl ether (V, see b)

Figures in parentheses are relative intensities
† Confirmed by high resolution mass measurement
m* denotes that a metastable transition was observed

TABLE 1. UV SPECTRA λ_{max} nm (ε_{max}) IN ETHANOL

(−)-Epicatechin (I)[10]	280	3,580
(−)-Epicatechin-3-gallate (II)[10,12]	279	14,000
(−)-Epicatechin-3.5-digallate (III)	282	23,800
(−)-Epigallocatechin (VII)[10]	271	1,450
(−)-Epigallocatechin-3-gallate (VIII)[10,12]	275	11,500
(−)-Epigallocatechin-3.5-digallate (IX)	283	26,600

(−)-Epigallocatechin digallate (IX) was obtained as an amorphous powder, judged to be homogeneous on the basis of its behaviour on two-dimensional paper chromatography. Its optical activity ($[\alpha]_D = -13°$) and spectroscopic properties suggested that it could be a derivative of the flavanol gallate type; the extinction coefficient (ε_{max} 26,600) indicated that it might be a digalloyl derivative (Table 1). Methylation yielded a decamethyl ether [$C_{29}H_{12}O_5(OMe)_{10}$ established by high resolution mass spectrometry] whose mass spectral fragmentation pattern (Fig. 1) demonstrated the presence of two trimethoxybenzoyl groups. These results were compatible with the partial structure $C_{15}H_8O(OMe)_4[OCOC_6H_2(OMe)_3]_2$ for the decamethyl ether, and its NMR spectrum (Table 2) clearly indicated that it was a digalloyl ester derived from (−)-epigallocatechin (VII); this proposal was confirmed by its tannase[19] hydrolysis which gave (−)-epigallocatechin-3-gallate (VIII) as an intermediate and (−)-epigallocatechin (VII) and gallic acid as final products. Comparison of its NMR spectrum with that of (−)-epigallocatechin-3-gallate (VIII) suggested that one galloyl residue should be associated with position 3 and the chemical shifts of the two *meta*-related protons (6 and 8) on ring A indicated that the second galloyl group was associated with either position 5 or position 7. Of these two possibilities for the location of the second galloyl group, position 5 was favoured because the signals (Table 2) associated with the quasi-equatorial proton (4) and the quasi-axial proton (4*) were clearly resolved and were amenable to first order analysis. This contrasts with the observation that the protons in positions 4 and 4* of 5-hydroxy- and 5-methoxy-derivatives of 2, 3- *cis*-flavan-3-ols usually exhibit approximate apparent chemical shift equivalence.[13,20] It was therefore proposed that the observable magnetic non-equivalence of the protons 4 and 4* in the natural digalloyl derivative of (-)-epigallocatechin (VII) requires the second galloyl group to be associated with a 5-galloyloxy-substituent. Furthermore, the generation of the flavylium cations (*m/e* 343 and 327) in the mass spectrum (Fig. 1) of the decamethyl ether (XI) was most easily interpreted in terms of loss of $(MeO)_3C_6H_2CO$ or $(MeO)_3C_6H_2CO_2$ from position 5 in association with intramolecular transfer of hydrogen from position 4. This interpretation also placed the second galloyloxy group in position 5. The proposed structure as (-)-epigallocatechin-3, 5-digallate (IX) for "Substance H" was confirmed by reaction of its decamethyl ether (XI) with aqueous methanolic sodium hydroxide. This yielded methyl 3,4,5-trimethoxybenzoate[21] and 7,3',4',5'-tetra-*O*-methyl-(−)-epigallocatechin (XIV) whose constitution (XIV) was clearly indicated by its mass spectral fragmentation pattern (Fig. 2) and by comparison (Table 2) of the chemical shifts of the ring-A protons (6 and 8) of flavan-3-ols and their derivatives.

The configurational formula (III) was similarly established for (−)-epicatechin-3,5-digallate. It was optically active ($[\alpha]_D = -9°$) and its UV spectrum (Table 1)

TABLE 2. 100 MHz NMR SPECTRAL DATA

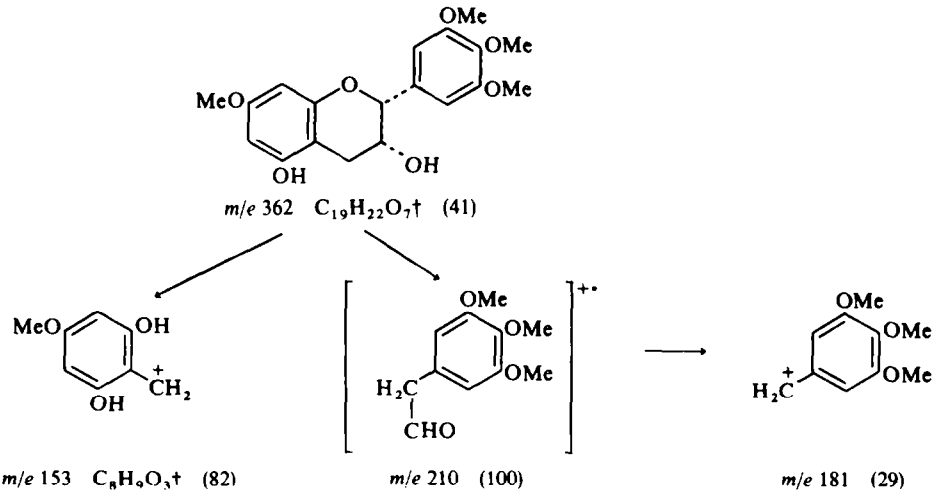
Compound	2	3	4,4*[c]	6	8	2′	5′	6′	2″, 6″	2‴, 6‴
(−)-Epigallocatechin-3,5-digallate (IX)[a]	4·88 s	4·46 m	6·92 7·20 dd *J* 16,4 dd *J* 16,2	3·57 d *J* 2·5	3·64 d *J* 2·5	3·37 s	—	3·37 s	2·99 s	2·76 s
Deca-*O*-methyl-(−)-epigallocatechin-3,5-digallate (XI)[b]	4·84 s	4·36 m	6·96 "dd"	3·42 d *J* 2·5	3·55 d *J* 2·5	3·32 s	—	3·32 s	2·84 s	2·58 s
(−)-Epigallocatechin-3-gallate (VIII)[a]	4·95 s	4·45 m	7·04 "t"	3·97 s	3·97 s	3·40 s		3·40 s	3·00 s	—
Octa-*O*-methyl-(−)-epigallocatechin-3-gallate (X)[b]	4·92 s	4·34 m	6·96 "d"	3·90 d *J* 2·5	3·76 d *J* 2·5	3·30 s	—	3·30 s	2·83 s	—
(−)-Epicatechin-3,5-digallate (III)[a]	4·81 s	4·51 m	6·89 7·17 dd *J* 16.4 dd *J* 16.2	3·56 d *J* 2·5	3·65 d *J* 2·5	2·90 d *J* 2	3·23 d *J* 8	3·10 dd *J* 8,2	2·98 s	2·77 s
Nona-*O*-methyl-(−)-epicatechin-3,5-digallate (V)[b]	4·81 s	4·39 m	6·97 "dd"	3·43 d *J* 2·5	3·56 d *J* 2·5	3·01 m	3·20 d *J* 8	3·01 m	2·85 s	2·59 s
(−)-Epicatechin-3-gallate (II)[a]	4·86 s	4·44 m	7·00 "t"	3·95 s	3·95 s	2·93 d *J* 2	3·25 d *J* 8	3·11 dd *J* 8,2	2·98 s	—
Hepta-*O*-methyl-(−)-epicatechin-3-gallate (IV)[b]	4·88 s	4·36 m	6·96 "d"	3·89 d *J* 2·5	3·75 d *J* 2·5	2·97 m	3·19 d *J* 8·5	2·97 m	2·83 s	—
7,3′,4′,5′-Tetra-*O*-methyl (−)-epigallocatechin (XIX)[b]	5·06 s	5·69 m	7·07 "d"	4·01 d *J* 2·5	3·84 d *J* 2·5	3·26 s	..	3·26 s	—	—

Chemical shifts are given on the τ scale. Multiplicities and coupling constants (*J* Hz) have been derived by first order analysis: s = singlet (broadened in some cases by additional coupling); d = doublet; dd = double doublet; m = multiplet (the centre of multiplets is quoted). The symbols "d", "t", and "dd" are used to refer to "doublets", "triplets", and "double doublets" associated with deceptively simple spectra. Deuteriation established the presence of signals assignable to hydroxyl groups; these are not included in the Table.

[a] Solvent CD_3COCD_3

[b] Solvent $CDCl_3$

[c] 4 and 4* refer to quasi-equatorial and quasi-axial protons respectively.[20]



For footnotes, refer to Fig. 1

FIG. 2. Mass spectrum of 7.3',4',5'-tetra-*O*-methyl-(−)-epigallocatechin (XIV)

suggested that it was a digalloyl derivative. Tannase hydrolysis gave (−)-epicatechin (I) and gallic acid as final products with (−)-epicatechin-3-gallate (II) as an observed intermediate. The NMR spectra (Table 2) of (−)-epicatechin-3,5-digallate (III) and its nonamethyl ether (V) and the mass spectrum (Fig. 1) of the nonamethyl ether (V) show a satisfying correlation with the corresponding spectra of (−)-epigallocatechin-3,5-digallate (IX) and its decamethyl ether (XI).

EXPERIMENTAL

NMR spectra were determined using a Varian HA-100 spectrometer with TMS as internal standard. Two-dimensional paper chromatography was carried out using Whatman No. 2 paper which was developed with solvent 1 (2% AcOH) and then with solvent 2 (*n*-BuOH–AcOH—H_2O; 4:1:5, upper phase). Column eluents were monitored for UV or visible absorption by passage through a Vitatron photometer unit connected to a chart-recorder.[8] Fractions were collected using an LKB 7000 automatic collector. Light petroleum refers to the fraction b.p. 60–80°. All evaporations were carried out under reduced pressure using a rotary film evaporator. Column fractions containing polyphenols in solution in organic solvents were evaporated to a small volume and after the addition of water were freed from organic solvents by further evaporation. The aqueous solutions thus obtained were then freeze-dried.

Separation of "Substance H" from an ethyl acetate extract of green tea. Cellulose powder (Whatman CF11, 200 g) was suspended in an excess of ethyl propionate–light petroleum (9:1) and distilled water (100 ml) was gradually added with shaking. A chromatographic column (75 × 3 cm) was packed with the cellulose slurry using a close-fitting Teflon plunger on a stainless steel rod and the column then eluted with ethyl propionate–light petroleum (9:1) until the absorbance of the eluent at 280 nm was below 0·05. A sample (5 g) of the EtOAc soluble polyphenols of green tea leaf, prepared according to Vuataz, Brandenberger, and Egli[10] and purified using moist diethyl ether as described by Gregory and Bendall,[22] was dissolved in ethyl propionate–light petroleum (9:1) (20 ml) and applied to the column which was then eluted with water saturated ethyl propionate–light petroleum (9:1) at a flow rate of 0·7 ml/min. The eluent fractions (4 ml each) were monitored by TLC using silica gel plates (Merck) developed in the solvent system HCOOH–EtOAc–$CHCl_3$ (3.8.10). Visualization was effected by spraying with a methanolic solution of Gibbs reagent (Merck, 2,6-dichloroquinone-*N*-chloroimide) which slowly revealed the flavanols as dark spots. "Substance H" eluted with (−)-epicatechin gallate between fractions containing (−)-epicatechin gallate and (−)-epigallocatechin gallate. The fractions containing "Substance H" were combined and freeze-dried giving a light brown, amorphous powder (88 mg). This crude material was purified by further chromatography using Sephadex LH20 (20 g) equilibrated in $CHCl_3$–MeOH (1:1) in a column (30 × 1·1 cm). The sample (85 mg) was dissolved in $CHCl_3$–MeOH (1:1) and applied to the column which was

eluted with the same solvent. Eluent fractions (2·5 ml each) were monitored by TLC. Fractions containing "Substance H", which eluted after (−)-epicatechin gallate and an orange-yellow band, were combined and freeze dried giving "Substance H" (20 mg) as a chromatographically homogeneous, white, amorphous powder. "Substance H" was found to be identical with (−)-epigallocatechin-3,5-digallate isolated from green tea as described below.

Isolation of flavanol digallates from green leaf extract. A sample (40 g) of EtOAc soluble polyphenols prepared according to Vuataz, Brandenberger, and Egli[10] was distributed between EtOAc and H_2O in a countercurrent distribution apparatus. After 72 upper phase transfers the contents of alternate tubes were monitored by paper chromatography and appropriate fractions were united to give combined fractions A–G. Fraction A (7 g) was found to contain (−)-epicatechin-3-gallate, (−)-epigallocatechin-3-gallate, (−)-epigallocatechin-3,5-digallate, and (−)-epicatechin-3,5-digallate.

A sample of the fraction A polyphenols (2·0 g) was applied to a column (100 × 2·5 cm) of Sephadex LH20 equilibrated in $CHCl_3$–MeOH (1 : 1) and was eluted with the same solvent. The fraction A polyphenol mixture contained traces of theaflavin and theaflavin gallates[5,6] which proved to be very useful for locating the other polyphenols since, under the conditions used with this chromatographic system, it was found that (i) theaflavin co-chromatographed with (−)-epicatechin-3-gallate, (ii) theaflavin monogallates co-chromatographed with (−)-epigallocatechin-3-gallate and (−)-epicatechin-3,5-digallate, and (iii) theaflavin digallate co-chromatographed with (−)-epigallocatechin-3,5-digallate. Thus, by continuously monitoring the visible absorption of the column eluent at 457 nm, an elution profile was obtained which enabled the collector fractions to be pooled into three main fractions. A_1, A_2, and A_3. Fraction A_1 (768 mg) contained crude (−)-epicatechin-3-gallate and was not further examined. Fraction A_2 (664 mg) contained mainly (−)-epigallocatechin-3-gallate together with theaflavin monogallates and (−)-epicatechin-3,5-digallate which were separated as described below. Fraction A_3 (163 mg) contained mainly (−)-epigallocatechin-3,5-digallate which was purified by further chromatography.

Isolation of (−)-epigallocatechin-3,5-digallate (IX). Fraction A_3 (163 mg) was applied to a column (38 × 2·3 cm) of Sephadex LH 20 equilibrated in $CHCl_3$–MeOH–light petroleum (1 : 2 : 1) and was eluted with the same solvent mixture. The UV absorption of the column eluent at 280 nm was continuously monitored and gave an elution profile showing three peaks. (−)-Epigallocatechin-3-gallate eluted first, followed by (−)-epigallocatechin-3,5-digallate, followed by theaflavin digallate. Fractions containing (−)-epigallocatechin-3,5-digallate were combined and freeze dried to give chromatographically homogeneous (−)-*epigallocatechin-3,5-digallate* (112 mg) as an off-white, amorphous powder. λ_{max}nm (ε) 283 (26,600), 207 (104,500): ν_{max} 3350, 1700, 1625 cm^{-1}; $[\alpha]_D^{23}$ −13°; R_f(solvent 1) 0·10; R_f(solvent 2) 0·61.

Isolation of (−)-epicatechin-3,5-digallate (III). Fraction A_2 (500 mg) was applied to a column (58 × 1·5 cm) of Sephadex LH20 equilibrated in $CHCl_3$–MeOH–light petroleum (1 : 2 : 1) and eluted with the same solvent mixture monitoring the UV absorption of the column eluent at 280 nm. The elution profile showed four peaks corresponding to (−)-epicatechin-3-gallate (very minor peak), (−)-epigallocatechin-3-gallate (major peak), (−)-epicatechin-3,5-digallate (minor peak), and theaflavin monogallates (minor peak). Fractions containing (−)-epicatechin-3,5-digallate were combined and freeze dried giving (−)-epicatechin digallate (37 mg) containing a trace of theaflavin monogallates as impurity. This material (66 mg) was further purified by one-dimensional prep. paper chromatography on Whatman 3MM papers in the solvent system *n*-BuOH–AcOH–H_2O (4 : 1 : 5, upper phase). The developed papers were examined under UV light and the dark purple bands corresponding to (−)-epicatechin-3,5-digallate were cut out and eluted by downward flow chromatography with propan-2-ol–AcOH–H_2O (4 : 1 : 4). The eluate was diluted with water and extracted with EtOAc. Material from the EtOAc extract was then freeze dried giving chromatographically homogeneous (−)-epicatechin-3,5-digallate (42 mg) as an off-white, amorphous powder. λ_{max} nm (ε) 282 (23,800), 204 (93,100); ν_{max} 3350, 1700, 1625 cm^{-1}; $[\alpha]_D$ −9°; R_f (solvent 1) 0·12; R_f(solvent 2) 0·75.

Tannase hydrolysis of (−)-epigallocatechin-3,5-digallate (IX). (−)-Epigallocatechin-3,5-digallate (60 mg) was dissolved in 0·1 *M*-NaOAc–AcOH buffer solution (pH 6·0; 40 ml) and treated with tannase[19] solution at 37°. Examination by two-dimensional paper chromatography[23] of the mixture after 15 min showed the presence of (−)-epigallocatechin-3,5-digallate (IX), (−)-epigallocatechin-3-gallate (VIII), and gallic acid; a second chromatogram after 5 hr showed the presence of only (−)-epigallocatechin (VII) and gallic acid. After 5 hr the solution was extracted with EtOAc and the residue (44 mg) was chromatographed on a Sephadex LH20 column. Elution with $CHCl_3$–MeOH–light petroleum (1 : 2 : 1) and monitoring at 280 nm of the eluate gave two fractions yielding gallic acid and (−)-epigallocatechin (VII) (16 mg), $[\alpha]_D$ −48° (EtOH) (lit. $[\alpha]_D$ −59·5°,[10] −56·3°[24]).

Tannase hydrolysis of (−)-epicatechin-3,5-digallate (III). (−)-Epicatechin-3,5-digallate (32 mg) similarly yielded (−)-epicatechin (10 mg), $[\alpha]_D$ = −55° (EtOH) (lit. $[\alpha]_D$ −65°[10]).

Methylation of (−)-epigallocatechin-3,5-digallate. (−)-Epigallocatechin-3,5-digallate (70 mg) in anhyd.

acetone (8 ml) was boiled under reflux (8 hr) with dimethyl sulphate (0·2 ml) and anhyd. K_2CO_3 (0·5 g). The mixture was then filtered, evaporated, fractionated by prep. TLC on silica plates, and eluted with $CHCl_3$ giving a gummy residue (59 mg) which separated from EtOH aq at $-10°$ giving (−)-*epigallocatechin*-3,5-*digallate decamethyl ether* (XI; 43 mg) as a buff-coloured, amorphous solid. (Found: M, 750·253. $C_{39}H_{42}O_{15}$ requires: M, 750·252); ν_{max} 1725, 1630, 1590 cm^{-1}; mass spectrum (Fig. 1).

Methylation of (−)-*epicatechin*-3,5-*digallate* (III). (−)-Epicatechin-3,5-digallate (III; 70 mg) was similarly methylated and after chromatography on silica plates yielded (−)-*epicatechin*-3,5-*digallate nonamethyl ether* (V; 56 mg) as a colourless gum. (Found: M, 720·241. $C_{38}H_{40}O_{14}$ requires: M, 720·242); ν_{max} 1725, 1630, 1590 cm^{-1}; mass spectrum (Fig. 1).

Alkaline hydrolysis of (−)-*epigallocatechin*-3,5-*digallate decamethyl ether* (XI). (−)-Epigallocatechin-3,5-digallate decamethyl ether (XI; 48 mg) was dissolved in MeOH (10 ml) and N_2 was bubbled through the solution. 2N-NaOH (1·0 ml) was added and the solution was kept (5 hr) at room temp. The solution was diluted with water and extracted with $CHCl_3$. The $CHCl_3$ extract was divided into neutral and acidic fractions by treatment with $NaHCO_3$ aq. The acidic fraction (4 mg) was identified as 3.4.5-trimethoxybenzoic acid. m.p. 167°.[25] The neutral fraction (37 mg) was fractionated by TLC on silica ($CHCl_3$–MeOH, 99:1). The high R_f component (10 mg), m.p. 82–83° (*n*-hexane) was identified as methyl 3,4,5-trimethoxybenzoate (lit.[21] m.p. 85°). The low R_f component (16 mg) was recrystallized from EtOH aq (95%) yielding 7.3′.4′.5′-*tetra*-O-*methyl*-(−)-*epigallocatechin* (XIV) as needles, m.p. 174°. (Found: M, 362·1355. $C_{19}H_{22}O_7$ requires: M, 362·1365); ν_{max} 3550, 3250, 1625, 1590; mass spectrum (Fig 2).

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