Journal of Natural Products
Vol. 52, No. 1, pp. 1-31, Jan-Feb 1989

NEW METHODS OF ANALYZING TANNINS[1]

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ABSTRACT.—Recently found antitumor, antiperoxidation, antivirus, and other activities of tannins, which are largely dependent on the structure of each tannin, enhanced the need for precise analyses of tannins. Centrifugal partition chromatography, combined with gel-column chromatography, facilitated preparative separation of tannins. In addition to fabms and high-performance gel permeation chromatography (hp-gpc), normal-phase hplc promptly indicated the extent of condensation of both hydrolyzable and condensed tannins and is particularly usable for the analysis of oligomeric hydrolyzable tannins. Components of the isomer mixture formed by a tannin due to the presence of a free anomeric hydroxyl group or of a hemiacetal group were separated by reversed-phase hplc. Fabms is applicable, without derivatization of each tannin, to the mol wt determinations of tannins that are highly polar, thermolabile, and involatile. In the ^{1}H- and ^{13}C-nmr spectra of various types of hydrolyzable tannins, the signals of glucose moieties were fully assigned based on ^{1}H-^{1}H and ^{1}H-^{13}C selective decoupling and 2D-nmr techniques. These data established a widely applicable way of determining the location and type of substituents on the sugar moiety in each molecule of hydrolyzable tannin. The absolute configurations of the chiroptical aromatic substituents were directly determined by the cd spectra. The esr measurements proved the radical scavenging effect in the inhibition of lipid peroxidation by tannins.

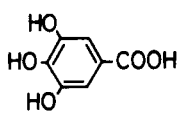
Although tannins, a large group of polyphenolic compounds widely distributed in plants, are often encountered in our lives, being contained in foods, beverages and medicinal plants, little has been known about the significance of their presence in these materials until recent years, except for some of their use such as antidiarrhetics and hemostatics (1). On the other hand, there has been a concept that tannin is toxic, and tannins accumulated in large amounts in pastures have been regarded as detrimental to cattle breeding (2). Some tannins were reported to be carcinogenic and hepatotoxic (3), although antitumor activity of certain plants due to tannins contained in them was also presented (4). However, these views often have been based on the results of administration of tannin materials in unusual ways or amounts or of a limited type of tannin, or of intractable mixtures of polyphenols such as those used for tanning leather. There were also reports that oral administration of tannin gave no evidence of toxic liver damage (5) and that tannins of various chemical structures did not show any mutagenic activity (6). It also should be remembered that large amounts of several kinds of tannins, including those contained in tea, red wine, and various fruits, are daily taken orally by an enormous number of people in the world, without showing any recognizable toxicity due to tannin.

Generally, tannins are traditionally classified into two large groups, hydrolyzable tannins and condensed tannins (7). These names are based on their hydrolysis and condensation occurring in the presence of acid or enzyme. Galloyl [**1**] and hexahydroxydiphenoyl (HHDP) [**2**] groups represent the polyphenolic part in the molecules of hydrolyzable tannins. Among them, those having the HHDP group have been named ellagitannins, e.g., casuarictin [**3**], as they produce ellagic acid [**4**] upon hydrolysis, and those having only the galloyl group are called gallotannins, e.g., pentagalloyl glucose [**5**]. Ellagic acid, which shows marked inhibitory effect on the mutagenic and carcinogenic activities of 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-

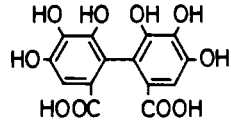
[1]Presented as a plenary lecture at the "New Directions and Methods in Natural Products Research" Symposium of the International Congress on Natural Products Research at Park City, Utah, July 17–21, 1988.

[*a*]pyrene (6,8), is, thus, generally a hydrolysis product from ellagitannins and not present in most of the fresh plants which were found to contain ellagic acid [**4**] in their extracts (9). Condensed tannins are condensates of flavan units, which are mostly (−)-epicatechin [**6**] or (+)-catechin [**7**], and some of them are galloylated. An example of these tannins is illustrated in structure **8**. Some hydrolyzable tannins condensed with flavan (10) or ascorbic acid (11) have been isolated from several plants. There are also caffeetannins, e.g., 3,5-di-*O*-caffeoylquinic acid [**9**], in which the polyphenolic group is the caffeoyl group (12).

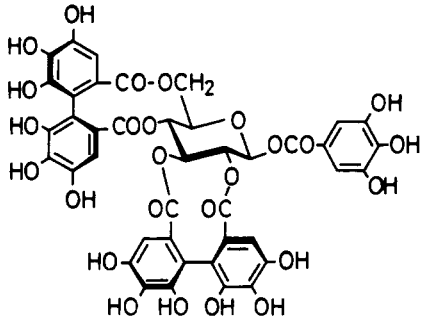
Recent isolation and structure determination of a large number of tannins enabled the investigation of their biological activities on the basis of the structural differences among tannin molecules. Among such biological activities are inhibitions of mutagens and carcinogens (6,8), tumor promotors (13), human immunodeficiency viruses (14,15), lipid peroxidation (16–18), lipoxigenase in arachidonate metabolism (19,20), autoxidation of several kinds of co-existing substances (21), reverse transcriptase (22) and other enzymes (23), histamine release from mast cell (24), hepatotoxicity (25), and also reduction of co-existing substances (26).



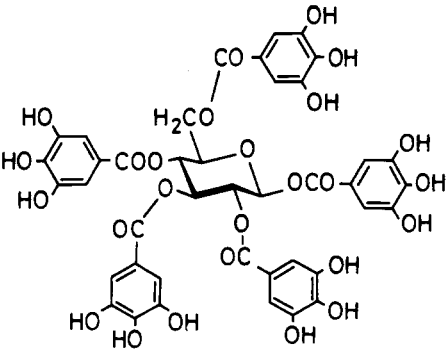
1 gallic acid



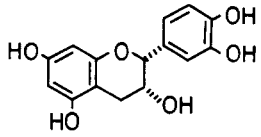
2 hexahydroxydiphenic acid



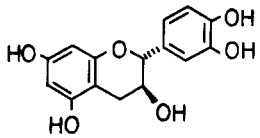
3 casuarictin



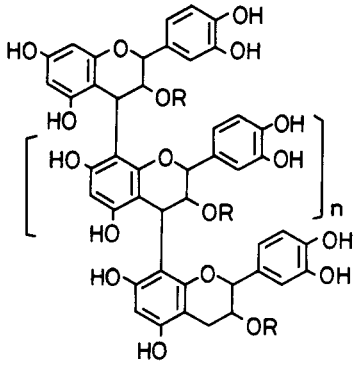
5 pentagalloyl glucose



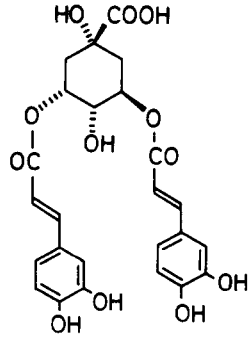
6 (−)-epicatechin



7 (+)-catechin



8 R=H or galloyl



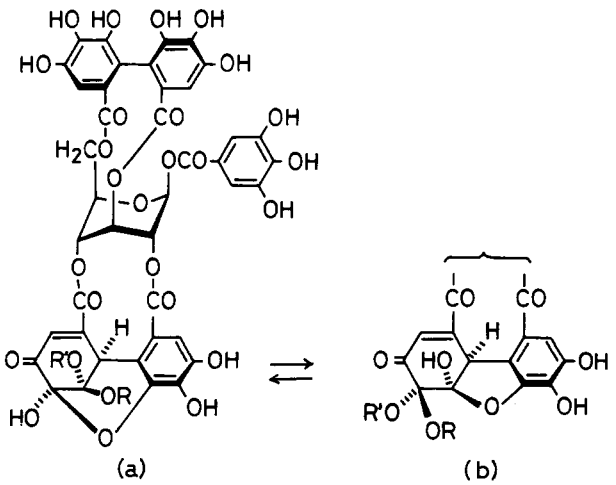
9 3,5-di-*O*-caffeoylquinic acid

The presence and strength of these activities differ depending on the structure of each tannin and on the structural type of tannin. Among several types of tannins, ellagitannins generally exhibit stronger inhibition on lipid peroxidation than the other types of tannins (27), and only some oligomeric hydrolyzable tannins show the host-mediated antitumor activity (28). The polyphenolic structure of the large molecule should be related to many biological activities of tannins, and the binding with protein and other substances, which is due to this structural feature of tannins, should be participating in the inhibitory actions of tannins on carcinogens, tumor promotors, and also several enzymes. Their property of forming stable free radicals, which is due to the presence of several phenolic hydroxyl groups in a molecule, is also regarded as the basis of their radical scavenging activities that inhibit peroxidation of lipids and other substances (27), although the strength of this activity of each tannin is not identical.

The development of methods of analysis of tannins, including chromatographic analyses and isolation and structure determination, as described in this review, brought marked advances in the determination of the biological activities mentioned above. The differences of the structures of tannins contained in each species of plant, found by the new methods of analysis, also produced data useful for discussing the correlations among plant chemosystematics, plant evolution, and occurrence of each tannin in plants (29,30).

It is noteworthy that hydrolyzable tannins having galloyl or HHDP groups are distributed along certain evolutionary routes in Choripetalae belonging to Dicotyledoneae (29,30).

QUANTITATION OF TANNINS BASED ON THEIR BINDING ACTIVITIES.—The hide-powder method of tannin quantitation, based on the binding of tannins to protein (31,32), an official method used by the leather industry, can be performed with simple apparatus. This method, however, requires a long time and a large amount of sample; it is not appropriate for the quantitation of the small amount of tannin in each fraction obtained from plant extraction in the laboratory. Among the other methods of tannin quantitation, the one originally proposed by Bate-Smith (33), which quantitates tannins by the colorimetry of the supernatant of the mixture of tannin sample and



10 R=R′=H geraniin
11 R=H, R′=Me (or Et)

SCHEME 1.

hemolyzed blood, based on the binding activities of tannins to protein, can be performed with a small amount of sample in a short time. We have improved this method (34) to be performed, with good reproducibility, with a sample containing a few milligrams of tannin of average binding activity (35). The RAG values of tannins have thus been obtained, based on the binding activity of geraniin [**10**] (36), a crystalline tannin which is the main component in the plants of *Geranium* species (37).

Improvement in reproducibility and a decrease of the sample amount to less than 1 mg were brought about by the use of methylene blue as the substrate in place of hemoglobin in the hemolyzed blood used for the determination of relative astringency based on the astringency of geraniin (RAG) value (35). The relative affinity to methylene blue based on the affinity of geraniin (RMBG) values obtained in this way for the tannins of mol wt up to 2000 are illustrated in Figure 1, which shows that the molecular weights of the polyphenols having binding activity are over 500, and that the RMBG values increase with the increase of the mol wt of each tannin up to about 1000. The increase of these values due to the increase of mol wt is no longer observed at molecular weights of over 1000, although these values of binding activity of tannins, when calculated on the basis of molar concentration of each tannin, increase even in this range of the mol wt.

There are some exceptions to this correlation of RMBG values and mol wt, for instance, ellagic acid [**4**], which is a hydrolysis product from ellagitannins, and (−)-epigallocatechin gallate [**12**], which is the main component responsible for the astrin-

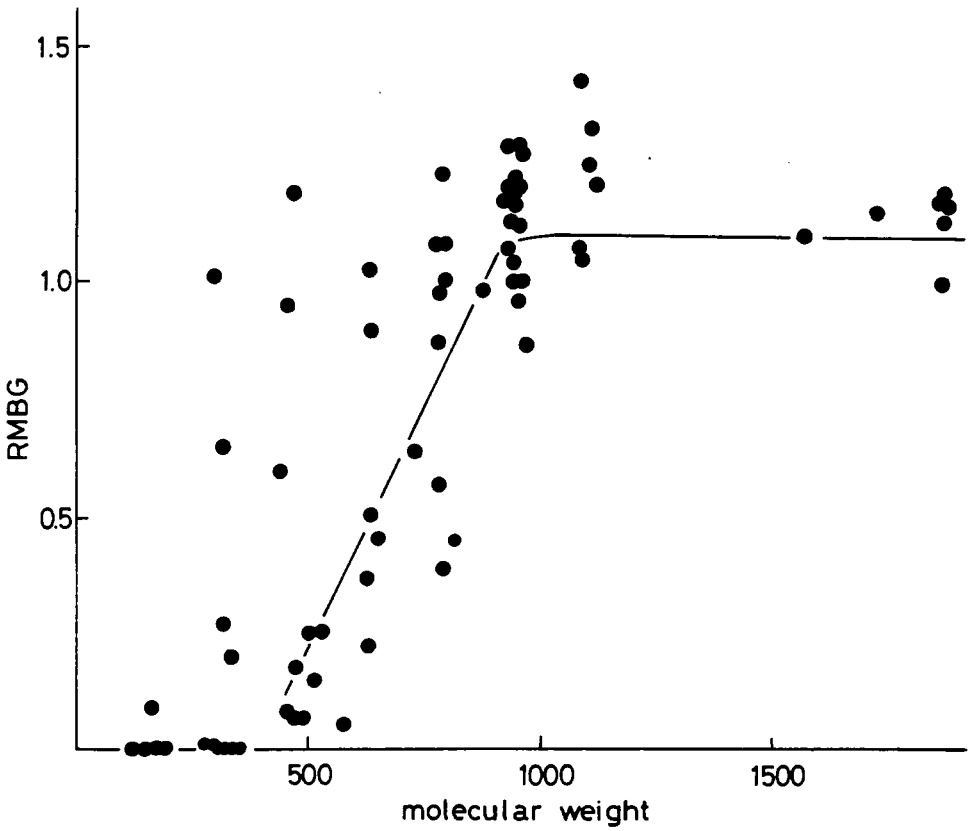
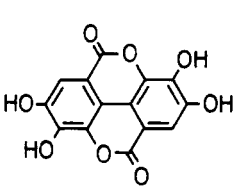
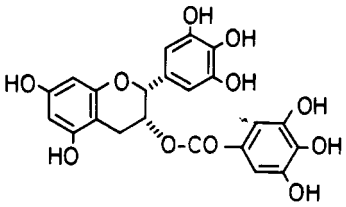


FIGURE 1. Correlation between RMBG values and molecular weights of tannins and related polyphenols.



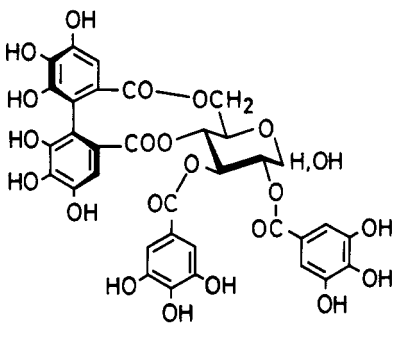
4 ellagic acid



12 (−)-epigallocatechin gallate

gent taste of green tea. It is noteworthy that marked biological activities, such as inhibition of carcinogenesis (6,8) and tumor promotion (13), are exhibited by these compounds.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY.—*Complication of the chromatograms of some tannins.*—Complications have been observed with reversed-phase hplc. An example is in the separation of the hplc peaks of anomers from each other in an equilibrium mixture of a hydrolyzable tannin having a free anomeric hydroxyl group. This is unlike the chromatograms of reducing carbohydrates which exhibit a single peak in spite of their formation of equilibrium mixtures of α and β anomers. These two peaks due to α and β anomers in the reversed-phase hplc are replaced by a single peak of different retention time after treatment with $NaBH_4$. As the hplc peak of tannin, in which the anomeric center is acylated, is not affected by the treatment with $NaBH_4$, the analysis after reduction with $NaBH_4$ is useful for discrimination of the anomer mixture forming tannins from the other compounds (38) (Figure 2).



tellimagrandin I [**13**]

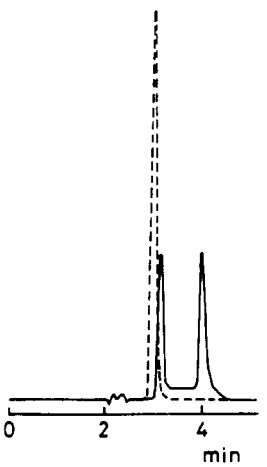


FIGURE 2. Hplc profile of tellimagrandin I [**13**]. Broken line represents the chromatogram obtained after the treatment with $NaBH_4$. Column: Merck LiChrosorb RP-18 (25 cm × 4 mm i.d.). Solvent: 0.01 M orthophosphoric acid-0.01 M potassium dihydrogenphosphate-MeCN (42.5:42.5:15).

Another example is a hydrolyzable tannin possessing a dehydrohexahydroxydiphenoyl (DHHDP) group in its molecule (dehydoellagitannin), which showed a complex peak when its alcoholic solution was injected onto the column. The chromatogram was more complex when a tannin had two or more DHHDP groups in a

molecule. This complication has been found to be due to the formation of acetals [**11**] in the alcoholic solutions as exemplified by geraniin [**10**] (38) (Scheme 1).

Determination of molecular weight.—Approximate molecular weights of tannins can be exhibited by hplc of methyl or acetyl derivatives on the gel permeation chromatography (gpc) column, and the gpc analysis of the tannins that are not alkylated or acylated can also be performed on the gpc column of weak adsorptivity as shown in Figure 3 (39). Rough estimation of the molecular size of both hydrolyzable and condensed tannins, particularly of oligomeric hydrolyzable tannins, has been facilitated by the application of normal-phase hplc. As illustrated in Figure 4, monomers, dimers, trimers, and tetramers in the extract of *Heterocentron roseum*, a Mexican melastomataceous plant, produce peaks with retention times which increase with increase in molecular size.

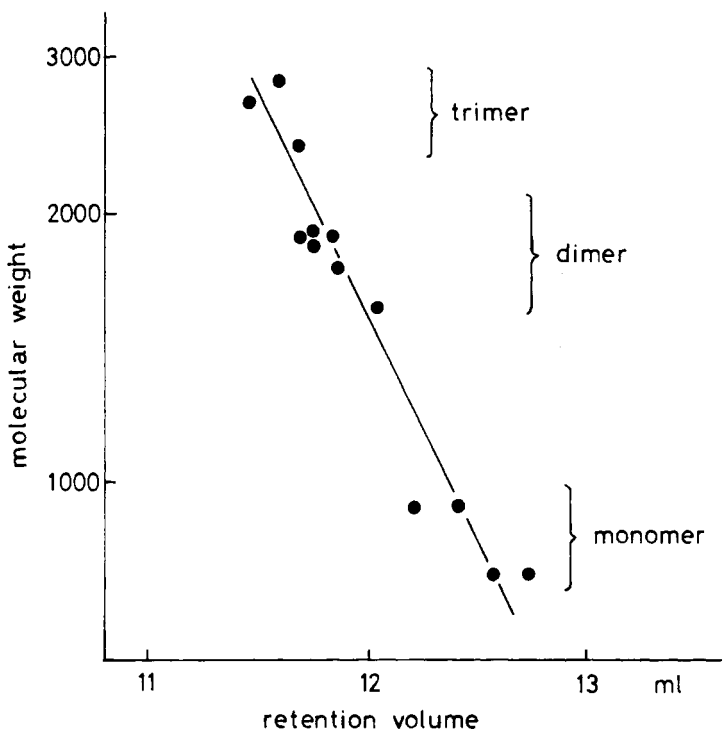


FIGURE 3. Correlation between molecular weight of hydrolyzable tannins and retention volume on hp-gpc.

Discrimination of type of each tannin by hplc.—The application of photodiode array detector to hplc of tannins in the plant extracts facilitated discrimination of ordinary tannins, caffeetannins, flavonoids, and other compounds, with sample amount comparable with those required for uv monitoring. This method is particularly efficacious for the investigation of the distribution of tannins and related polyphenols of each type in plants (40).

Differences in stability of tannins exhibited by hplc.—Hydrolyzable tannins are generally easily hydrolyzed when they are extracted with boiling H_2O from the plants, as observed by hplc analysis during the hot-H_2O extraction of geraniin from *Geranium thunbergii* (41) (Scheme 2). However, the ester bondings in the isolated hydrolyzable tannins

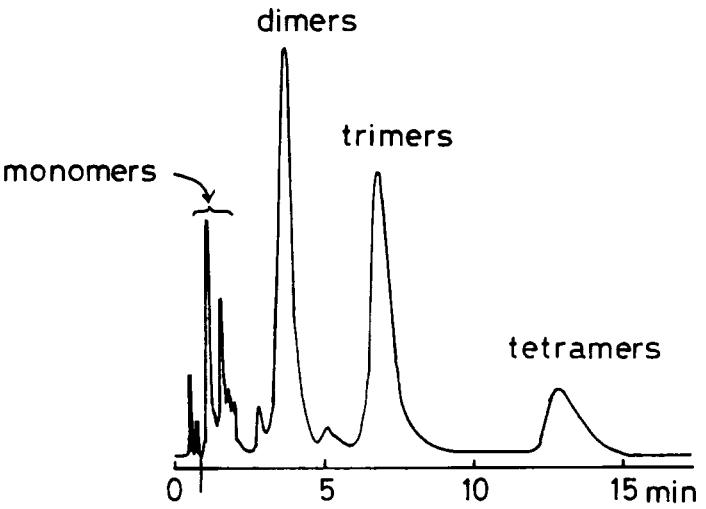


FIGURE 4. Hplc of mixture of hydrolyzable tannins containing monomers and oligomers, extracted from *Heterocentron roseum*. Column: Develosil 60-5 (15 cm × 4 mm i.d.). Solvent: hexane-MeOH-THF-HCOOH (60:45:15:1) containing oxalic acid 500 mg per 1.2 liters.

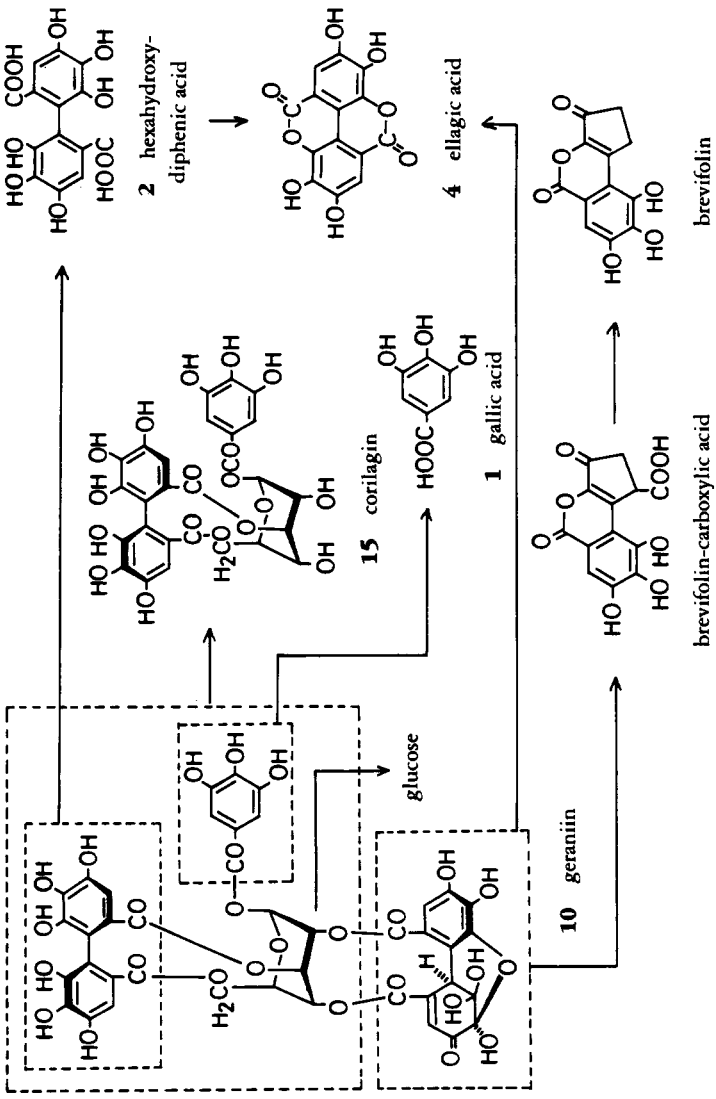
are fairly stable and are not immediately hydrolyzed in hot H_2O, except for some oligomeric hydrolyzable tannins (42) described in a foregoing part of this review, although lability of depside linkages in gallotannins, which are methanolyzed at room temperature at pH 6, has been demonstrated (43).

Caffeetannins and labiataetannins having caffeoyl group in their molecules, represented by 3,5-di-*O*-caffeoylquinic acid [**9**] and other caffeoyl derivatives of quinic acid contained as the main components in *Artemisia* species (12), and rabdosiin, which is a dimer of rosmarinic acid [**14**] (Figure 5) having a lignan skeleton (44), behave in quite different ways. The example of the hplc analysis upon boiling-H_2O extraction of rosmarinic acid [**14**] from *Perillae folium* shows the stability of this polyphenolic compound. However, this compound is quickly decomposed when the plant is dried under direct sunshine or at elevated temperature as in Figure 5 (45).

When isolated tannins are kept in the air at room temperature, hydrolyzable tannins are generally fairly stable, and most of them can be kept without change of color, while condensed tannins and the structural units composing them, catechin and epicatechin, and so on, are slowly oxidized in the air to give reddish colors.

CHROMATOGRAPHIC ISOLATION OF TANNINS.—Properties of tannins that are apt to bind with protein and other various materials and are often hydrolyzed in aqueous solution prevent the use of several kinds of solid support which are favorable for the chromatographic separation of the other types of compound. However, most of the problems due to these properties of tannins have been overcome by selection of appropriate solid support and solvent systems for the chromatography and also by applying new methods of countercurrent chromatography.

Droplet countercurrent chromatography and centrifugal partition chromatography.—The glass tubes of outer diameter 5 mm allowed formation of round droplets of the moving phase mainly composed of *n*-BuOH, which induced practical separation of components in the tannin mixtures by droplet countercurrent chromatography (dccc) (46). However, it still required a fairly long time for the development, often longer than 2 days for


2 hexahydroxy-diphenic acid
4 ellagic acid
15 corilagin
1 gallic acid
glucose
10 geraniin
brevifolin-carboxylic acid
brevifolin

SCHEME 2.

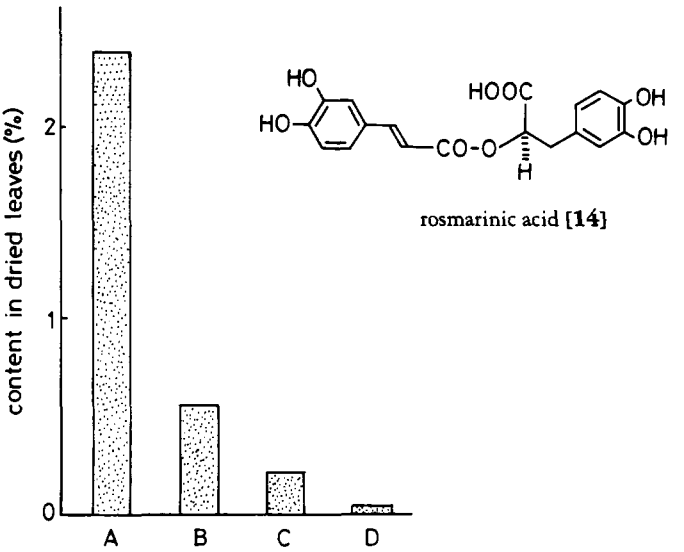


FIGURE 5. Contents of rosmarinic acid [**14**] in *Perillae folium* dried in different ways: (A) under direct sunshine (3 days); (B) in the shade (4 h); (C) in an oven at 60° (2 h); (D) in an oven at 80° (2 h).

the separation of the components in a fraction, and was not usable for separation of several oligomeric hydrolyzable tannins.

The application of centrifugal partition chromatography (cpc), which is a countercurrent chromatography performed in a centrifuge, markedly shortened the time of development, usually to one-fourth or one-fifth that of dccc (47). This shortening of the developing time also reduced the amount of developing solvent, and, hence, noticeably reduced the total time of the chromatographic separation including the time of evaporating solvents. This method was found particularly beneficial for the separation of oligomeric hydrolyzable tannins, which often requires a long developing time for the normal-phase development of dccc and gives poor separation of each component upon reversed-phase development.

An example of good separation of oligomeric hydrolyzable tannins in a plant extract is as follows.

EtOAc extract from *Lythrum anceps* contains a variety of tannins including the dehydroellagitannins granatins A [**16**] and B [**17**] (47), *C*-glucosidic hydrolyzable tannins (castalagin [**18**] and vescalagin [**19**]), and also dimeric ellagitannin (oenothein B [**20**]). Although the dehydroellagitannins among them have been separated from the others by dccc using the solvent system *n*-BuOH–*n*-PrOH–H_2O (4:1:5) within a reasonable time, separation of the latter two by this method was almost impracticable, since they transfer poorly to the mobile phase. However, the separation of these tannins by cpc with normal-phase development using the same solvent system was achieved within a quarter of the time required for dccc. Oenothein B [**20**] and castalagin [**18**] were obtained from fractions III and IV, respectively, in this cpc, although vescalagin [**19**] was retained in the stationary phase (47) (Figure 6).

Heterocentron roseum is rich in oligomeric hydrolyzable tannins, and most of them were isolated by a single column chromatography on a vinyl polymer resin (Toyopearl HW-40) of the *n*-BuOH extract obtained from aqueous Me_2CO homogenate of the leaves (48). However, a labile trimer, nobotanin J [**21**] which is gradually decomposed into pedunculagin [**22**] and a dimer **23** when kept in an aqueous (or MeOH) solution

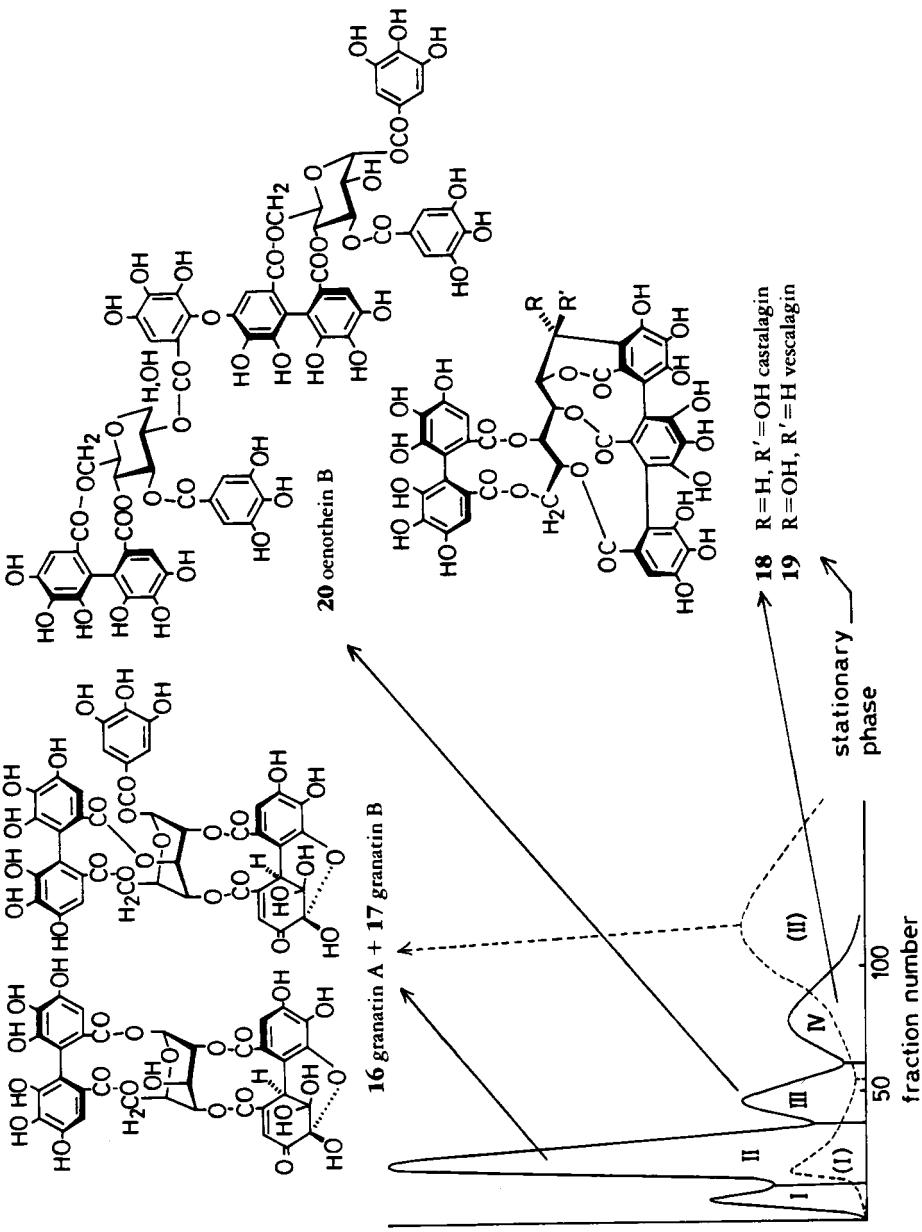
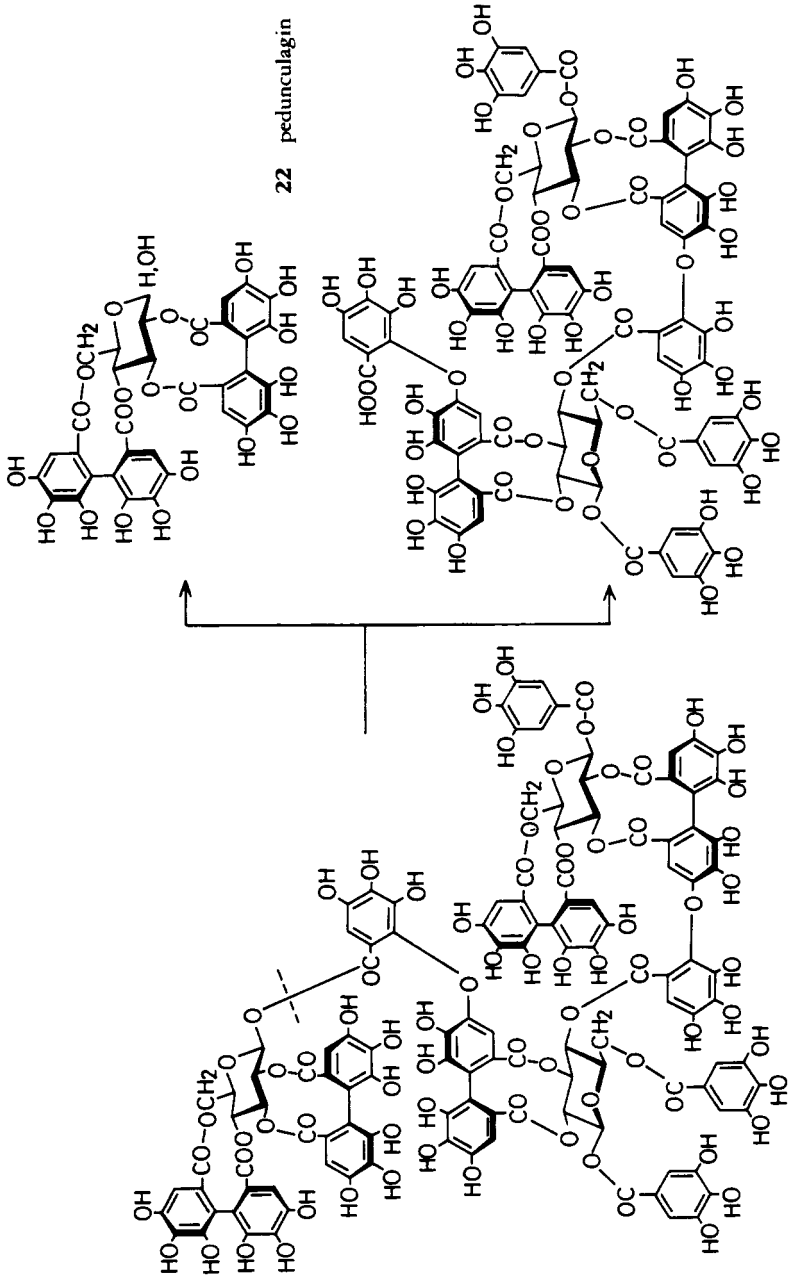


FIGURE 6. Centrifugal partition (—) and droplet countercurrent (- - -) chromatograms of tannins in a fraction from *Lythrum anceps* leaf extract.



SCHEME 3.

(Scheme 3), could not be purified even by repeated chromatography on the resin or gel columns, as it was always contaminated by **22** and **23** which should be produced on a solid support during the development. Nobotanin J [**21**] was successfully purified by cpc with normal-phase development using the solvent system *n*-BuOH–*n*-PrOH–H_2O (4:1:5) without this unfavorable degradation. A crude tetramer (nobotanin K [**24**]) obtained by the cc on Toyopearl HW-40 mentioned above was also difficult to purify by the rechromatography on the resin column. This is due to the high polarity and strong adsorptivity of nobotanin K [**24**] and accompanying tetramers of related structures. The reversed-phase cpc with *n*-BuOH–*n*-PrOH–H_2O (4:1:5) of the tetramer fraction resulted in efficient purification of each tetramer within several hours, as illustrated in Figure 7 (42).

Highly water-soluble derivatives of tannins were also effectively separated with this technique. Geraniin makes a condensate with ascorbic acid in high yield when kept at room temperature in a weakly acidic solution, and this condensate, elaeocarpusin [**25**], is also present in the H_2O-soluble portion of the extract from *Geranium* species of plants (11) (Scheme 4). Isolation of this condensate from the aqueous solution was effected by normal-phase cpc using the solvent mixture *n*-BuOH–*n*-PrOH–H_2O (4:1:5) followed by chromatography on a Sephadex LH-20 column (47).

Chromatographic separation on gel columns.—As most tannins are strongly adsorbed on Si gel, this is not a suitable material for the preparative separation of tannins, although it can be used for analytical hplc when developed with acid-containing solvent mixtures (49,50).

This property of tannins also makes separation on the gel columns different from that of the other types of compounds. The separation of tannins on the columns of hydroxypropylated dextran gel, such as Sephadex LH-20 (51), is induced by the difference in adsorptivity of each tannin on the gel rather than by gel filtration.

The disadvantage of this gel, in addition to its lability, is its strong adsoption of several types of tannins, particularly of condensed tannins of large molecules, which are not recovered from the column with organic solvents and thus shorten the gel life for reuse. A vinyl polymer gel such as Toyopearl HW-40 (TOSOH, Japan) or Diaion HP-20 (Mitsubishi Chemical, Japan), which can be washed with alkali to recover the original activity of the resin, allows remarkably prolonged use for the separation of tannins.

Combined use of these resins with the new method of countercurrent chromatography usually induces good separation of each component in the tannin mixtures.

STRUCTURAL ANALYSES.—Although chemical degradation, such as complete hydrolysis with acid, partial hydrolysis with hot H_2O (37) or enzyme, or thiol degradation (51), is often necessary for elucidating chemical structures of tannins, structural studies of tannins have been facilitated by advances in various spectroscopic techniques, especially in nmr spectroscopy.

Mass spectral analysis.—Field desorption (fd) and fabms are of significant value in the structural analysis of tannins that are highly polar and thermally labile and of poor volatility. The predominant molecular ion species observed in the mass spectra of hydrolyzable tannins, which are not derivatized, are often $[M + Na]^+$ and/or $[M + K]^+$ in addition to the protonated molecular ion $[M + H]^+$. The former ions should arise from a minute amount of inorganic impurities, usually NaCl or KCl. Therefore, addition of a small amount of a sodium halide or potassium halide, as cation donors to the sample matrix, is often effective for detection of such a molecular ion species. Examples of application of these new techniques to the structural studies of tannins follow.

Of two possible structures, i.e., hemiacetal form (a) and *gem*-diol form (b) at the DHHDP group of geraniin [**10**] (Scheme 1), the former structure was supported by the

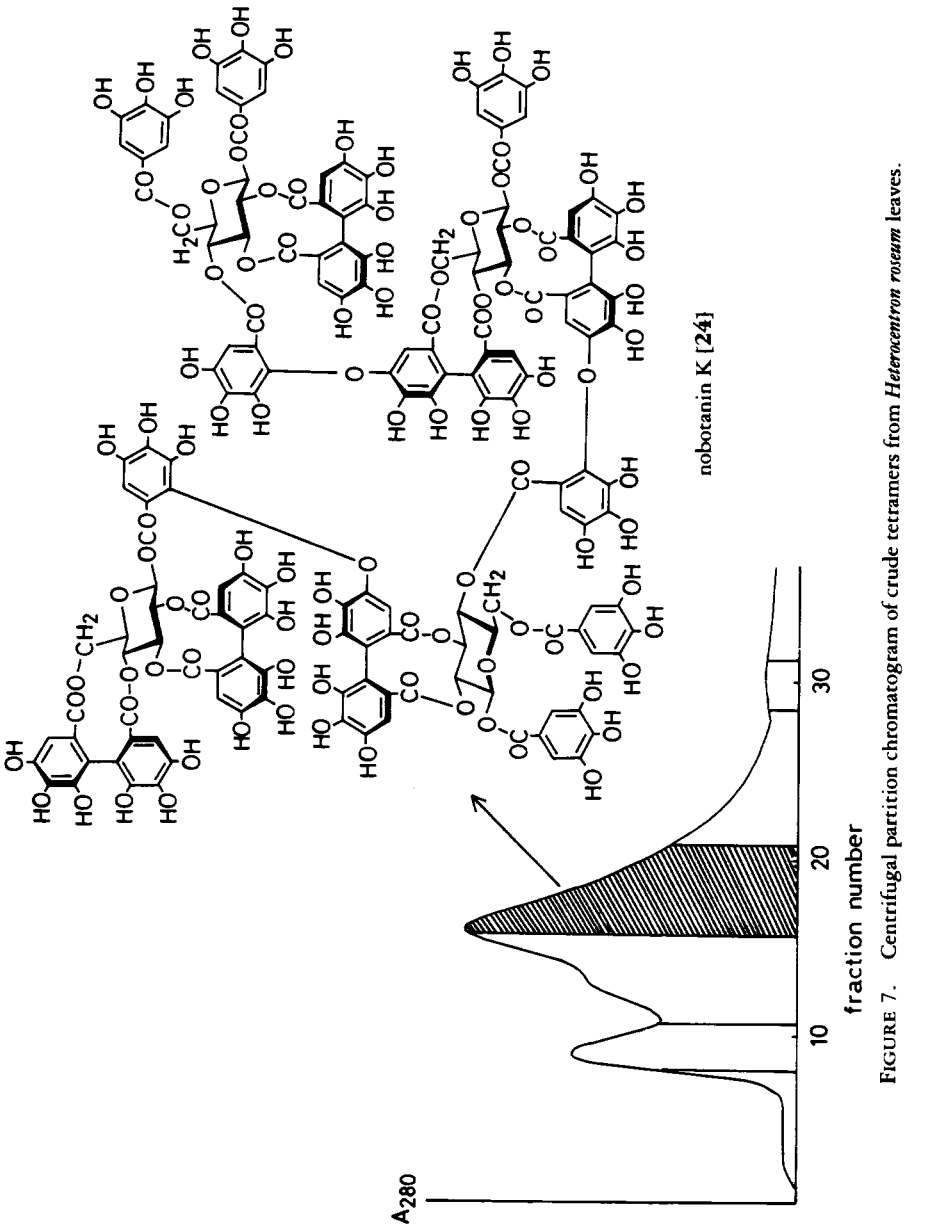
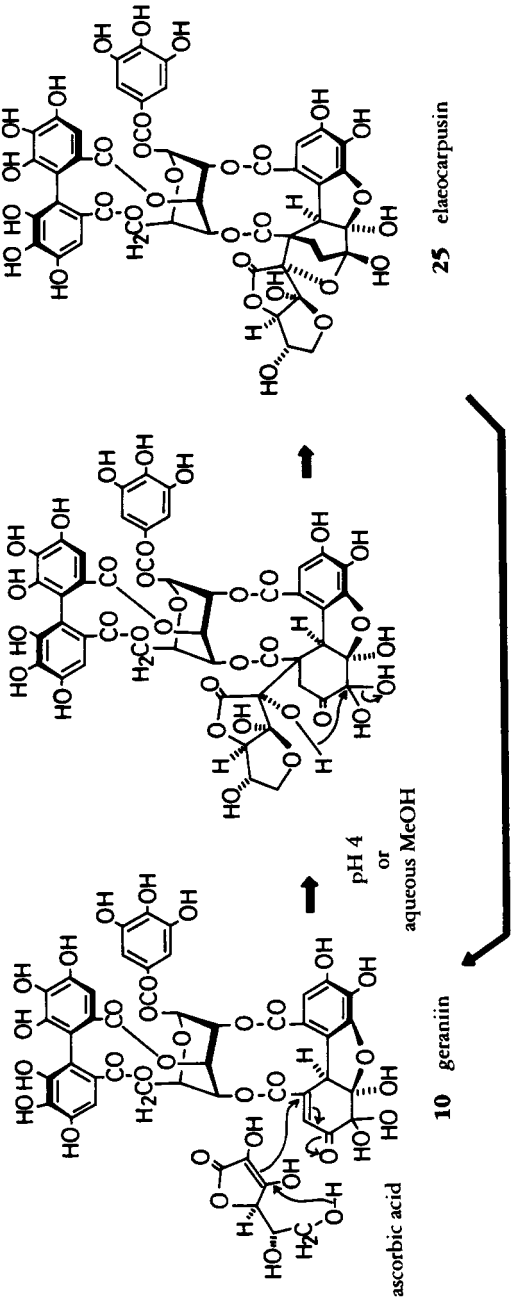
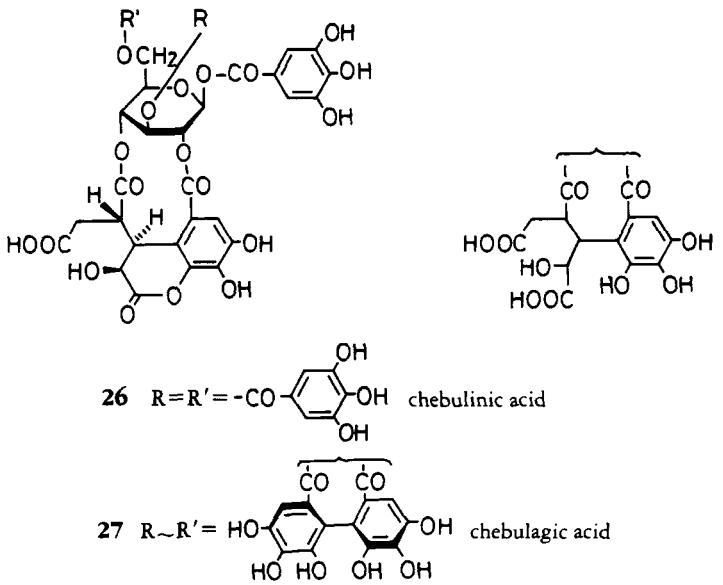


FIGURE 7. Centrifugal partition chromatogram of crude tetramers from *Heterocentron roseum* leaves.


ascorbic acid
10 geraniin
pH 4
or
aqueous MeOH
pH 7
25 elaeocarpusin

SCHEME 4.

prominent peak at *m/z* 975 due to $[M + Na]^+$ in the fd mass spectrum (39). Similarly, evidence for the monolactone structures of chebulinic acid [**26**] (52) and chebulagic acid [**27**] (52), for which dicarboxylic acid structures at their chebulic acid moiety had previously been proposed (53), was obtained by their fabms which showed $[M + Na]^+$ ion peaks at *m/z* 977 and 979, respectively (38).

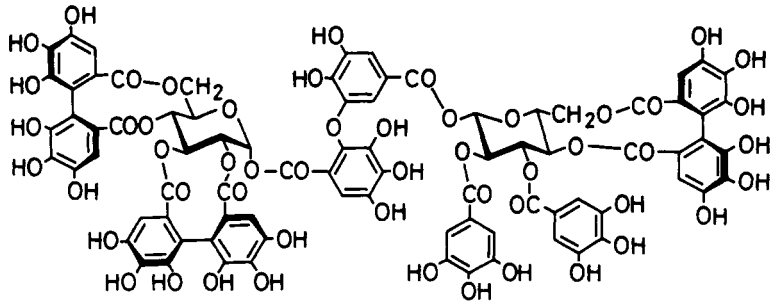


The fabms technique was also successfully applied to the determination of molecular weights of dimeric hydrolyzable tannins gemin A [**28**] ($[M + H]^+$ 1873) (54) (Figure 8), rugosin D ($[M + Na]^+$ 1898) (55), nobotanin F ($[M + Na]^+$ 1895; $[M + K]^+$ 1911) (56), and others.

The negative fabms of tannins of this class also gives a prominent peak due to the $[M - H]^-$ ion and is often preferable to the positive fabms. It is advantageous to measure both positive and negative fabms for discrimination of the molecular ion species in the spectra.

^{1}H- and ^{13}C-nmr spectral analyses.—The structural information of complicated tannins has been supplied to a large extent by nmr spectroscopy. As accumulation of spectral data for various structures of the tannins in each class should greatly facilitate structural analysis of new compounds based on the spectral comparisons, we have carried out a systematic assignment of ^{1}H and ^{13}C signals of the hydrolyzable tannins possessing glucose cores, which are most widely distributed in the plant kingdom and are rich in structural variety. Particularly, full assignment of the ^{13}C resonances of the glucose moiety has been performed in order to establish a means of determining the location of the acyl groups on the glucose residue (Scheme 5).

The ^{1}H- and ^{13}C-nmr spectra of hydrolyzable tannins, complemented by the findings from acid hydrolysis, usually provide information concerning the nature and number of polyphenol groups, e.g., galloyl, HHDP, valoneoyl, dehydrodigalloyl (DHDG) and DHHDP groups. These polyphenolic groups are recognized in the ^{1}H-nmr spectra as a 2H singlet (galloyl), two 1H singlets (HHDP), three 1H singlets (valoneoyl), and two 1H doublets (J = 2 Hz) accompanied by a 1H singlet (DHDG) in the aromatic region. The presence of a DHHDP group in a molecule can be deduced from three 1H singlets at ca. δ 5.2, 6.4, and 7.1, which are often accompanied by doublets (J = 2 Hz) around δ 4.8 and 6.1 which are due to methine and vinyl protons in the five-



gemin A [**28**]

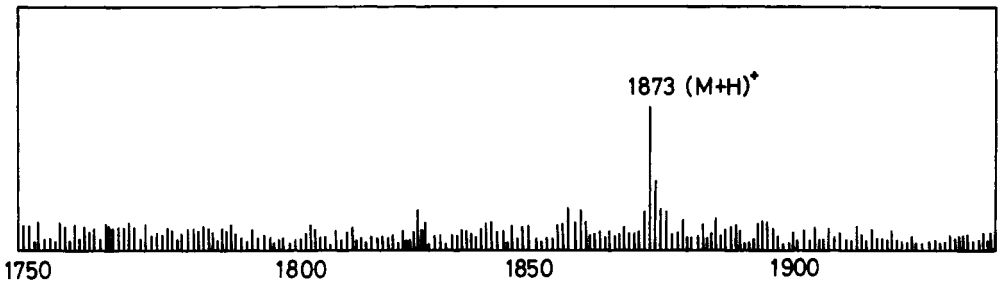


FIGURE 8. Fab mass spectrum of gemin A [**28**].

and six-membered hemiacetal structures in the equilibrium mixture, as exemplified by geraniin [**10**] (36). Complementary information concerning these polyphenolic groups can be also obtained from the ^{13}C-nmr spectra (58).

Based on the conformational differences of their glucose residues, monomeric hydrolyzable tannins having a glucose residue are classified into three groups, i.e., those having a $^4C_1$ glucopyranose core, those having $^1C_4$ and related boat conformation, and those having open-chain glucose.

TABLE 1. ^{13}C Resonances of the Glucose Moieties in Hydrolyzable Tannins (50.1 MHz, from TMS in Me_2CO-d_6).

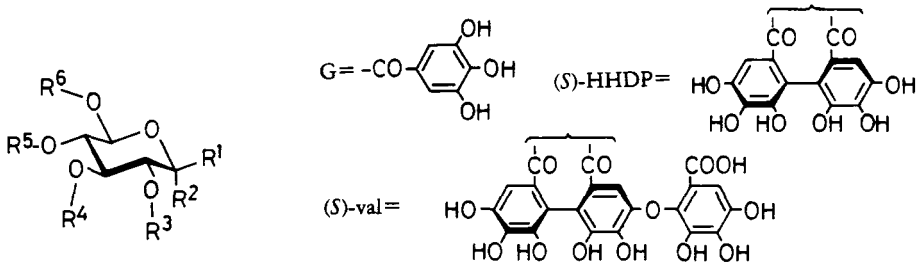
Compound	Carbon					
	C-1	C-2	C-3	C-4	C-5	C-6
1,2,3,4,6-Penta-*O*-galloyl-glu[a] [**5**]	93.4	71.9	73.5	69.5	74.1	62.9
1,2,3,6-Tetra-*O*-galloyl-glu [**29**]	93.5	71.9	76.0	69.5	76.1	63.7
1,2,4,6-Tetra-*O*-galloyl-glu [**30**]	93.4	73.8	73.3	71.7	74.0	63.1
1,2,3-Tri-*O*-galloyl-glu [**31**]	94.2	72.5	76.7	69.8	79.2	62.4
1,2,6-Tri-*O*-galloyl-glu [**32**]	93.6	73.9	75.5	71.2	76.0	64.0
1,3,6-Tri-*O*-galloyl-glu [**33**]	95.6	72.3	78.8	69.4	75.8	64.0
Casuarictin [**3**]	92.4	76.0	77.3	69.3	73.5	63.1
Rugosin A [**34**]	94.2	72.2	73.6	71.2	73.6	63.6
Rugosin C [**35**]	92.8	76.4	77.6	69.8	74.0	63.5
Tellimagrandin II [**36**]	93.8	71.8	73.3	70.8	73.1	63.1
Strictinin [**37**]	95.9	74.7	75.6	72.8	73.2	63.7
Potentillin [**38**]	90.7	74.1	76.0	69.1	71.0	63.2
Sanguiin H-4[b] [**39**]	91.4	74.5	79.1	68.0	76.6	61.8
Gemin A [**28**]	90.6	74.1	75.9	68.9	71.2	63.1
	93.9	71.5	73.1	70.6	73.1	63.0

[a]glu = β-D-glucopyranose.
[b]Measured in MeOH-d_4.

Hydrolyzable tannins with $^4C_1$ glucose residue.—Tannins of this type include gallotannins and most of the ellagitannins (monomers and oligomers).

The ^{13}C resonances of the glucose moiety in the tri- and tetragalloylglucoses, as well as ellagitannin monomers in which the anomeric center is acylated, have been assigned by the ^1H-^{13}C single frequency selective decoupling (PSD) technique or the ^1H-^{13}C COSY measurements on a high resolution nmr instrument which provided a fairly well-resolved signal of each glucose proton in the ^1H-nmr spectra (200–500 MHz). Based on these assignments (Table 1), the following valuable findings for the structural analysis of new tannins were obtained (59,60).

General acylation shifts can be applicable to a series of polygalloylglucoses and ellagitannins, although the shift values are variable depending on the position of the acyl group (Scheme 5). The mean values of galloylation shifts are: α-carbon, +0.2–1.2



	R^1	R^2	R^3	R^4	R^5	R^6
1,2,3,6-Tetra-*O*-galloyl-glu[a] [**29**]	O-G	H	G	G	H	G
1,2,4,6-Tetra-*O*-galloyl-glu [**30**]	O-G	H	G	H	G	G
1,2,3-Tri-*O*-galloyl-glu [**31**]	O-G	H	G	G	H	H
1,2,6-Tri-*O*-galloyl-glu [**32**]	O-G	H	G	H	H	G
1,3,6-Tri-*O*-galloyl-glu [**33**]	O-G	H	H	G	H	G
Rugosin A [**34**]	O-G	H	G	G	(*S*)-val	
Rugosin C [**35**]	O-G	H	(*S*)-HHDP		(*S*)-val	
Tellimagrandin II [**36**]	O-G	H	G	G	(*S*)-HHDP	
Strictinin [**37**]	O-G	H	H	H	(*S*)-HHDP	
Potentillin [**38**]	H	O-G	(*S*)-HHDP		(*S*)-HHDP	
Sanguiin H-4 [**39**]	H	O-G	(*S*)-HHDP		H	H

SCHEME 5.

ppm, β-carbons, −1.4–−2.8 ppm, δ- and γ-carbons, almost unaffected. The mean values of hexahydroxydiphenoylation shifts are: α-carbons, +1.3–1.7 ppm, β-carbons, ca. 3 ppm. These shift values can be used as the additivity parameters in assigning the individual carbon resonances.

Significant differences between the effect on the β-carbon induced by galloylation at C-2 (C-1, −0.2 ppm) and that induced by hexahydroxydiphenoylation at C-2 and C-3 (C-1, −3.5 ppm) led to a diagnostic value of the C-1 chemical shift, which is useful in distinguishing the substituent at C-2, as summarized in Scheme 6 (59). It is noteworthy that these diagnostic values are independent of the substituents at C-3–C-6. These findings thus provide a basis for facile assignment of the substitution mode at C-1 and C-2 in the molecules of newly isolated tannins of this class.

Reflecting the difference of the effects on the α- and β-carbons upon galloylation and hexahydroxydiphenoylation, sequences of the individual signals of glucose in hydrolyzable tannins differ from each other (Table 2).

The ^1H-^{13}C long-range 2D-nmr spectra often give useful information concerning

[a]glu = β-D-glucopyranose.

TABLE 2. Relative Positions of the ^{13}C Resonances of the $^4C_1$-Glucose Residue in Hydrolyzable Tannins Having Different Acyl Groups.

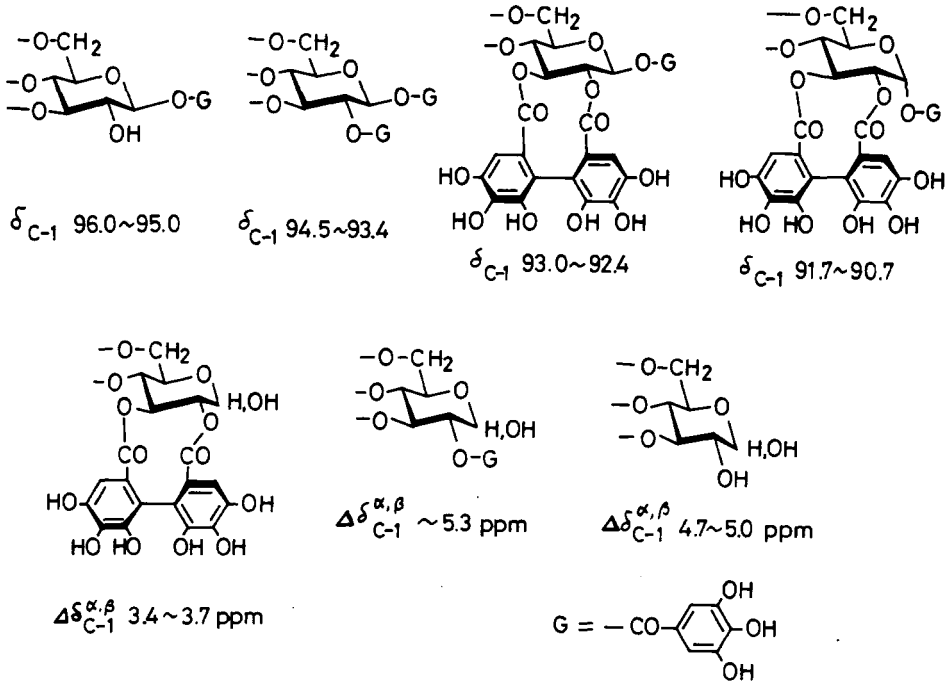
Compound	Sequence of carbon signals
	Lower field ←
Pentagalloylglu[a] [**5**]	C-1 ← C-5 ← C-3 ← C-2 ← C-4 ← C-6
Casuarictin [**3**]	C-1 ← C-3 ← C-2 ← C-5 ← C-4 ← C-6
Tellimagrandin II [**36**]	C-1 ← C-3 ← C-5 ← C-2 ← C-4 ← C-6

[a]glu = β-D-glucopyranose.

locations of acyl groups on the glucose residue. As shown in Figure 9, signals due to H_B and H_C of a valoneoyl group in rugosin A [**34**] were correlated with those of H-6 and H-4 in the glucose core, through their three-bond long-range couplings (J_{CH} = 10 Hz) with the ester carbonyl carbon signals, to establish the orientation of the valoneoyl group on the glucose residue (61,62).

Because most oligomeric hydrolyzable tannins isolated from various plants to date have the glucose cores in the $^4C_1$ conformation, the ^{13}C-nmr data of the monomers described above have been applied to effect facile structural analysis of the oligomers, mainly by simple addition and subtraction of signals in each spectrum, between the oligomer under investigation and biogenetic monomeric units. For example, gemin A [**28**] is regarded as a dimer produced by intermolecular C-O oxidative coupling between galloyl groups at C-1 of potentillin [**38**] and tellimagrandin II [**36**] (54). Comparison of **28** with the monomeric units of **38** and **36** allowed precise characterization of the monomeric constituents of the dimer. Analogous correlations have been observed in the other oligomers and their monomeric units (59).

The ^{13}C-nmr spectral analysis was also applied to the tannins existing as anomer mixtures (60), such as tellimagrandin I [**13**] and pedunculagin [**22**], which show two peaks in their respective hplc charts, as described before. Accumulated data for the tan-



SCHEME 6.

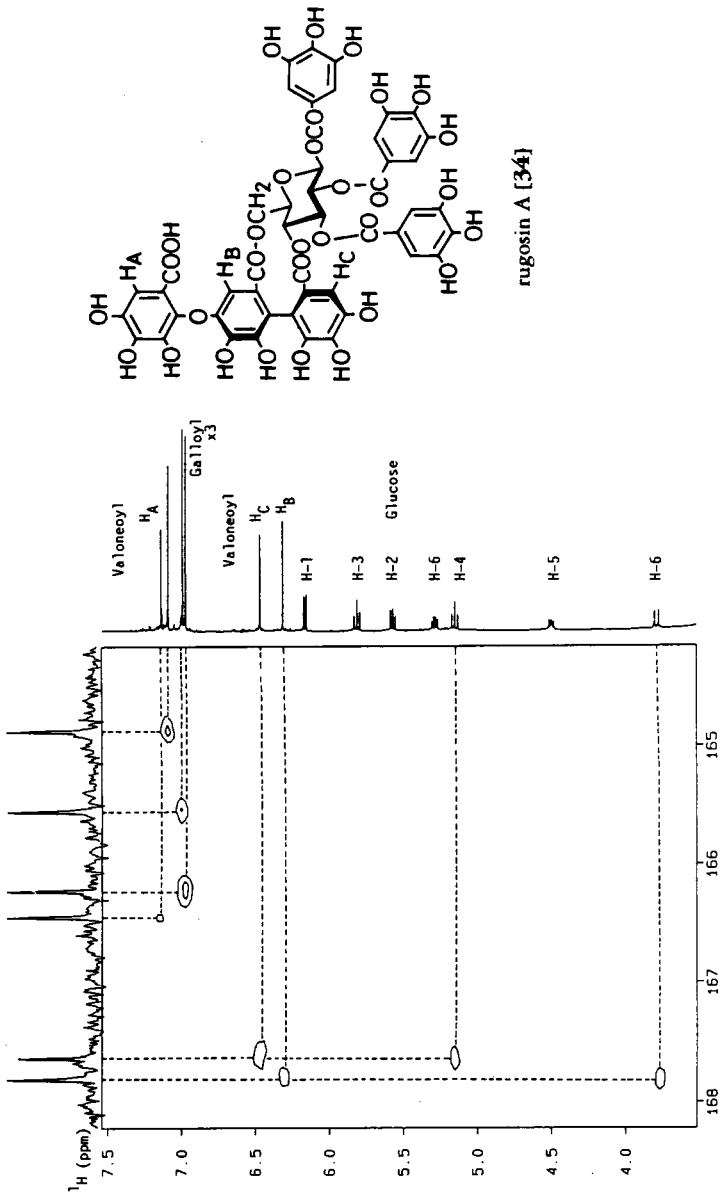


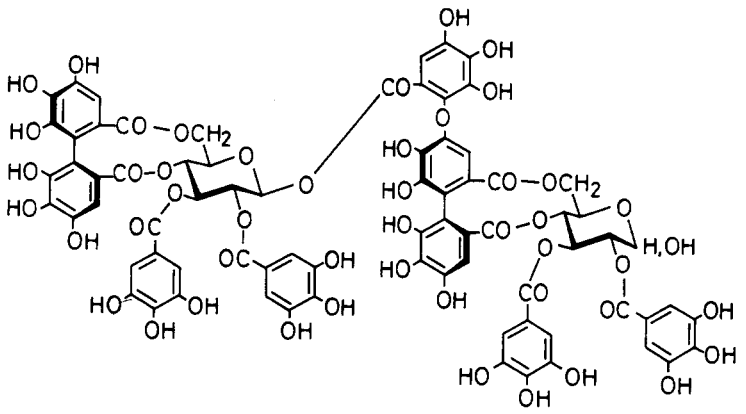
FIGURE 9. Contour map of the ^{1}H-^{13}C long-range 2D nmr spectrum of rugosin A [34].

TABLE 3. ^{13}C-nmr Spectral Data for Glucose Residue in Hydrolyzable Tannins Existing as α,β-Anomer Mixture (100 MHz).

Compound	α-Anomer						β-Anomer					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
Tellimagrandin I [**13**]	91.2	72.9	71.1	71.1	67.2	63.5	96.7	74.1	73.5	71.1	72.0	63.5
Pedunculagin [**22**]	91.8	75.6	75.8	69.9	67.4	63.6	95.4	78.3	77.6	69.6	72.5	63.6
Gemin D[a]	94.0	72.1	74.0	71.0	67.5	63.8	98.4	74.8	75.9	71.3	72.0	63.8
Rugosin E [**40**]												
Glucose core L[b]							93.1	71.6	73.1	70.5	72.8	62.9
Glucose core R[c]	91.1	72.8	71.2	71.0	66.7	63.4	96.5	74.0	73.5	71.0	71.7	63.4

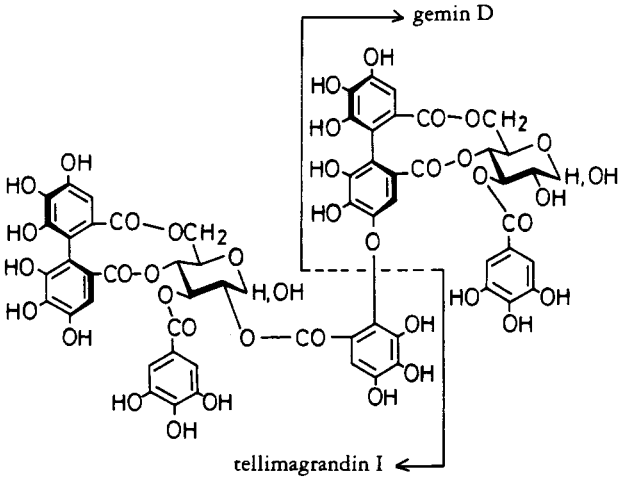
[a]Gemin D = 3-*O*-galloyl-4,6-(*S*)-hexhydroxydiphenoyl-D-glucose.
[b]Left glucose core in formula **40**. The signals of the glucose core L of both anomers overlap because of the anomerization of the glucose core R.
[c]*Right glucose core in formula* **40**.

nins of this class indicate that the difference ($\Delta\delta_{C\text{-}1}^{\alpha,\beta}$) between the chemical shifts of the α- and β-anomeric carbon signals also gives information concerning the substituent (galloyl, HHDP, or hydroxyl groups) at C-2 (59) (Scheme 6). Although the ^{13}C-nmr spectra of dimeric and trimeric hydrolyzable tannins existing in two to eight anomeric mixtures are complicated, information obtainable from the above-mentioned diagnostic values and comparisons of the signals with those in fully assigned spectra of the probable monomeric precursors allow assignment of the locations of the acyl groups in these oligomers, without performing degradative experiments (Table 3). The application of this method of structural analysis to camptothin A [**41**] (61,63) (Scheme 7), which was recently isolated from *Camptotheca acuminata*, is shown in Figure 10.



40 rugosin E

Hydrolyzable tannins with $^1C_4$ glucose residue.—This type of tannins includes geraniin [**10**] (36), corilagin [**15**] (64), chebulagic acid [**27**] (52), and others. These tannins have ester linkages bridged at O-3–O-6 and/or O-2–O-4, which causes conformational change of the glucopyranose core from $^4C_1$ to $^1C_4$ or related boat conformation, as evidenced from the change of coupling pattern of each proton in the ^{1}H-nmr spectra. The



41 camptothin A

SCHEME 7.

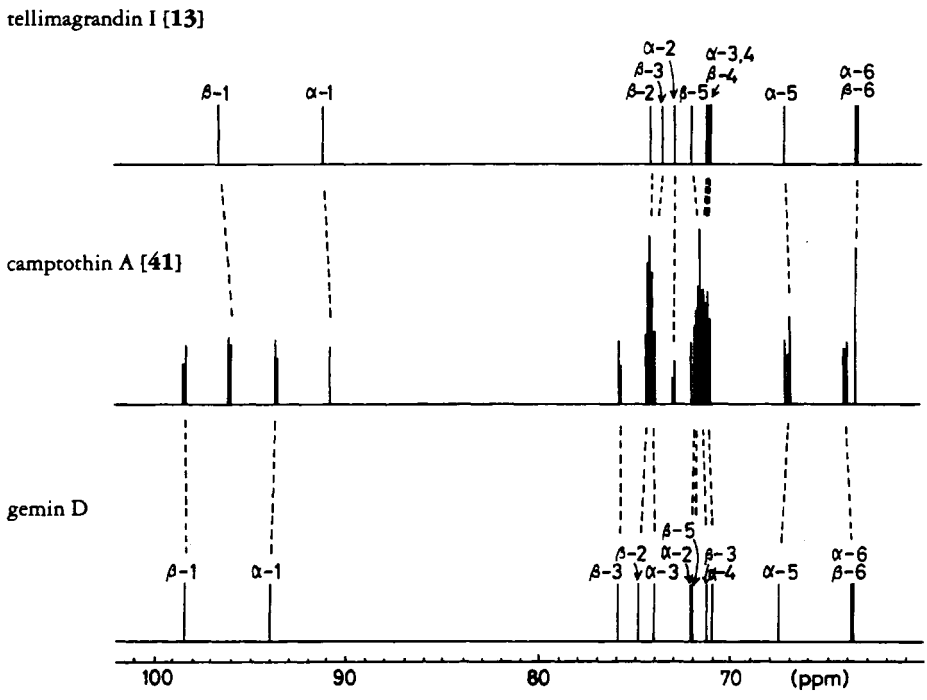


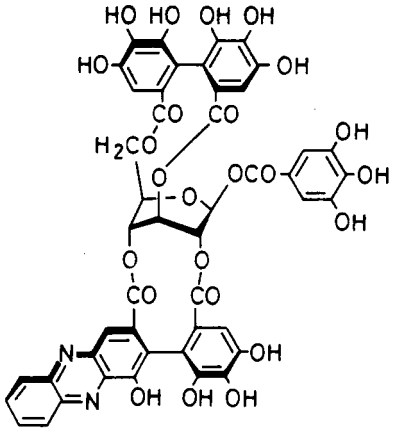
FIGURE 10. ^{13}C-nmr spectral comparison of camptothin A [**41**] with tellimagrandin I [**13**] and gemin D.

assignment of each proton of the glucose residues of this type of tannin is often difficult because of small vicinal couplings of H-1–H-4 and the presence of long-range coupling due to W-arrangements between H-1–H-3 and H-3–H-5. However, the nOe and exchange spectroscopy (NOESY), which does not show the cross peaks for the long-range couplings, is advantageous for straightforward assignments of each proton (65) (Table 4).

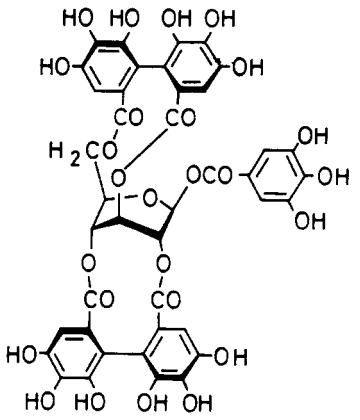
Two dimeric tannins, euphorbins A [**44**] and B, composed of geraniin [**10**] and pentagalloylglucose [**5**], have recently been isolated from *Euphorbia hirta* (66). Although their ^{1}H- and ^{13}C-nmr spectra are complicated by the existence of a geraniin part as the equilibrium mixture of six- and five-membered hemiacetal forms, their phenazine derivatives produced by condensation with *o*-phenylenediamine showed simplified spectra. The ^{13}C resonances of each glucose residue in the phenazine derivative

TABLE 4. ^{13}C-nmr Spectral Data for Glucose Residue Adopting $^{1}C_4$ or a Related Boat Conformation in Hydrolyzable Tannins and Related Compounds.

Compound	C-1	C-2	C-3	C-4	C-5	C-6
Corilagin [**15**]	94.2	68.8	70.4	62.2	75.5	64.3
Geraniin [**10**]						
(a) form	90.8	69.9	63.3	65.9	72.6	63.6
(b) form	91.8	70.4	62.3	66.8	73.1	63.8
Chebulagic acid [**27**]	91.5	70.5	61.7	66.1	73.5	63.9
Geraniin phenazine [**42**]	91.6	76.6	68.7	67.6	76.8	65.2
1-*O*-Galloyl-2,4;3,6-bis-*O*-hexahydroxydiphenoyl-β-D-glucose [**43**]	91.9	76.0	67.7	67.7	76.9	65.3



42 geraniin phenazine



43 1-*O*-galloyl-2,4;3,6-bis-*O*-hexahydroxydiphenoyl-β-D-glucose

45 of euphorbin A coincided well with those of **42** and **5** to effect elucidation of their structures (Figure 11).

C-*Glucosidic ellagitannins*.—In some species of plants, hydrolyzable tannins possessing the $^4C_1$ glucose cores are metabolized into the *C*-glucosidic tannins having glucose residue of open chain form (40,57), as shown in the structures of casuarinin [**46**] (58) and stachyurin [**47**] (58). Some of them are further metabolized into complex tannins (e.g., guavin A) by condensation with catechin or its analogs. The glucose carbon signals of these tannins have also been completely assigned with the aid of 2D nmr spectroscopy (65) (Table 5). The characteristic feature of the spectra of these *C*-glucosidic tannins is the chemical shift of the C-2 signal, which appears at the lowest field among the glucose carbon signals and is dependent on the configuration at C-1. Thus, it is observed at ca. δ 76 when the configuration at C-1 is β and at ca. 81 when the configuration is α, and can be utilized in differentiating the stereochemistry at C-1 of the open-chain glucose core in the newly isolated *C*-glucosidic tannins.

Circular dichroism (cd) spectral analysis.—Determination of the chirality of the HHDP group and related biphenyl groups is one of the main problems in structural analysis of ellagitannins. The dextrorotatory dimethyl hexamethoxydiphenate [**48**] was established to have the (*R*) configuration (36) on the basis of the identity of its dihydroxy congener, which was prepared by the $LiAlH_4$ reduction of **48**, with that derived from schizandrin in which the absolute configuration of biphenyl group was determined to be (*R*) (67). The absolute configuration of the HHDP group was determined by measurement of the specific rotation of **48** obtained by methanolysis of methylated tannins. However, accumulated cd data of a series of tannins and related compounds, for which the absolute configuration of the HHDP group had been determined by the above-mentioned chemical method, brought a convenient empirical rule for determining the absolute structures of ellagitannins and also of gallotannins without performing chemical degradations (68).

The cd spectra of (*R*)-**48** and (*S*)-**48** exhibited strong Cotton effects at 225 and 250 nm, of the sign opposite to each other (Figure 12). These Cotton effects are associated with the biphenyl conjugation bands in the uv spectra and are indicative of the absolute configuration of the HHDP group: the positive 250 nm Cotton effect corresponds to the *R* configuration and the negative one to the *S* configuration. The cd spectra of ellagitannins of known stereostructures such as tellimagrandin I [**13**], corilagin [**15**], pedunculagin [**22**], and tellimagrandin II [**36**] exhibited three Cotton effects around

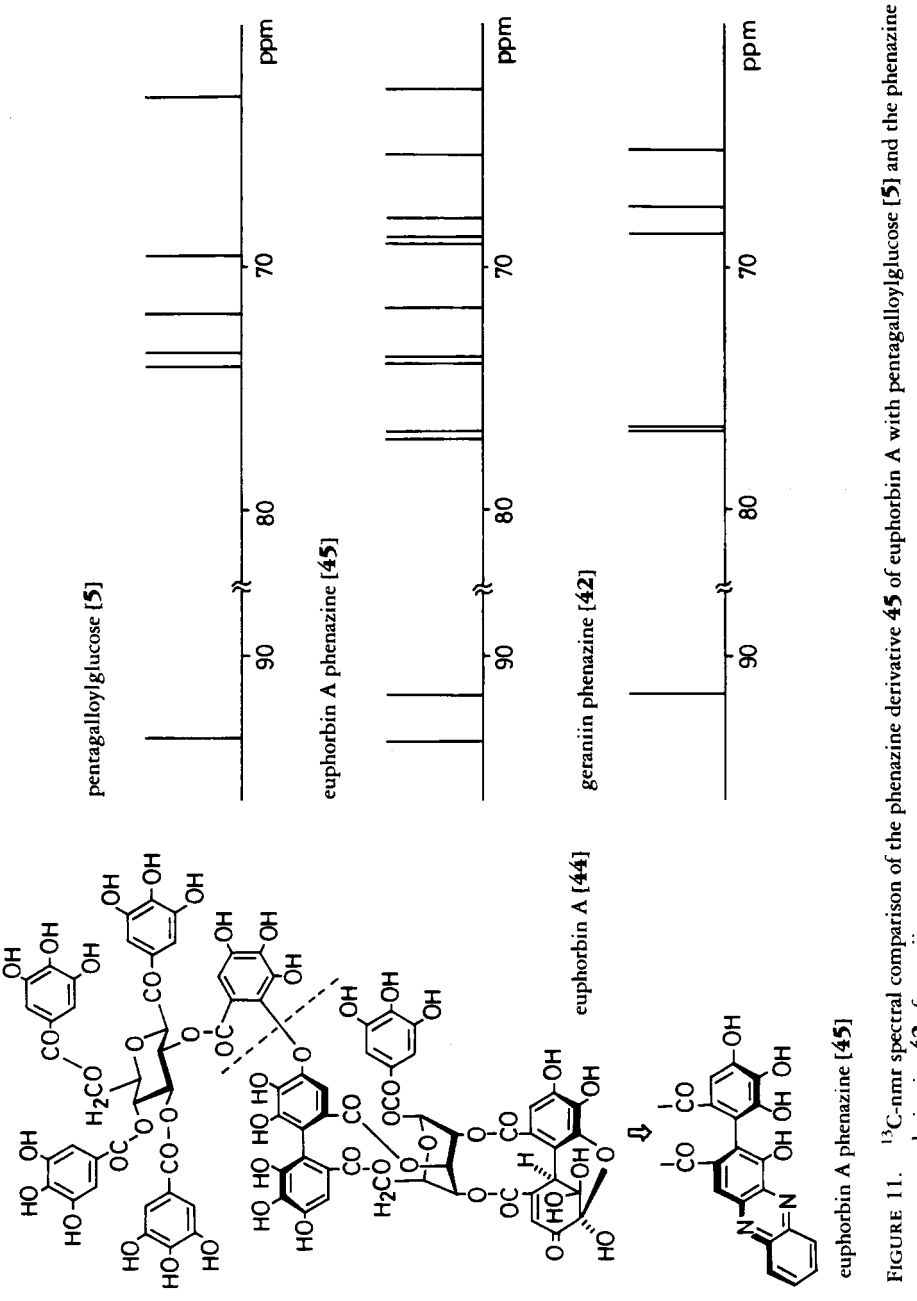


FIGURE 11. ^{13}C-nmr spectral comparison of the phenazine derivative **45** of euphorbin A with pentagalloylglucose [**5**] and the phenazine derivative **42** of geraniin.

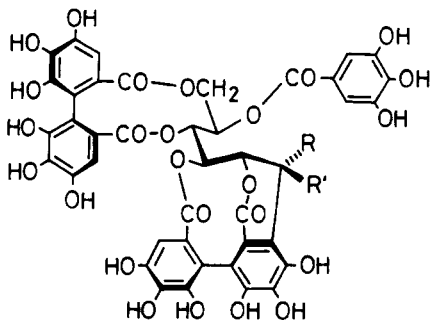
TABLE 5. ^{13}C-nmr Spectral Data for Glucose Residue in C-Glucosidic Tannins and Complex Tannins.

Compound	C-1	C-2	C-3	C-4	C-5	C-6
Casuarinin [**46**][a]	67.6	76.7	69.8	74.2	71.2	64.6
Stachyurin [**47**]	65.5	81.0	70.9	73.3	72.0	64.5
Casuariin	68.5	77.1	70.8	77.1	68.5	67.2
Guavin A[a]	46.5	81.2	75.8	71.2	71.7	64.4

[a]Assignments are based on the ^{1}H-^{13}C correlation spectra.

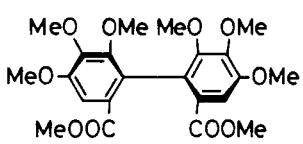
235, 265, and 285 nm (Figure 13) among which the former two of opposite signs are associated with the chirality of the HHDP group, although they show small bathochromic shifts due to decrease of the twist angle.

The Cotton effect around 285 nm, with somewhat smaller amplitude, is regarded as one of the split-type Cotton effects centered near 275 nm, arising from the intramolecular charge transfer transition between adjacent galloyl groups and/or HHDP group(s). This assignment was confirmed by comparison with the cd spectra of a series of positional isomers of tri- and tetragalloylglucoses as well as pentagalloylglucose [**5**], which showed split Cotton effect centered near 273 nm, which is analogous to that of methyl 2,3-di-*O*-benzoyl-α-D-glucoside, with red shift of ca. 40 nm due to the

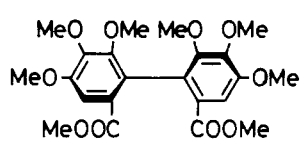


46 R=H, R′=OH casuarinin
47 R=OH, R=H stachyurin

polyphenolic structure (Table 6). The sign of the first Cotton effect around 285 nm is consistent with the sum of the chiralities among the galloyl groups, which are expected to be effected on the basis of the exciton chirality rule (69). It is noteworthy that the 235 nm peak was absent in the cd spectra of these galloylglucoses. The Cotton effect around 235 nm is, therefore, diagnostic for the absolute configuration of the HHDP and/or valoneoyl group in the ellagitannins, i.e., positive for the (*S*) configuration and negative for the (*R*) configuration. This correlation apparently exists regardless of the presence or absence of the galloyl groups or the conformation of the glucose residue. The amplitude of this peak also reflects the number of HHDP (or valoneoyl) groups in a molecule, as indicated by the cd spectra of monomeric and oligomeric ellagitannins (Table 6).



(*R*)-**48**



(*S*)-**48**

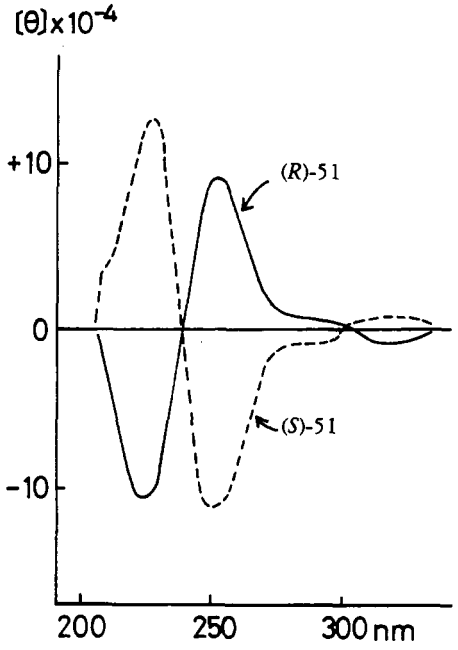


FIGURE 12. Cd spectra of (*R*)-**51** and (*S*)-**51** in MeOH.

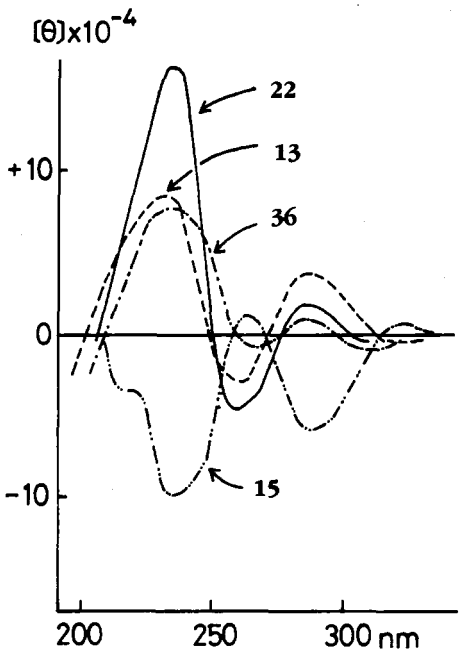


FIGURE 13. Cd spectra of tellimagrandin I [**13**], corilagin [**15**], pedunculagin [**22**] and tellimagrandin II [**36**] in MeOH.

The absolute configuration at the methine center of the DHHDP group in the dehydroellagitannin molecules has also been empirically determined by the cd spectra as follows (70).

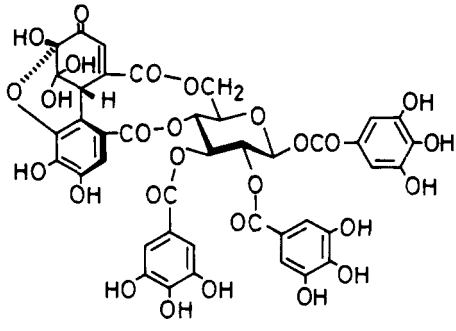
The absolute configuration at C-1′ of the DHHDP group in a dehydroellagitannin molecule was first established for geraniin [**10**] (*R* configuration) (36) and isoterchebin [**49**] (*S* configuration) (71), based on the specific rotation of the products from methanolysis after methylation of the phenazine derivatives **42** and **51** produced from each tannin.

TABLE 6. Cd Spectral Data[a] of Hydrolyzable Tannins and Related Compounds.

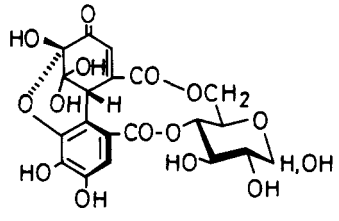
Compound	Cotton effect, $[\theta] \times 10^{-4}$ (nm)		
R-Dimethyl hexamethoxydiphenate [**48**]	+4.8 (250)	−5.5 (225)	
S-Dimethyl hexamethoxydiphenate [**48**]	−5.6 (250)	+6.7 (225)	
2,3-*O*-[(*S*)-HHDP[b]]-D-glucose	−0.1 (287)	−2.7 (262)	+10.2 (235)
4,6-*O*-[(*S*)-HHDP]-D-glucose	+0.2 (286)	−2.2 (264)	+ 8.9 (237)
3,6-*O*-[(*R*)-HHDP]-D-glucose	−0.4 (290)	+2.4 (256)	− 8.5 (236)
Corilagin [**15**]	−6.6 (284)	+1.7 (262)	−10.3 (237)
Tellimagrandin II [**36**]	+0.8 (285)	−1.0 (264)	+ 8.1 (235)
Pedunculagin [**22**]	+2.1 (282)	−5.4 (259)	+16.8 (233)
Casuarictin [**3**]	+0.7 (281)	−5.3 (261)	+20.2 (234)
Rugosin A [**34**]	+4.9 (281)	−2.3 (256)	+15.8 (224)
Methyl 2,3-*O*-dibenzoyl-α-D-glucoside		+5.1 (233)	− 1.6 (217)
Methyl 2,3-*di*-*O*-*galloyl*-α-D-*glucoside*	+3.0 (284)	−1.7 (260)	+ 3.1 (215)
1,2,3-Tri-*O*-galloyl-β-D-glucose [**31**]	+1.1 (280)	−0.1 (257)	
Gemin A [**28**]	+6.5 (283)	−6.8 (262)	+33.5 (235)
Rugosin E [**40**]	+9.6 (282)	−5.3 (258)	+22.4 (224)

[a]Measured in MeOH.
[b]HHDP = hexahydroxydiphenoyl.

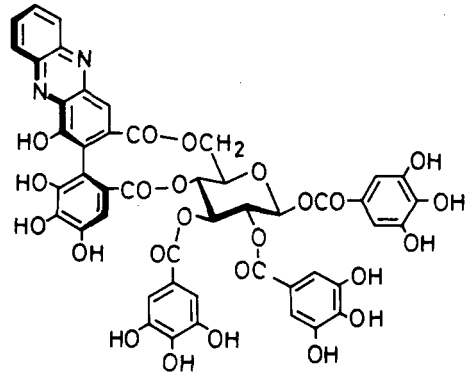
The cd spectrum of 4,6-*O*-[(1′*S*)-DHHDP]-D-glucose [**50**], which was prepared by degalloylation of isoterchebin [**49**] with tannase, exhibited positive Cotton effects at 375 and 237 nm along with negative one at 206 nm. These three Cotton effects are ascribable to the n-π* transition, and the first (K-band) and second π-π* transition of α,β-unsaturated ketone, respectively (72). The positive Cotton effects at 375 and 237 nm are consistent with those of paspalicine (73), whose stereochemistry markedly resembles conformation I of the DHHDP group in isoterchebin [**49**] (Scheme 8).



49 isoterchebin

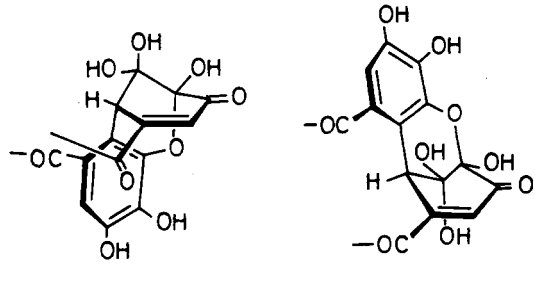


50 4,6-*O*-[(1′*S*)-DHHDP]-D-glucose



51 isoterchebin phenazine

The cd spectrum of geraniin [**10**], which has an HHDP group and a galloyl group and also a (1′*R*)-DHHDP group in the molecule, showed three Cotton effects of the signs opposite to those of **50** at similar wavelength, as shown in Table 7. The peak around 230 nm in **50** is overlapped by one of the Cotton effects due to the HHDP group in **10**. The prominent Cotton effect around 200 nm, of the sign opposite to that at 230 nm, and the peak near 350 nm are available for correlating the cd spectra with the absolute configuration at C-1′ of the DHHDP group. This correlation was successfully applied to the other dehydroellagitannins (70).



(I) (II)

SCHEME 8.

TABLE 7. Cd Spectral Data of Dehydroellagitannins and Related Compounds.

Compound	C-1′	Cotton effect, $[\theta] \times 10^{-4}$ (nm)				
Geraniin [**10**]	(*R*)	−1.0 (361)	− 6.2 (291)	+4.5 (261)	−7.1 (236)	+10.3 (196)
Isoterchebin [**49**]	(*S*)	+0.8 (372)			+4.6 (228)	− 7.4 (210)
4,6-*O*-DHHDP[a]-D-glucose [**50**]	(*S*)	+0.9 (375)			+2.6 (237)	− 7.1 (206)
Geraniin phenazine [**42**]			−14.2 (280)	+8.8 (250)		− 5.4 (219)
Isoterchebin phenazine [**51**]			+ 6.2 (275)	−3.4 (241)		+ 5.1 (219)

[a]DHHDP = dehydrohexahydroxydiphenoyl.

The absolute configuration at C-1′ of the DHHDP group was confirmed by the cd spectra of the phenazine derivatives, prepared by condensation of tannins with *o*-phenylenediamine. The (*S*)-phenazine derivative **51** from **49** showed significant Cotton effects, which are positive at 275 nm and negative at 241 nm, while the (*R*)-phenazine derivative **42** from **10** gave Cotton effects of opposite signs (Table 7). This correlation was observed without exception in the cd spectra of the other phenazine derivatives (70). As the absolute configurations, (*R*) and (*S*), of the phenazine moiety were proved to be the same as the (*R*) and (*S*) configurations at C-1′ in the DHHDP group of the original tannins, the strong Cotton effects around 245 and 280 nm in the cd spectra of dehydroellagitannins directly indicate the stereochemistry of the DHHDP group.

These cd spectral correlations are useful for facile determination of the absolute configurations in a newly isolated hydrolyzable tannins.

Electron spin resonance (esr) spectral analysis.—Tannins and related polyphenols have recently been found to exhibit significant inhibitory effects on lipid peroxidation in rat liver mitochondria and microsomes (16), Cu(II)-catalyzed autoxidation of ascorbic acid (21), and lipoxigenase in arachidonate metabolism (19,20), as described before. These inhibitory effects of tannins were associated with the radical scavenging activity of tannins by the esr spectra as follows. In an experimental model system of lipid peroxidation, autoxidation of methyl linoleate that was initiated by photoirradiation in the presence of 2,2′-azobisisobutylonitrile was significantly inhibited by tannins, while polyphenols with low molecular weight, such as gallic acid [**1**], (+)-catechin [**7**], etc., showed minor inhibitory effects. Kinetic study for this autoxidation indicated that the inhibitory activities of tannins are dependent on both the type of phenolic groups and their number in the molecule, and tannins act as radical scavenger of chain radical reaction of autoxidation (27). The strength of radical scavenging activity of the phenolic group was in the order HHDP group > galloyl group > DHHDP group. This radical scavenging mechanism was verified by the formation of stable radicals of tannins, which was exhibited by the esr measurements. The esr spectrum of geraniin [**10**], which has a galloyl, an HHDP and a DHHDP group in a molecule, exhibited a doublet signal which is analogous to that of pedunculagin [**22**], and, hence, ascribable to that of the HHDP group. In situ esr measurement, under the inhibition condition of autoxidation mentioned above, showed similar signals of geraniin [**10**], again indicating that the HHDP group forms the most stable radical and acts as an effective radical scavenger of the chain-carrying peroxy radicals (27). Inhibitory effects of caffeetannins and related compounds on the lipoxigenase-dependent peroxidation of linoleic acid were also revealed to be operated by similar radical scavenging mechanisms, by detection of stable radicals derived from the one-electron oxidation of dihydroxyphenyl group in the esr spectrum (18).

ACKNOWLEDGMENTS

Some parts of the work presented here have been carried out under collaboration with Prof. Y. Fujita, Faculty of Pharmaceutical Sciences, Okayama University (esr), Prof. T. Shingu, Faculty of Pharmaceutical Sciences, Kobe Gakuin University (nmr), Prof T. Koga, Daiichi College of Pharmacy, and Dr. N. Toh, Faculty of Engineering, Kyushu Kyoritsu University (cd). A part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Sciences and Culture, Japan. We are grateful to them for their assistance.

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