Tannins and Related Compounds. CXIV.[1] Structures of Novel Fermentation Products, Theogallinin, Theaflavonin and Desgalloyl Theaflavonin from Black Tea, and Changes of Tea Leaf Polyphenols during Fermentation

Fumio HASHIMOTO, Gen-ichiro NONAKA* and Itsuo NISHIOKA

Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan. *Received November 18, 1991*

Continuing chemical examination of black tea has led to the isolation of three novel fermentation products, theogallinin (1), theaflavonin (10) and desgalloyl theaflavonin (11). The structure of 1 was established on the basis of physico-chemical evidence to be a condensation product linked through pyrogallol–pyrogallol rings in theogallin (2) and (−)-epigallocatechin 3-*O*-gallate (3), while 10 and 11 were characterized as B,B′-linked bisflavonoids formed by an oxidative coupling of isomyricitrin (12) and tea catechins [3 and (−)-epigallocatechin (4)]. Furthermore, high performance liquid chromatography analyses of the changes of tea polyphenols during fermentation have revealed that original tea catechins are more rapidly transformed by endogenous phenol oxidase to theasinensins (*e.g.* 6, 8) and oolongtheanin (13) than the formerly known black tea pigments, theafiavins.

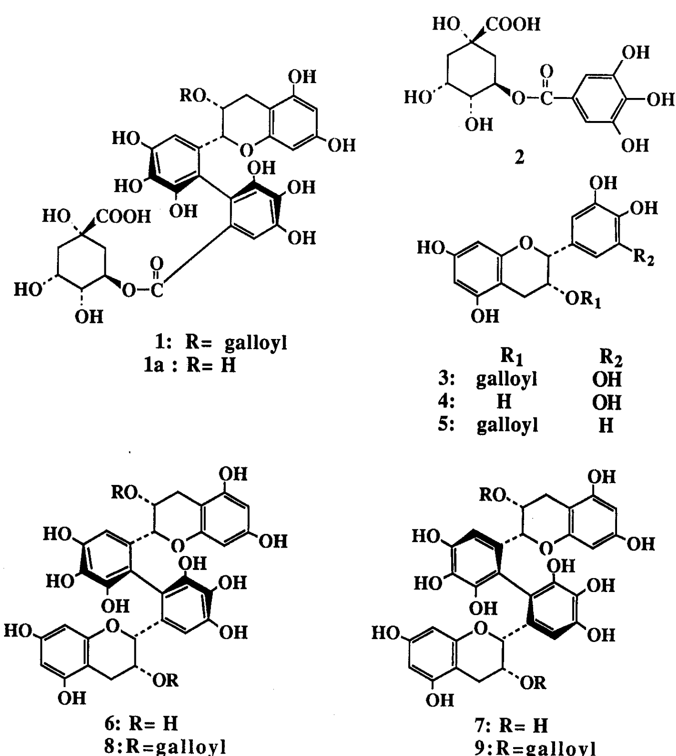
Keywords black tea; tea polyphenol; fermentation; theogallinin; theaflavonin; desgalloyl theaflavonin; flavan-3-ol; theogallin; isomyricitrin; oxidative coupling

To clarify the mechanism of oxidation of tea leaf polyphenols in the fermentation process, we have been undertaking chemical examinations of the constituents of green tea,[2] oolong tea[3] (semi-fermented) and black tea[4] (fully fermented), as well as their fresh original plant materials.[5] So far, by comparing the patterns and the contents of each component polyphenol, we have found that the oxidation occurs predominantly not only through the condensation of catechol and pyrogallol rings in two flavan-3-ol molecules or in a flavan-3-ol and gallic acid, leading to the formerly known red pigments, theaflavins and theaflavic acids, but also through an intermolecular coupling of two pyrogallol rings to form theasinensins, oolongtheanin and theaflagallins. In continuing our chemical studies on tea polyphenols, we have isolated three new fermentation products named theogallinin (**1**), theaflavonin (**10**) and desgalloyl theaflavonin (**11**) from black tea. Furthermore, high-performance liquid chromatographic (HPLC) examination of the changes of the polyphenols during fermentation has revealed that the original tea polyphenols are rapidly converted to theasinensins and oolongtheanin by the action of endogenous polyphenol oxidase. This paper presents full details of these studies.

Results and Discussion

Structure of Theogallinin (1) Theogallinin (**1**) was isolated as a pale brown amorphous powder from the aqueous acetone extract of a commercial blend of Indian and Ceylonese black teas by a combination of Sephadex LH-20, MCI-gel CHP 20P and Bondapak Porasil B chromatographies. The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** exhibited flavan C-ring signals at δ 2.54 (dd, J=18, 4 Hz, H-4), 2.90 (br d, J=18 Hz, H-4), 4.59 (br s, H-2) and 5.38 (m, H-3), of which the small coupling constant of the H-2 signal and the lowfield shift of the H-3 signal suggested the presence of a 3-*O*-acylated 2,3-*cis* flavan-3-ol framework. In addition, the spectrum showed signals due to two methylenes (δ 1.55—2.44, 4H, m) and three oxygen-bearing methines (δ 3.10, dd, J=10, 3 Hz; δ 4.07, m; δ 5.19, m), the chemical shifts and the coupling patterns being closely correlated with those of theogallin (**2**).[6] In the aromatic region, the observation of a two-proton singlet at δ 7.13 and two one-proton singlets at δ 7.03 and 7.21 suggested the presence of a gallic acid ester group and two di-substituted pyrogallol rings, respectively. The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** indicated the presence of thirty-six carbon atoms, corresponding to the total carbon number of (−)-epigallocatechin 3-*O*-gallate (**3**) plus **2**. Furthermore, the signal pattern in **1** was quite similar to that of the combined spectra of **3** and **2**, but differed slightly in the chemical shifts of the flavan C-2′/C-6′ and the galloyl C-2/C-6 signals appearing unequivalently.[2] These findings suggested that **1** consists of (−)-epigallocatechin 3-*O*-gallate and theogallin moieties, each being connected through a carbon to carbon bond at the B-ring and the galloyl group.

On enzymatic hydrolysis with tannase, **1** yielded a



© 1992 Pharmaceutical Society of Japan

NII-Electronic Library Service

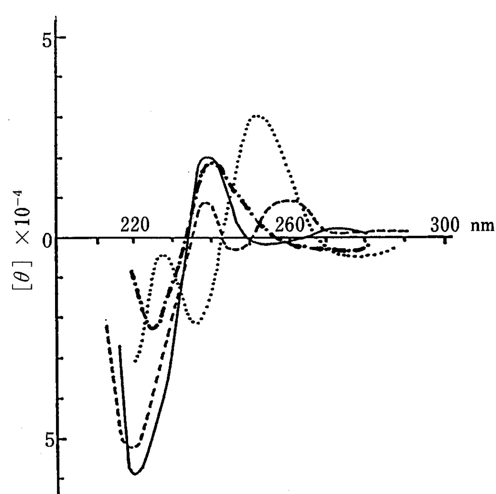
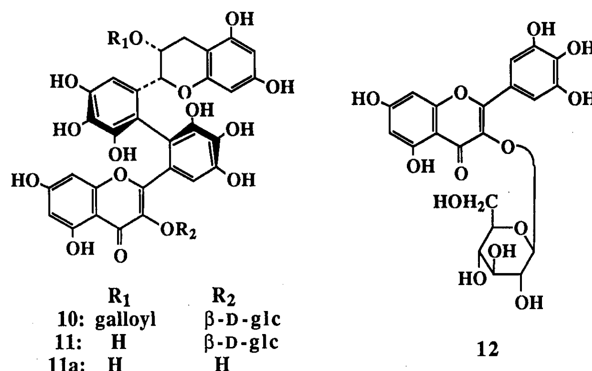


Fig. 1. CD Spectra (in MeOH) of **1a**, **6**, **7** and **11a**
---, **1a**; —·—, **11a**; ——, **6**; ------, 7.



hydrolysate (**1a**) and gallic acid. The ^{1}H-NMR spectrum of **1a** showed the upfield shift (δ 4.06) of the flavan H-3 signal, and the signal patterns were similar to those of (−)-epigallocatechin (**4**) plus **2**, except for the above-mentioned B-ring and galloyl signals.

To confirm the structure of **1**, an attempt was made to prepare the desgalloyl derivative (**1a**). When a mixture of **2** and **4** was treated with potassium ferricyanide in a weakly alkaline medium, **1a** was formed in a small quantity, together with theasinensin C (**6**).[2] This fact clearly indicated that the asymmetric centers in the flavan and the quinic acid moieties possess the same absolute configurations as those of **6** and **2**.

The atropisomerism of the biphenyl bond was determined as follows. Comparison of the circular dichroism (CD) spectrum of **1a** with those of theasinensins C (**6**) and E (**7**) having *R*- and *S*-configurations, respectively, showed that the signs of the Cotton effects in **1a** were in good accord with those of **6** rather than **7** (Fig. 1). Thus, the chirality of the biphenyl bond was concluded to be in the *R*-series.

Based on these physico-chemical findings, the structure of theogallinin was determined to be as represented by the formula **1**. Theogallinin is regarded as a fermentation product formed by oxidative coupling of theogallin (**2**) and (−)-epigallocatechin 3-*O*-gallate (**3**). The absence of a diastereoisomer having the *S*-biphenyl configuration indicates that the coupling reaction proceeds stereospecifically.

Structures of Theaflavonin (10) and Desgalloyl Theaflavonin (11) Theaflavonin (**10**) and desgalloyl theaflavonin (**11**) were obtained as yellowish brown amorphous powders from the relatively earlier fraction of the first Sephadex LH-20 chromatography. The ultraviolet (UV) spectra of **10** and **11** were closely correlated, showing absorption maxima similar to those of flavonoids (see Experimental). In contrast, the fact that **10** and **11** were positive (orange coloration) to the anisaldehyde–sulfuric acid reagent[7] indicated the presence of a flavan-3-ol moiety in the molecule. The ^{1}H-NMR spectrum of **10** exhibited flavan C-ring signals at δ 2.66 (dd, J=18, 4 Hz, H-4), 2.94 (br d, J=18 Hz, H-4), 4.96 (br s, H-2) and 5.54 (m, H-3), whose chemical shifts and coupling patterns were similar to those of **3**. The *meta*-coupled flavan A-ring signals and a two-proton galloyl singlet appeared at δ 6.01 and 6.10 (each d, J=2 Hz), and 7.08 (s), respectively. Furthermore, the observation of additional *meta*-coupled doublets at δ 6.24 and 6.55 (J=2 Hz) and a sugar anomeric signal at δ 4.79 (d, J=6 Hz) suggested the presence of a flavonol glycoside moiety. The ^{13}C-NMR spectrum of **10** displayed forty-three carbon signals, among which seven (six aromatics and one carboxyl) and six aliphatics were attributable to the galloyl ester group and the sugar carbons, respectively. The remaining thirty carbons correspond to the flavonol and flavan-3-ol nuclei. The chemical shift (δ 179.1) of the carbonyl signal further supported the presence of the flavonol skeleton.[8] In the ^{1}H-NMR spectrum, the B-ring signals of the flavonol and flavan-3-ol moieties appeared as one-proton singlets at δ 7.08 and 6.92, suggesting that these units possess pyrogallol rings and are linked at the respective C-2′ positions through a carbon to carbon bond.[2]

The ^{1}H- and ^{13}C-NMR spectra of **11** closely resembled those of **10**, except for the absence of the galloyl group and the upfield shift of the flavan H-3 and C-3 signals (δ *ca.* 4.2 and 65.2). The desgalloyl structure of **11** was confirmed unequivocally by tannase hydrolysis of **10**, which afforded **11** and gallic acid.

On enzymatic hydrolysis with crude hesperidinase, **11** furnished a hydrolysate (**11a**) and glucose. The ^{13}C-NMR spectrum of **11a** showed the upfield shift of the flavonol C-3 signal by 0.7 ppm as compared with that of **11**, and the neighboring C-2 and C-4 atoms resonated in fairly upfield regions (−10.9 and −2.3 ppm, respectively). On the basis of these findings, the location of the glucose moiety was concluded to be at the flavonol C-3 position, and the flavonol unit was therefore considered to be isomyricitrin (myricetin 3-*O*-β-D-glucoside) (**12**).[9] The presence of the myricetin moiety was further confirmed by electron-impact mass spectrometry (EI-MS) of the undecamethyl ether of **11a**, which exhibited, together with the molecular ion at m/z 776, a fragment peak at m/z 401 arising from the hexamethyl myricitrin residue.

As for the chirality of the biphenyl bond, comparison of the CD spectrum of **11a** with those of theasinensins C (**6**) and E (**7**) (Fig. 1) indicated it to be in the *R*-series. Accordingly, the structures of theaflavonin and desgalloyl theaflavonin were concluded to be as shown by the formulae **10** and **11**, respectively. Taking into account the co-existence of isomyricitrin (**12**), (−)-epigallocatechin (**4**) and its 3-*O*-gallate (**3**) in fresh tea leaves, **10** an **11** are also considered to be formed by oxidative coupling during the

NII-Electronic Library Service

fermentation steps. It should be noted that to the best of our knowledge, **10** and **11** are the first bisflavonoids linked through a carbon–carbon bond at the B-rings of flavonol and flavan-3-ol nuclei.

Changes of Polyphenols during Fermentation The fresh leaves of *Camellia* (*C.*) *sinensis* var. *assamica*, which is the original plant material of black tea, show rapid browning immediately after harvest. This phenomenon has been considered to be due to the much higher activities of endogeneous polyphenol oxidase than those of other original tea materials. As for the enzymatic oxidation reaction, detailed studies have been made on the black tea pigments, theaflavins,[10] but only a few reports have appeared on the formation of theasinensins and other products.[11] Therefore, to clarify the mechanism of the transformation of tea polyphenols, we have attempted to examine the changes of the overall patterns of polyphenols in the fermentation processes.

The fresh leaves of *C. sinensis* var. *assamica* were allowed to undergo spontaneous fermentation on standing in air. At intervals of 0.5—4 h, the reaction was stopped by heating, and extraction with aqueous acetone yielded polyphenol mixtures, which were analyzed by HPLC. Figure 2 shows typical chromatograms obtained after 0, 1, 4 and 12 hs' fermentation, and the contents of polyphenols in each step are summarized in Table II and Fig. 3. From these data, it is evident that in contrast to the relatively small changes in the yields of polyphenol extracts, the decreases of the flavan-3-ol levels, *e.g.* peaks 6[(—)-epicatechin 3-*O*-gallate (**5**)]and 11 [(−)-epigallocatechin 3-*O*-gallate (**3**)], are quite significant. Furthermore, the conversion of the flavan-3-ols into theasinensins proceeded very quickly and was maximal after 2 h. On the other hand, the contents of theaflavins (peaks 25—29) reached the maxima after 4 h. Among proanthocyanidins, the amounts of compounds

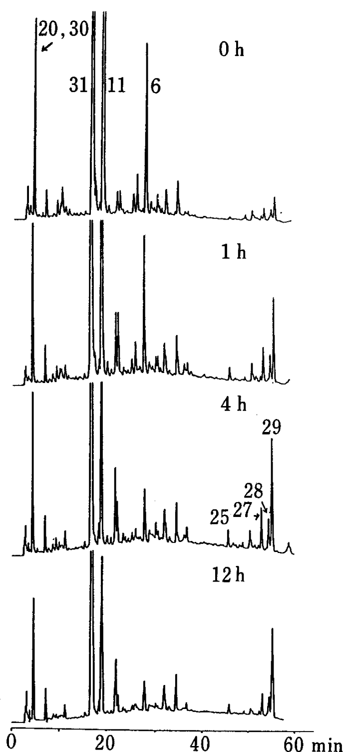


Fig. 2. HPLC Profiles (Condition A) after Fermentation for 0, 1, 4 and 12 h.

Peak numbers correspond to compounds listed in Table I.

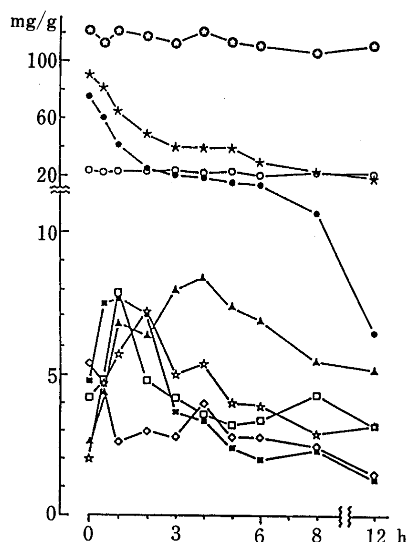


Fig. 3. Changes of Polyphenols during Fermentation

O, extracts; ★, total polyphenols; ●, flavan-3-ols; ◇, proanthocyanidins; ■, hydrolyzable tannins; ☆, theasinensins; ▲, theaflavins and theaflagallins; □, gallic acid; ○, caffeine.

Table I. Retention Times (min) of Standard Compounds

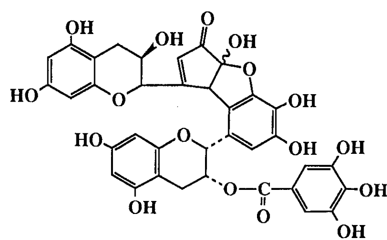
Peak No.	Compounds	Conditions A	B	C
1	(−)-Epiafzelechin[3a]	22.0	18.4	
2	(−)-Epiafzelechin 3-*O*-gallate[3a]		38.2	
3	(+)-Catechin[3a]	11.3		
4	(+)-Gallocatechin[3a]	5.8		
5	(−)-Epicatechin[3a]	16.6		
6	(−)-Epicatechin 3-*O*-gallate (ECG)[3a]	26.7		
7	(−)-Epicatechin 3-*O*-(3′-*O*-methyl)-gallate[3a]		36.2	
8	(−)-Epicatechin 3,5′-di-*O*-gallate[2]		44.7	
9	(−)-Epigallocatechin[3a]		8.0	
10	(−)-Epigallocatechin 3-*O*-*p*-coumaroate[2]		42.2	
11	(−)-Epigallocatechin 3-*O*-gallate (EGCG)[3a]	18.0		
12	(−)-Epigallocatechin 3,5′-di-*O*-gallate[2]	30.2		
13	Procyanidin B-2 3,3′-di-*O*-gallate[3b]	27.6		
14	EGCG-(4β-8)-ECG[3b]	22.4	30.0	
15	Prodelphinidin B-2 3,3′-di-*O*-gallate[3b]	17.2		
16	Procyanidin B-3[3b]		11.5	
17	Prodelphinidin B-4[3b]		7.4	
18	Prodelphinidin B-4 3′-*O*-gallate[3b]		15.8	
19	Procyanidin C-1[3b]	20.4		
20	Theogallin[6]			4.2
21	1,4,6-Tri-*O*-galloyl-β-D-glucose[5a]		24.2	
22	Strictinin[5a]	13.0		
23	Theasinensin A[2]		28.6	
24	Theasinensin B[2]	8.8		
25	Epitheaflagallin 3-*O*-gallate[4]	44.5		
26	Theaflavin[4]	48.9		
27	Theaflavin 3-*O*-gallate[4]	51.2		
28	Theaflavin 3′-*O*-gallate[4]	52.6		
29	Theaflavin 3,3′-di-*O*-gallate[4]	54.0		
30	Gallic acid			5.3
31	Caffeine	15.8		

Condition A: column, Nucleosil $5C_{18}$ (4×250 mm), solvent, CH_3CN–H_2O (0.05 M H_3PO_4); column temp., 40 °C; flow rate, 0.9 ml/min. Condition B: column, Nucleosil $5C_{18}$ (4×250 mm); solvent, [CH_3CN–tetrahydrofuran (3:2)]–H_2O (0.05 M H_3PO_4); column temp., 45 °C; flow rate, 0.9 ml/min. Condition C: column, Cosmosil $5C_{18}$ (4×250 mm); solvent, CH_3CN–H_2O (0.05 M H_3PO_4); column temp., 20 °C; flow rate, 1.0 ml/min.

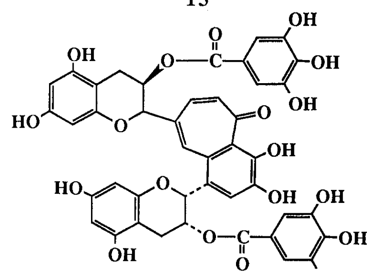
NII-Electronic Library Service

Table II. Contents of Polyphenols in Fermented Tea Leaf

Peak No.	Compounds	Contents (mg/g)/times (h)									
		0	0.5	1	2	3	4	5	6	8	12
	Flavan-3-ols										
1	(−)-Epiafzelechin	–	–	–	–	–	–	–	–	–	–
2	(−)-Epiafzelechin 3-*O*-gallate	0.5	0.4	0.4	+	+	+	+	+	–	–
3	(+)-Catechin	+	+	+	+	+	+	+	+	–	–
4	(+)-Gallocatechin	2.1	1.3	0.8	0.5	0.3	0.5	0.3	0.4	0.2	+
5	(−)-Epicatechin	1.2	0.8	0.6	0.2	+	+	+	+	–	–
6	(−)-Epicatechin 3-*O*-gallate (ECG)	8.2	6.7	5.7	4.1	2.7	2.5	2.0	1.9	1.4	0.8
7	(−)-Epicatechin 3-*O*-(3′-*O*-methyl)-gallate	0.8	0.8	0.8	+	+	+	+	+	+	+
8	(−)-Epicatechin 3,5′-di-*O*-gallate	–	–	–	–	–	–	–	–	–	–
9	(−)-Epigallocatechin	8.5	4.4	3.1	1.9	1.9	2.0	1.4	1.7	1.5	0.8
10	(−)-Epigallocatechin 3-*O*-*p*-coumaroate	+	+	+	+	+	+	+	+	–	–
11	(−)-Epigallocatechin 3-*O*-gallate (EGCG)	53.3	45.3	29.5	17.9	15.0	12.7	11.1	9.4	7.4	4.7
12	(−)-Epigallocatechin 3,5′-di-*O*-gallate	0.6	0.5	0.7	0.7	0.5	0.6	0.4	0.4	0.2	0.2
	Subtotal	75.2	60.2	41.6	25.3	20.4	18.3	15.2	13.8	10.7	6.5
	Proanthocyanidins										
13	Procyanidin B-2 3,3′-di-*O*-gallate	1.3	1.4	+	+	+	+	+	+	+	+
14	EGCG-(4*β*-8)-ECG	+	+	+	+	+	+	+	+	+	+
15	Prodelphinidin B-2 3,3′-di-*O*-gallate	–	–	–	–	–	–	–	–	–	–
16	Procyanidin B-3	2.1	2.2	1.5	1.5	1.5	1.6	1.4	1.4	1.2	0.4
17	Prodelphinidin B-4	0.8	0.4	1.1	1.5	1.3	2.4	1.4	1.4	1.3	1.1
18	Prodelphinidin B-4 3′-*O*-gallate	1.2	0.7	+	+	+	+	+	+	–	–
19	Procyanidin C-1	+	–	–	–	–	–	–	–	–	–
	Subtotal	5.4	4.7	2.6	3.0	2.8	4.0	2.8	2.8	2.5	1.5
	Hydrolyzable tannins										
20	Theogallin	4.6	7.2	7.5	6.1	3.7	3.3	2.4	2.0	2.3	1.3
21	1,4,6-Tri-*O*-galloyl-*β*-D-glucose	0.1	0.1	+	+	+	+	+	+	+	+
22	Strictinin	0.1	0.2	0.2	0.1	+	+	+	+	+	+
	Subtotal	4.8	7.5	7.7	6.2	3.7	3.4	2.4	2.0	2.3	1.3
	Theasinensins										
23	Theasinensin A	1.4	3.2	4.2	5.6	3.9	4.1	3.1	3.0	2.3	2.6
24	Theasinensin B	0.6	1.5	1.5	1.6	1.1	1.3	0.9	0.9	0.6	0.6
	Subtotal	2.0	4.7	5.7	7.2	5.0	5.4	4.0	3.9	2.9	3.2
	Theaflavins and theaflagallin										
25	Epitheaflagallin 3-*O*-gallate	0.2	0.2	0.3	0.3	0.4	0.3	0.4	0.3	0.2	0.2
26	Theaflavin	0.6	0.4	0.8	0.4	0.6	0.8	0.6	0.7	0.3	0.3
27	Theaflavin 3-*O*-gallate	0.7	1.0	1.6	1.5	1.7	2.0	1.5	1.5	1.0	1.0
28	Theaflavin 3′-*O*-gallate	0.8	1.3	1.7	1.3	1.9	1.9	1.8	1.7	1.4	1.3
29	Theaflavin 3,3′-di-*O*-gallate	0.3	1.5	2.4	2.8	3.3	3.4	3.1	2.7	2.6	2.4
	Subtotal	2.6	4.4	6.8	6.3	7.9	8.4	7.4	6.9	5.5	5.2
	Total	90.0	81.5	64.4	48.0	39.8	39.5	39.2	29.4	23.9	17.7
	Others										
30	Gallic acid	4.2	4.8	7.9	4.8	4.2	3.6	3.2	3.4	4.3	3.2
31	Caffeine	23.7	22.0	23.0	22.8	23.7	21.8	23.0	20.3	22.6	21.7
	Extracts (10 mg/g)	12.0	11.2	12.1	11.8	11.2	12.2	11.3	11.1	10.7	11.2



13



14

having galloyl group(s), *e.g.* B-2 3,3′-di-*O*-gallate[7] and B-4 3′-*O*-gallate,[5b]) quickly decreased, whereas almost no changes were observed in procyanidins B-3 and B-4. This fact, coupled with the observation that the theogallin level increased after 1 h, suggested the presence of gallic acid transferase in tea leaves. In addition, it was confirmed that caffeine (peak 31) remains intact in the fermentation processes.

Next, to examine whether theasinensins are actually derived from the original flavan-3-ols, enzymatic reactions were performed by using acetone precipitates[12] obtained from the leaves of *C. sinensis* var. *assamica*. Incubation of (−)-epigallocatechin 3-*O*-gallate (**3**), the major tea leaf polyphenol, with the crude enzyme preparation for 24 h afforded several oxidation products. HPLC analysis (Fig. 4) of these products clearly indicated, among others, the formation of theasinensins A (**8**) and D (**9**) and oolongtheanin (**13**). Since **13** is considered from its structural

NII-Electronic Library Service

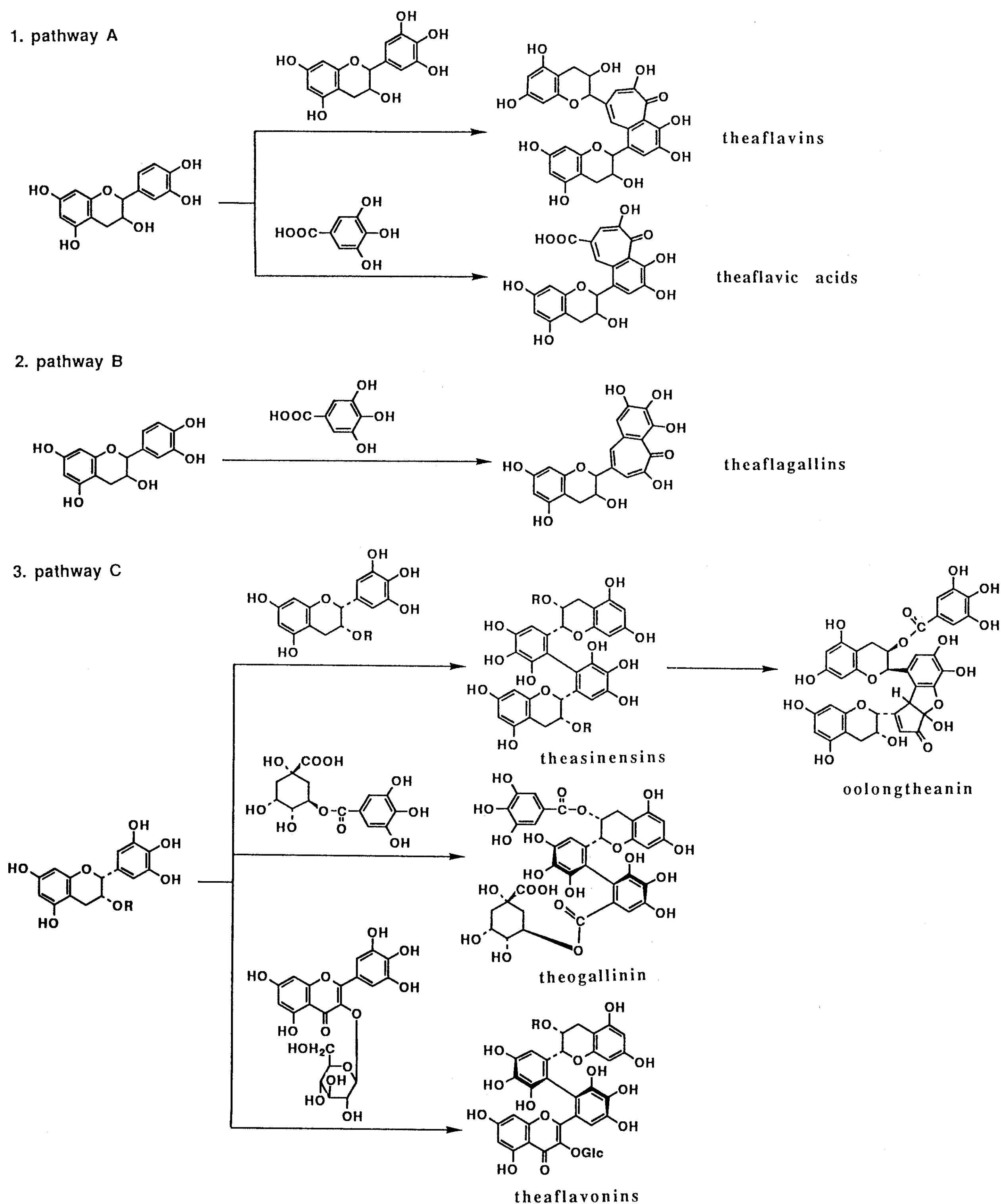


Chart 1. Enzymatic Conversion of Polyphenols in Tea Leaves

features to be formed by an intermolecular coupling of two flavan pyrogallol rings, oxidation of the aromatic ring, decarboxylation, *etc.*, **8** and **9** are regarded as intermediates in the formation of **13**. On the other hand, enzymatic reaction employing a mixture of (−)-epigallocatechin 3-*O*-gallate (**3**) and (−)-epicatechin 3-*O*-gallate (**5**) produced theaflavin 3,3′-di-*O*-gallate (**14**), together with compounds **8**, **9** and **13**. These findings confirmed unequivocally that theasinensins and oolongtheanin are formed enzymatically from **3** by an oxidative coupling of two pyrogallol rings, whereas theaflavin (**14**) is produced by an oxidative condensation of catechol and pyrogallol rings. Furthermore, the former oxidation pathways were found to predominate over the production of theaflavins.

Owing to the occurrence of small quantities of theogallinin (**1**) and theaflavonins (**10**, **11**) in tea leaves, we could not confirm the enzymatic production of **1**, **10** and **11**, but these compounds are considered to be derived by

NII-Electronic Library Service

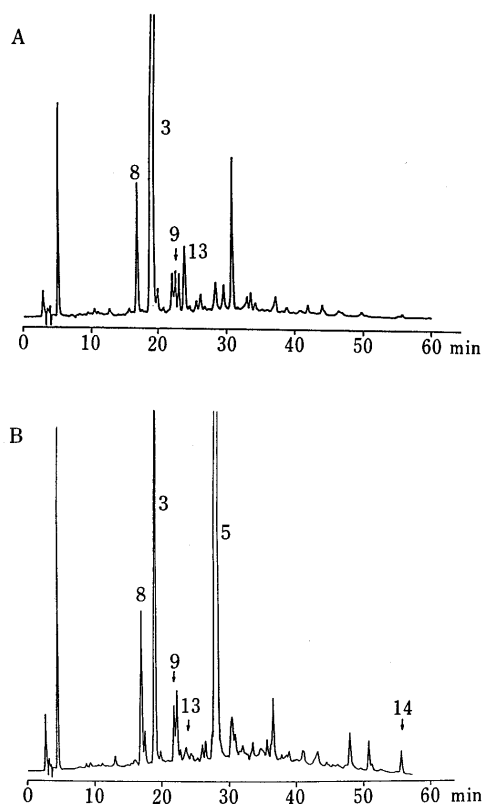


Fig. 4. HPLC (Condition A) of Enzymatic Reaction Products (after 24 h)

A, from **3**; B, from a mixture of **3** and **5**.

a pyrogallol–pyrogallol coupling analogous to those of theasinensins (Chart 1).

Experimental

Details of the instruments and chromatographic conditions used in this study are essentially the same as described in the previous paper,[13] except for the following. HPLC was conducted on a Toyo Soda CCPM apparatus equipped with a UV-8000 spectrophotometer.

Isolation of 1, 10 and 11 from Black Tea A commercial blend (2.0 kg) of Indian and Ceylonese black teas (Nittoh) was extracted five times with 80% aqueous Me_2CO at room temperature. After removal of Me_2CO by concentration under reduced pressure, the solution was extracted with ether. The aqueous layer was chromatographed over Sephadex LH-20 with water containing increasing proportions of MeOH to afford five fractions, of which fraction II was rechromatographed over Sephadex LH-20 with ethanol. Purification of the first eluted compound by chromatography over Sephadex LH-20 with 80% aqueous MeOH gave theaflavonin (**10**) (23 mg). The second fraction was repeatedly chromatographed over MCI-gel CHP 20P and Bondapak Porasil B with water containing increasing amounts of MeOH to furnish desgalloyl theaflavonin (**11**) (35 mg) and theogallinin (**1**) (111 mg).

Theogallinin (1) A pale brown amorphous powder, $[\alpha]_D^{19}$ $-79.2°$ (c=0.5, Me_2CO). FAB MS m/z: 801 $(M+H)^+$. *Anal.* Calcd for $C_{36}H_{32}O_{21}\cdot 7H_2O$: C, 46.65; H, 5.00. Found: C, 46.26; H, 4.71. ^{1}H-NMR (Me_2CO-d_6+D_2O) δ: 1.55—2.44 [4H, m, theogallin (T)-2,6-H], 2.54 [1H, dd, J=18, 4 Hz, flavan (FA)-4-H], 2.90 (1H, br d, J=18 Hz, FA-4-H), 3.10 (1H, dd, J=10, 3 Hz, T-4-H), 4.07 (1H, m, T-5-H), 4.59 (1H, br s, FA-2-H), 5.19 (1H, m, T-3-H), 5.38 (1H, m, FA-3-H), 5.93, 6.08 (each 1H, d, J=2 Hz, FA-6,8-H), 7.03, 7.21 (each 1H, s, FA-6′-H, T-6′-H), 7.13 (2H, s, galloyl H). ^{13}C-NMR (Me_2CO-d_6+D_2O)δ: 28.8 (FA-4-C), 38.1, 39.4 (T-2, 6-C), 69.1 (FA-3-C), 71.9, 72.3 (T-3, 5-C), 72.4 (T-4-C), 76.0 (T-1-C), 77.0 (FA-2-C), 95.9, 96.4 (FA-6,8-C), 98.4 (FA-4a-C), 108.6 (FA-6′-C), 110.0 (galloyl 2,6-C), 111.5 (T-6′-C), 116.5, 117.5 (FA-2′-C, T-2′-C), 121.6 (galloyl 1-C), 122.7 (T-1′-C), 129.3 (FA-1′-C), 133.4 (FA-4′-C), 138.1 (T-4′-C), 139.1 (galloyl 4-C), 144.3, 144.9, 145.2, 145.4 (FA-3′, 5′-C, T-3′, 5′-C), 145.8 (galloyl 3,5-C), 157.0 (FA-5,7,8a-C), 167.3, 168.5 (–COO–), 180.1 (–COOH). CD (c=0.0027, MeOH)$[\theta]^{20}$ (nm): 0 (217), −114000 (228), 0 (241), +15800 (245), 0 (254), −25000 (275).

Tannase Hydrolysis of 1 A solution of **1** (10 mg) in water (2 ml) was incubated with tannase at room temperature for 20 min. The reaction mixture was applied to an MCI-gal CHP 20P column, and elution with water containing increasing amounts of MeOH yielded gallic acid and the hydrolysate (**1a**) (4 mg) as a pale brown amorphous powder, $[\alpha]_D^{23}$ $-25.8°$ (c=0.1, Me_2CO). FAB MS m/z: 671 $(M+Na)^+$. *Anal.* Calcd for $C_{29}H_{28}O_{17}\cdot 7/2H_2O$: C, 48.95; H, 4.96. Found: C, 48.82; H, 4.72. ^{1}H-NMR (Me_2CO-d_6+D_2O) δ: 1.60—2.00 (4H, m, T-2, 6-H), 2.34 (1H, dd, J=17, 4 Hz, FA-4-H), 2.68 (1H, br d, J=17 Hz, FA-4-H), 3.06 (1H, m, T-4-H), 4.06 (2H, m, FA-3-H, T-5-H), 4.48 (1H, br s, FA-2-H), 5.15 (1H, m, T-3-H), 5.92, 6.06 (each 1H, br s, FA-6,8-H), 6.96, 7.20 (each 1H, s, FA-6′-H, T-6′-H). ^{13}C-NMR (Me_2CO-d_6+D_2O) δ: 29.0 (FA-4-C), 37.8, 39.2 (T-2, 6-C), 65.2 (FA-3-C), 71.7, 72.2, 73.9 (T-3,4,5-C), 77.0 (FA-2-C, T-1-C), 96.8 (FA-6,8-C), 99.5 (FA-4a-C), 108.6 (FA-6′-C), 111.6 (T-6′-C), 116.0, 116.5 (FA-2′-C, T-2′-C), 122.1 (T-1′-C), 130.4 (FA-1′-C), 133.2 (FA-4′-C), 138.0 (T-4′-C), 144.3, 144.8, 145.0, 145.4 (FA-3′, 5′-C, T-3′, 5′-C). 156.3, 156.7, 156.9 (FA-5,7,8a-C), 168.6 (–COO–), 180.1 (–COOH). CD (c=0.0034, MeOH) $[\theta]^{24}$ (nm): 0 (217), −23600 (225), 0 (234), +19200 (240), 0 (260), −2950 (270).

Preparation of 1a An aqueous solution of potassium ferricyanide (1.5 g) and sodium bicarbonate (1 g) was added to an ice-cooled solution of **2** (520 mg) and **4** (580 mg) in water (100 ml). After stirring at room temperature for 1 h, the reaction mixture was directly subjected to MCI-gel CHP 20P chromatography. Elution with water and then with water–MeOH (4 : 1) afforded products, which were found to be identical with the tannase hydrolysate (**1a**) and theasinensin C (**6**) by comparisons of physical and spectral data.

Theaflavonin (10) A yellowish brown amorphous powder, $[\alpha]_D^{22}$ $-179.4°$ (c=1.0, Me_2CO). FAB MS m/z: 937 $(M+H)^+$. *Anal.* Calcd for $C_{43}H_{36}O_{24}\cdot 2H_2O$: C, 53.09; H, 4.14. Found: C, 53.45; H, 4.22. ^{1}H-NMR (Me_2CO-d_6+D_2O) δ: 2.66 (1H, dd, J=18, 4 Hz, FA-4-H), 2.94 (1H, br d, J=18 Hz, FA-4-H), 3.30—4.00 (6H, in total, m, glucose-H), 4.79 (1H, d, J=6 Hz, anomeric H), 4.96 (1H, br s, FA-2-H), 5.54 (1H, m, FA-3-H), 6.01, 6.10 (each 1H, d, J=2 Hz, FA-6,8-H), 6.24, 6.55 [each 1H, d, J=2 Hz, flavonol (FO)-6,8-H), 6.92, 7.08 (each 1H, s, FA-6′-H, FO-6′-H), 7.08 (2H, s, galloyl H). ^{13}C-NMR (Me_2CO-d_6+D_2O) δ: 21.7 (FA-4-C), 62.3, 70.7, 75.2, 76.5, 77.2, 106.3 (glucose-C), 69.3 (FA-3-C), 77.4 (FA-2-C), 95.5, 96.1, 96.8 (FA-6,8-C, FO-8-C), 99.0, 99.8 (FA-4a-C, FO-6-C), 105.3 (FO-4a-C), 108.1 (FA-6′-C), 110.2 (galloyl 2,6-C), 112.3 (FO-6′-C), 113.8, 115.4 (FA-2′-C, FO-2′-C), 121.6, 121.8 (FO-1′-C, galloyl 1-C), 130.6 (FA-1′-C), 133.4 (FA-4′-C), 136.7 (FO-3-C), 137.5 (FO-4′-C), 139.1 (galloyl 4-C), 144.0, 145.3, 145.7 (FA-3′, 5′-C, FO-3′,5′-C), 145.9 (galloyl 3,5-C), 157.4, 158.0 (FA-5,7,8a-C, FO-8a-C), 161.2 (FO-2-C), 161.9 (FO-5-C), 165.7 (FO-7-C), 167.2 (–COO–), 179.1 (FO-4-C). UV λ_{max}^{MeOH} nm (log ε): 253 (4.42), 260 sh (4.41), 301 (4.02), 345 (4.08); $\lambda_{max}^{MeOH+NaOAc}$ nm (log ε): 261 (4.40), 300 (3.99), 346 (4.06); $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 266 (4.50), 312 (4.06), 405 (4.14); $\lambda_{max}^{MeOH+AlCl_3+HCl}$ nm (log ε): 267 (4.37).307 sh (3.98). CD (c=0.0021, MeOH) $[\theta]^{20}$ (nm): 0 (213), −143000 (222), 0 (243), +11200 (253), 0 (265), −21100 (276).

Desgalloyl Theaflavonin (11) A yellowish brown amorphous powder, $[\alpha]_D^{18}$ $-63.9°$ (c=1.0, Me_2CO). FAB MS m/z: 785 $(M+H)^+$. *Anal.* Calcd for $C_{36}H_{32}O_{20}\cdot 2H_2O$: C, 53.87; H, 4.27. Found: C, 53.91; H, 4.55. ^{1}H-NMR (Me_2CO-d_6+D_2O) δ: 2.50 (1H, dd, J=17, 4 Hz, FA-4-H), 2.75 (1H, br d, J=17 Hz, FA-4-H), 3.20—4.20 (7H in total, m, FA-3-H, glucose-H), 4.79 (1H, br s, FA-2-H), 4.80 (1H, d, J=8 Hz, anomeric H), 6.00 (2H, br s, FA-6,8-H), 6.28, 6.49 (each 1H, d, J=2 Hz, FO-6,8-H), 6.84, 7.10 (each 1H, s, FA-6′, FO-6′-H). ^{13}C-NMR (Me_2CO-d_6+D_2O) δ: 29.7 (FA-4-C), 62.0, 70.3, 75.0, 77.3, 77.4, 106.1 (glucose-C), 65.2 (FA-3-C), 77.0 (FA-2-C), 95.5, 95.1, 96.4 (FA-6, 8-C, FO-8-C), 99.6 (FA-4a-C, FO-6-C), 105.1 (FO-4a-C), 108.0 (FA-6′-C). 112.5 (FO-6′-C), 113.4, 115.4 (FA-2′-C, FO-2′-C), 121.1 (FO-1′-C), 131.3 (FA-1′-C), 132.8 (FA-4′-C). 136.5 (FO-3-C), 137.2 (FO-4′-C), 144.1, 145.0, 145.6 (FA-3′,5′-C, FO-3′,5′-C), 157.0, 157.3, 157.8 (FA-5, 7, 8a-C, FO-8a-C), 161.2 (FO-2-C), 161.8 (FO-5-C), 165.3 (FO-7-C), 179.1 (–COO–), 179.1 (FO-4-C). UV λ_{max}^{MeOH} nm (log ε): 254 sh (4.34), 261 sh (4.34), 297 (4.00), 345 (3.91); $\lambda_{max}^{MeOH+NaOAc}$ nm (log ε): 262 (4.34), 300 (4.00), 341 (3.99); $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 268 (4.45), 311 (4.07), 406 (4.06); $\lambda_{max}^{MeOH+AlCl_3+HCl}$ nm (log ε): 268 (4.33), 304 sh (3.97). CD (c=0.0030, MeOH) $[\theta]^{20}$ (nm): 0 (218), −57300 (224), 0 (241), +19800 (256).

Methylation of 10 A mixture of **10** (25 mg), dimethyl sulfate (0.4 ml) and anhydrous potassium carbonate (0.5 g) in dry Me_2CO (15 ml) was heated under reflux for 3 h. After removal of the inorganic salts by filtration, the product was purified by silica gel chromatography with benzene–Me_2CO (6 : 1) to yield the tridecamethyl ether (6 mg) as a pale yellow

NII-Electronic Library Service

amorphous powder, $[\alpha]_D^{27}$ $-161.7°$ ($c=0.4$, Me_2CO). FD MS m/z: 1119 $(M+H)^+$, 956, 163. *Anal.* Calcd for $C_{56}H_{62}O_{44} \cdot 2H_2O$: C, 58.23; H, 5.76. Found: C, 58.00; H, 6.00. ^{1}H-NMR (Me_2CO-d_6+D_2O) δ: 2.70—3.20 (2H, m, FA-4-H), 3.47—4.05 (6H in total, m, glucose-H), 3.51—4.01 (OCH_3), 4.69 (1H, d, $J=8$ Hz, anomeric H), 5.02 (1H, br s, FA-2-H), 5.86 (1H, m, FA-3-H), 6.14 (2H, br s, FA-6, 8-H), 6.42, 6.47 (each 1H, d, $J=2$ Hz, FO-6, 8-H), 7.15, 7.47 (each 1H, s, FA-6′-H, FO-6′-H), 7.28 (2H, s, galloyl H).

Tannase Hydrolysis of 10 A solution of **10** (50 mg) in water was treated with tannase at room temperature for 30 min. The mixture was worked up as above to yield a partial hydrolysate, which was found to be identical with desgalloyl theaflavonin (**11**) by comparisons of physical and spectral data.

Crude Hesperidinase Hydrolysis of 11 A solution of **11** (30 mg) in water (5 ml) was shaken with crude hesperidinase at room temperature for 2 h. The reaction mixture was treated with ethanol, and the resulting precipitates were removed by filtration. The filtrate was concentrated to dryness under reduced pressure, and the residue was chromatographed over Sephadex LH-20 with ethanol. The first fraction was analyzed by GLC after preparing the trimethylsilyl derivative with bis-trimethylsilyl acetamide (20 ml) and pyridine (0.1 ml), and glucose was detected [t_R (min): 9.2, 14.1. Column, 1.5% SE-50 (2 m × 4 mm); N_2 flow rate, 40 ml/min; column temperature, 150 °C. t_R (min): 7.2, 10.3. Column, 1.5% OV-1 (2 m × 4 mm); N_2 flow rate, 40 ml/min; column temperature, 150 °C]. The second fraction was further chromatographed over silica gel with chloroform–MeOH–water (7 : 3 : 0.5) to give the hydrolysate (**11a**) (16 mg) as a pale brown amorphous powder, $[\alpha]_D^{23}$ $-40.0°$ ($c=0.3$, Me_2CO). FAB MS m/z: 623 $(M+H)^+$. *Anal.* Calcd for $C_{30}H_{22}O_{15} \cdot 2H_2O$: C, 54.71: H, 3.98. Found: C, 55.16; H, 4.39. ^{1}H-NMR (Me_2CO-d_6+D_2O) δ: 2.42 (1H, dd, $J=17$, 4 Hz, FA-4-H), 2.72 (1H, dd, $J=17$, 2 Hz, FA-4-H), 4.16 (1H, m, FA-3-H), 4.77 (1H, br s, FA-2-H), 5.90, 5.97 (each 1H, d, $J=2$ Hz, FA-6, 8-H), 6.17, 6.33 (each 1H, d, $J=2$ Hz, FO-6, 8-H), 6.77, 6.97 (each 1H, s, FA-6′-H, FO-6′-H). ^{13}C-NMR (Me_2CO-d_6+D_2O) δ: 29.0 (FA-4-C), 65.1 (FA-3-C), 77.3 (FA-2-C), 94.9, 95.6, 96.1 (FA-6, 8-C, FO-8-C), 98.6, 99.3 (FA-4a-C, FO-6-C), 104.3 (FO-4a-C), 107.6 (FA-6′-C), 111.0 (FO-6′-C), 113.1, 115.1 (FA-2′-C, FO-2′-C), 121.9 (FO-1′-C), 131.2 (FA-1′-C), 132.6 (FA-4′-C), 135.8 (FO-3-C), 137.1 (FO-4′-C), 144.8, 145.4, 145.7 (FA-3′, 5′-C, FO-3′, 5′-C), 150.3 (FO-2-C), 157.0, 157.3, 157.7 (FA-5, 7, 8a-C, FO-8a-C), 161.4 (FO-5-C), 164.6 (FO-7-C), 176.8 (FO-4-C). CD ($c=0.0003$, MeOH) $[\theta]^{23}$ (nm): -52200 (219), 0 (235), $+8700$ (239), 0 (243), -3100 (247), 0 (251), $+9300$ (260), $+600$ (276), 2500 (297).

Methylation of 11a A mixture of **11a** (13 mg), dimethyl sulfate (0.2 ml) and anhydrous potassium carbonate (0.5 g) in dry Me_2CO was refluxed with stirring for 3 h. The reaction mixture was worked up as described above to give the undecamethyl ether (7 mg) as a white amorphous powder, $[\alpha]_D^{27}$ $-87.1°$ ($c=0.3$, Me_2CO). FAB MS m/z: 777 $(M+H)^+$. *Anal.* Calcd for $C_{41}H_{44}O_{15} \cdot 2H_2O$: C, 60.51; H, 5.95. Found: C, 60.82; H, 5.67. ^{1}H-NMR ($CDCl_3$) δ: 2.50 (1H, dd, $J=18$, 5 Hz, FA-4-H), 2.88 (1H, br s, $J=18$ Hz, FA-4-H), 3.69—3.94 (OCH_3), 4.31 (1H, m, FA-3-H), 4.75 (1H, br s, FA-2-H), 6.04 (2H, br s, FA-6,8-H), 6.24, 6.35 (each 1H, d, $J=2$ Hz, FO-6,8-H), 7.11, 7.12 (each 1H, s, FA-6′-H, FO-6′-H).

Fermentation of Tea Leaves Fresh leaves (5.0 g each) of *C. sinensis* var. *assamica* were crumpled, and after addition of water (1 ml), the wet leaves were kept at room temperature. Samples fermented for 0.5, 1, 2, 3, 4, 5, 6, 8, and 12 h were heated at 95 °C in 80% aqueous Me_2CO (50 ml) for 1 min, and the whole was left standing at room temperature for 24 h. Extraction of each sample was further repeated three times, and the combined extracts were concentrated to 100 ml under reduced pressure. An aliquot (10 ml) of this solution was concentrated to dryness, and the residue dissolved in 80% aqueous MeOH was applied to a pre-column of Toyopak ODS M. Elution with 80% aqueous MeOH and then with water gave a polyphenolic mixture, which was dissolved in 80% aqueous MeOH (5 ml) and analyzed by HPLC.

Preparation of Crude Enzymes A mixture of fresh leaves (400 g) of *C. sinensis* var. *assamica* and polyamide (200 g) was homogenized in 0.01 M potassium phosphate solution (pH 7.0) under cooling. After removal of the insolubles by filtration through gauze, the solution was centrifuged (4500 rpm), and the supernatant was treated with Me_2CO at -20 °C. The resulting precipitates were collected by filtration, dissolved in 0.01 M citric acid–0.02 M potassium phosphate buffer (pH 5.6) (100 ml) and used for enzymatic reaction.

Enzymatic Conversion of Flavan-3-ols Compound **3** (or a mixture of **3** and **5**) (10 mg) was incubated in the above crude enzyme solution (1 ml) at 30 °C for 24 h. The mixture was heated at 95 °C for 1 min, and treated with MeOH (4 ml). After removal of the precipitates by filtration, the filtrate was concentrated to dryness. The residue was dissolved in 80% aqueous MeOH (3 ml), and the solution was used for HPLC analysis.

HPLC Analysis Chromatographic conditions were as follows. Condition A: column, Nucleosil 5 C_{18} (4.0 mm i.d. × 250 mm) (Nagel); solvent, acetonitrile-water (0.05 M potassium phosphate); column temperature, 40 °C; flow rate, 0.9 ml/min; detection, 280 nm. Condition B: column, Nucleosil 5 C_{18} (40 mm i.d. × 250 mm); solvent, [acetonitrile–tetrahydrofuran (3 : 2)]–water (0.05 M potassium phosphate); column temperature, 45 °C; flow rate, 0.9 ml/min; detection 280 nm. Condition C: column, Cosmosil 5 C_{18} (4.0 mm i.d. × 250 mm) (Nakarai Chem., Ltd.); solvent, acetonitrile–water (0.05 M phosphoric acid); column temperature 20 °C; flow rate, 1.0 ml/min; detection, 280 nm. For elution of the compounds, the following linear gradient systems were employed. Conditions A; 8—31% acetonitrile in water/0—55 min, B; 8—33% acetonitrile in water/0—60 min, and C; 5—12% acetonitrile in water/0—6 min.

Acknowledgements The authors are grateful to Mr. I. Nagaoka, Ibusuki Subtropical Plant Experimental Station of Kyushu University, and to Mr. Y. Takubo, Saga Research Institute of Tea, for providing fresh tea leaves. They are also indebted to Mr. Y. Tanaka, Miss Y. Soeda and Mr. R. Isobe for ^{1}H-NMR, ^{13}C-NMR and MS measurements.

References

1) Part CXIII: T. Tanaka, H. Fujisaki, G. Nonaka and I. Nishioka, *Heterocycles*, **33**, 375 (1992).
2) G. Nonaka, O, Kawahara and I. Nishioka, *Chem. Pharm. Bull.*, **31**, 3906 (1983).
3) *a*) F. Hashimoto, G. Nonaka and I. Nishioka, *Chem. Pharm. Bull.*, **35**, 611 (1987); *b*) *Idem*, *ibid.*, **36**, 1676 (1988); *c*) *Idem*, *ibid.*, **37**, 3255 (1989).
4) G. Nonaka, F. Hashimoto and I. Nishioka, *Chem. Pharm. Bull.*, **34**, 61 (1986).
5) *a*) G. Nonaka, R. Sakai and I. Nishioka, *Phytochemistry*, **23**, 1753 (1984). *b*) F. Hashimoto, G. Nonaka and I. Nishioka, *Chem. Pharm. Bull.*, **37**, 77 (1989).
6) H. Nishimura, G. Nonaka and I. Nishioka, *Phytochemistry*, **23**, 262 (1984).
7) G. Nonaka, I. Nishioka, T. Nagasawa and H. Oura, *Chem. Pharm. Bull.*, **29**, 2862 (1981).
8) P. K. Agrawal, R. S. Thakur and M. C. Bansal, "Carbon-13 NMR of Flavonoids," ed. by P. K. Agrawal, Elsevier, Amsterdam, 1989.
9) Y. Takino, H. Imagawa and H. Yoshida, *Agric. Biol. Chem.*, **26**, 699 (1962).
10) E. A. H. Roberts, "The Chemistry of Flavonoid Compounds," ed. by T. A. Geissman, The MacMillan Co., New York, 1962, p. 468; A. Robertson D. S. Bendall, *Phytochemistry*, **22**, 883 (1983); Y. Takino, A. Ferretti, V. Flanagan, M. A. Gianturco and M. Vogel, *Can. J. Chem.*, **45**, 1949 (1967).
11) G. W. Sanderson, J. E. Berkowitz, H. Co and H. N. Graham, *J. Food Sci.*, **37**, 399 (1972); M. Nakagawa and H. Torii, *Agric. Biol. Chem.*, **29**, 278 (1965).
12) T. Tanaka, T. Sueyasu, G. Nonaka and I. Nishioka, *Chem. Pharm. Bull.*, **32**, 2676 (1984).
13) G. Nonaka, S. Nakayama and I. Nishioka, *Chem. Pharm. Bull.*, **37**, 2030 (1989).

NII-Electronic Library Service