Chem. Pharm. Bull. **39**(10) 2638—2643 (1991) Vol. 39, No. 10

Enzymatic Sulfation of Polyphenols Related to Tannins by Arylsulfotransferase

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This report discusses a novel type of arylsulfotransferase (AST) which was derived from human intestinal bacterium sulfated polyphenolic compounds when *p*-nitrophenyl sulfate (PNS) was taken as a donor substrate. (+)-Catechin, (±)-catechin, (−)-epicatechin and (−)-epicatechin gallate were better substrates than tyramine. (−)-Epigallocatechin and (−)-epigallocatechin gallate were slightly worse substrates than tyramine. Although gallic acid was a bad substrate, alkyl gallate esters were better substrates than tyramine. The degree of acceptor specificity increased in proportion to the length of the alkyl group up to the carbon number of five. Pedunculagin, geraniin and corilagin were less effective than tyramine. Rosmarinic acid and penta-*O*-galloyl-β-D-glucose were similarly well sulfated. Two products, 4′-monosulfate and 4′,5-disulfate of (+)-catechin, were detected at a two-fold molar excess of PNS over (+)-catechin. When (+)-catechin-4′-monosulfate as an acceptor was enzymatically sulfated with PNS as a donor, only the 4′,5-disulfate was produced. Thus, arylsulfotransferase was useful for the convenient preparation of sulfate esters of polyphenols at their specific hydroxyl groups.

Keywords arylsulfotransferase; (+)-catechin; polyphenol; sulfate conjugation; intestinal bacteria

Introduction

Sulfate conjugation is widely known as one of the major metabolic pathways for the detoxification of endogenous and exogenous phenolic compounds. Arylsulfotransferase (EC 2.8.2.1) was first discovered in guinea pig liver,[1] and thereafter was found in the brain, kidney, intestinal epithelial cells and so on.[2-4] The enzyme catalyzes the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to aliphatic and phenolic alcohol groups, and forms sulfate conjugates. A similar type of enzyme was also found in the plant kingdom.[5]

Tannins are polyphenolic compounds of various chemical structures and are divided into two main classes, hydrolyzable tannins and condensed tannins. Several studies have been reported on their complexation with proteins or metal ions, and on their biological activities which involved the inhibitory effect of (−)-epigallocatechin gallate on protein kinase C[6] and carcinogenesis.[7]

We discovered a novel type of arylsulfotransferase from the human intestinal bacterium, *Eubacterium* A-44.[8] This enzyme catalyzes the transfer of sulfate groups from phenol sulfate esters, but not from PAPS, to other phenols with strict specificity. The bacterial enzyme is several thousand-fold more active than the enzymes from mammalian and plant origins, and is expected to be useful for the preparation of sulfated phenols.

We have already reported that this enzyme effectively sulfates polyphenols, such as chalcones, xanthones and flavones.[9] Therefore, human intestinal bacteria may play an important role in the sulfation of flavones and tannins ingested as components of vegetable food. The present paper describes the enzymatic sulfation of monomeric polyphenols such as gallic acid and catechin derivatives, and also of some condensed tannins, by the bacterial arylsulfotransferase, and the position of sulfated hydroxyl groups of (+)-catechin used as a typical example.

Materials and Methods

Chemicals *p*-Nitrophenyl sulfate (PNS) was purchased from Sigma Chemicals, Co. (U.S.A). Tyramine was purchased from Nacalai Tesque (Japan) and diethylaminoethyl (DEAE)-cellulose was from Brown Co. (U.S.A). General anaerobic medium (GAM) broth was from Nissui Seiyaku Co., Ltd. (Japan). Methyl, ethyl, *n*-propyl, *n*-butyl and isoamyl gallate were supplied from Fuji Chemical (Japan), and lauryl and stearyl gallate were purchased from Tokyo Kasei (Japan). Rosmarinic acid (from *Perilla frutescens* BRITTON var. *crispa* DECNE.),[10] pedunculagin (from *Casuarina stricta* AIT.),[11] pentagalloylglucose (=1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, from gallotannin mixture in Chinese gall),[12] geraniin (from *Geranium thunbergii* SIEB. *et* ZUCC.)[13] and corilagin (from *G. thunbergii*)[13] were prepared according to cited references.

Gallic acid, ellagic acid, chlorogenic acid, (+)-catechin and (±)-catechin were purchased from Sigma Chemical, Co. (U.S.A.). (−)-Epicatechin was purchased from Aldrich Chemical Co., Inc. (U.S.A.), (−)-epicatechin gallate, (−)-epigallocatechin and (−)-epigallocatechin gallate were from Kurita Ind., Ltd. (Japan). All other chemicals were of analytical reagent grade.

Partial Purification of Arylsulfotransferase *Eubacterium* A-44, isolated from human feces, was cultured under the same procedure as described in our previous report,[8] except that 1 mM PNS was added to the culture medium as an inducer in place of phenolphthalein disulfate.[14] The crude extract of the enzyme was prepared according to the method described in our previous report[15] and did not exhibit any arylsulfatase activity when PNS was used as a substrate. The extract was applied to a column of DEAE-cellulose and partially purified under the same procedure.[9] The resulting enzyme solution (12.6 units/ml, 29.8 mg protein/ml) was used for the sulfation of polyphenols. The assay method and the definition of the enzyme activity were described in our previous reports.[8,15]

Enzymatic Sulfation of Polyphenols Tannins and related polyphenols were dissolved in dimethylsulfoxide (DMSO). A reaction mixture contained 0.17 ml of 1 mM acceptor, 0.034 ml of 200 mM PNS, 0.048 ml of 25 mM L-ascorbic acid, 0.048 ml of 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) and 0.7 ml of 0.1 M Tris–HCl buffer (pH 8.0). The reaction was started by the addition of 0.1 ml of arylsulfotransferase preparation (12.6 units/ml) to the reaction mixture, which was incubated for 15 min at 37 °C. The final concentration of EDTA (48 μM) had little effect on the enzyme activity. The initial velocity of the sulfation reaction was measured by monitoring absorbance at 405 nm and the acceptor activity was obtained by comparison with the enzymatic activity using tyramine as a control acceptor.

Enzymatic Preparation of Sulfated (+)-Catechin A reaction mixture contained 17 ml of 20 mM (+)-catechin, 3.4 ml of 200 mM PNS, 4.8 ml of 25 mM L-ascorbic acid, 4.8 ml of 1 mM EDTA-2Na, 70 ml of 0.1 M Tris–HCl buffer (pH 8.0) and 10 ml of arylsulfotransferase preparation (12.6 units/ml). The mixture was incubated for 24 h at 37 °C. The reaction mixture was lyophilized and the resulting residue was subjected to preparative paper partition chromatography (PPC) through the same procedure in our previous report,[9] except that *n*-BuOH–EtOH–H_2O (5:1:4, v/v/v) was used as a solvent system in place of *n*-BuOH–AcOH–H_2O (4:1:2, v/v/v).

Structural Determination of Catechin Sulfates Nuclear magnetic resonance (NMR) spectra were measured on a JEOL JNM-GX 400 spec-

trometer in acetone-d_6, dimethylsulfoxide-d_6, or methanol-d_4 solutions using tetramethylsilane as an internal standard. Fast atom bombardment mass spectrum (FAB-MS) was obtained with a JEOL JMS-SX 102 spectrometer.

Analysis of the Reaction Products by Two-Dimensional Cellulose Thin-Layer Chromatography (TLC) Electrophoresis The reaction products were spotted on a cellulose TLC plate (Merck, 10 × 20 cm) and developed with *n*-BuOH–EtOH–H_2O (5 : 1 : 4, v/v/v). The plate was air-dried and subjected to high voltage electrophoresis for 60 min in an HCOOH–AcOH H_2O buffer (33 : 147 : 1820, v/v/v), pH 1.9 at 1.5 kV by electrophoretic combined equipment (Bio-Rad Power Supply Model 3300, Pharmacia Flat Bed Apparatus FBF-3000, mgw Lauda RMT, RM6). After electrophoresis, the plate was dried, spots were detected under a ultraviolet (UV) lamp (365 nm), and then their mobilities were determined.

Results

Bacterial arylsulfotransferase was used for the sulfation of several polyphenols. Some of them tended to be oxidized spontaneously by atmospheric oxygen during the sulfation reaction. Therefore, our spectrophotometric analysis needed to be carried out in the presence of an antioxidant, such as ascorbic acid, which prevented their oxidation most effectively. Because iron chelated ascorbic acid is known to be an oxidant, 48 μM EDTA was added to the reaction mixture in order to eliminate the influence of such undesired oxidation and coloration. Inhibition of the enzyme activity by EDTA at the final concentration was negligible.

Polyphenols in the present investigation, except for gallic acid, ellagic acid and chlorogenic acid, were rapidly sulfated with PNS as a donor substrate, (Table I). (+)-Catechin as well as (±)-catechin, (−)-epicatechin and (−)-epicatechin gallate were better substrates than tyramine, which is used as an acceptor substrate in a standard assay. (−)-Epigallocatechin and (−)-epigallocatechin gallate were slightly worse substrates than tyramine. Alkyl gallate esters, such as methyl-, ethyl-, *n*-propyl-, *n*-butyl-, isoamyl- and lauryl gallate, were better substrates than tyramine. The degree of the acceptor specificity increased in proportion to the length of alkyl group up to the carbon number of five. Lauryl gallate was also sulfated as well, but stearyl gallate was less sulfated among the alkyl gallate esters examined. Pedunculagin, geraniin and corilagin were less effective than tyramine. Rosmarinic acid and penta-*O*-galloyl-β-D-glucose were similarly well sulfated. Numerous condensed tannins are composed of catechin and analogs in various combinations, though there are many exceptions in certain plant species. We had already reported the chemical structures of enzymic sulfated quercetin as a typical flavonoid example.[9] Therefore, in the present study we investigated the position of sulfated hydroxyl groups of catechin as a component of tannins.

A two-fold molar excess of PNS over catechin was incubated in the reaction mixture for 24 h at 37 °C for the preparation of sulfated (+)-catechin. The reaction products were concentrated *in vacuo* until the concentration was appropriate for PPC. Two new spots were detected as products with low mobility in *n*-BuOH–EtOH–H_2O compared with catechin, PNS and *p*-nitrophenol (PNP) (lane 5, in Fig. 1). They were designated in terms of product I (of higher *Rf* value) and product II (of lower *Rf* value). PNP derived from a donor substrate, PNS, was clearly observed along with a small amount of remaining PNS, while no PNP was detected in the absence of the enzyme (lane 4, in Fig. 1). These results suggest that products I and II were

TABLE I. Sulfation of Tannins

Acceptors	Activity (%)
Tyramine	100
Gallic acid[a)]	3.4
Ellagic acid[a)]	6.6
Chlorogenic acid[a)]	2.9
(+)-Catechin[a)]	437
(±)-Catechin[a)]	241
(−)-Epicatechin[a)]	231
(−)-Epicatechin gallate[b)]	194
(−)-Epigallocatechin[b)]	78.8
(−)-Epigallocatechin gallate[b)]	84.0
Methyl gallate	281
Ethyl gallate	420
n-Propyl gallate	438
n-Butyl gallate	467
Isoamyl gallate	487
Lauryl gallate	273
Stearyl gallate	32.4
Pedunculagin	31.3
Corilagin	35.2
Rosmarinic acid	159
Penta-*O*-galloyl β-D-glucose	182
Geraniin	24.2

A reaction mixture contained 0.17 ml of 1 mM acceptor (*a*) 20 mM, *b*) 2 mM) under the standard assay conditions as described in Materials and Methods. Activity for tyramine as an acceptor at the corresponding concentration was taken as 100.

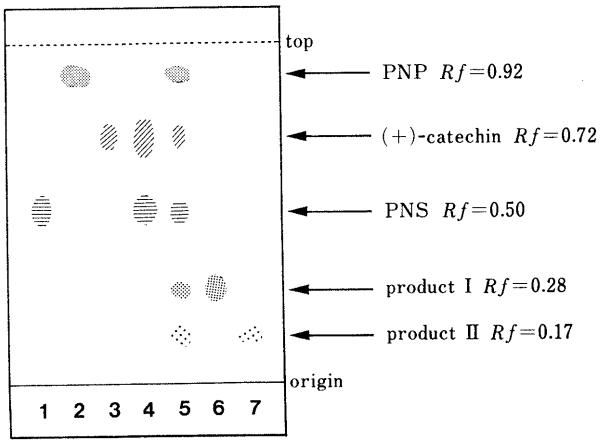

Fig. 1. Paper Partition Chromatogram of Reaction Products

Preparation and analysis of the reaction products were described in Materials and Methods.

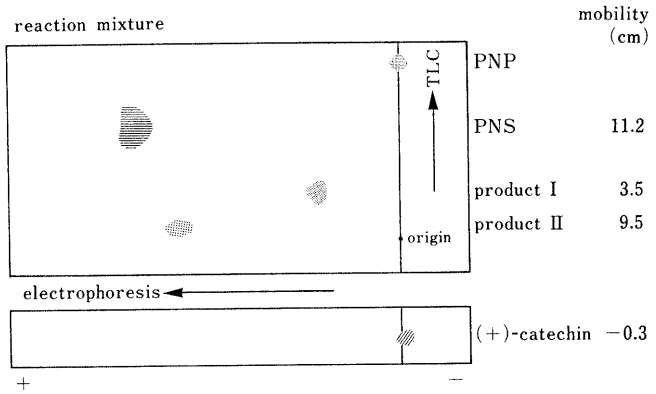

Fig. 2. TLC and Electrophoresis of Reaction Mixture

Solvent system; TLC, *n*-BuOH–EtOH–H_2O (5 : 1 : 4, v/v/v); high voltage thin-layer electrophoresis, 1000 V, 60 min, pH 1.9, HCOOH–AcOH–H_2O (33 : 147 : 1820, v/v/v).

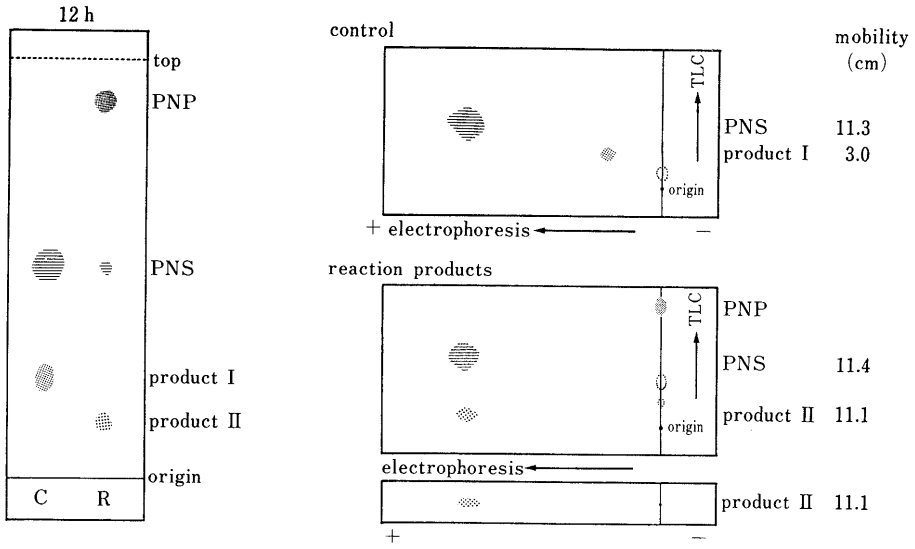

Fig. 3. Sulfation of Product I

C, control, product I+PNS; R, reaction product, product I+PNS+AST. TLC and electrophoresis were carried out under the same procedure described in Fig. 2.

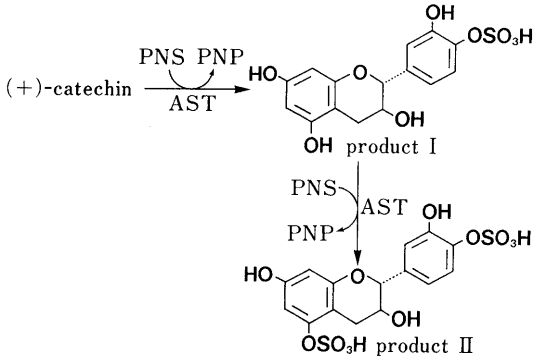

Chart 1. Successive Sulfation of (+)-Catechin by Arylsulfotransferase

sulfated catechin. A mixture of products I and II was prepared in a large scale, and subjected to preparative PPC in the same solvent system. Each band was cut out and extracted with water. The extract was concentrated to dryness. The products were analyzed by PPC with the same development and it was confirmed that they were well separated (lanes 6 and 7, in Fig. 1).

It is known that sulfation causes a change in the polarity of the compound. Thus, the determination of the number of sulfates by electrophoretic experiment would be important to structural elucidation. Two-dimensional development combining cellulose TLC and electrophoresis provided a clearer separation (Fig. 2). Product II was more mobile in the electrophoretograms than product I, although (+)-catechin and PNP were not mobile. The mobilities of products I and II were 3.5 and 9.5 cm to anode, respectively, under the present conditions. Based on these results, it appeared that product II contained more sulfate groups than product I. Therefore, it is assumed that product I is an intermediate between (+)-catechin and product II. Product II was produced from product I when the isolated product I was incubated again for 12 h under the same reaction conditions (Fig. 3). These results suggested that product I was first formed as an intermediate and then product II was formed when PNS was in excess in molar over catechin (Chart 1).

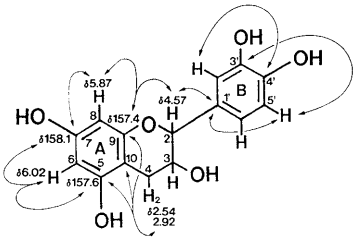

Fig. 4. Significant Long-Range Correlations Observed in the ^{1}H–^{13}C Long-Range COSY Spectrum of (+)-Catechin (**1**) in Acetone-d_6

Prior to analyzing the structure of products I and II, we carried out a re-examination of the proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra of (+)-catechin by means of two-dimensional (2-D) NMR techniques and found that the previous assignments[16,17] of ^{13}C-signals for C-5, C-7 and C-9 must be revised.

In the ^{1}H–^{13}C long-range correlation spectroscopy (COSY) spectrum of (+)-catechin, the quaternary carbon at δ 157.4 (C-9) showed long-range correlations with 4-H_2 (δ 2.45 and 2.92) and 2-H (δ 4.57) and also with the aromatic proton at δ 5.87 (8-H), while the quaternary carbon at δ 157.6 (C-5) exhibited long-range correlations with 4-H_2 (δ 2.54 and 2.92) and with the aromatic proton at δ 6.02 (6-H) (see Fig. 4). It followed that the carbon signals at δ 157.4 and δ 157.6 are unambiguously assigned to C-9 and C-5, respectively, and also the proton signals at δ 5.87 and δ 6.02 to 8-H and 6-H, respectively. On the other hand, the quaternary carbon at δ 158.1 correlated with both 8-H and 6-H, and were thus assigned reasonably to C-7. Some other significant long-range correlations observed are also shown by arrows in Fig. 4. Complete assignments of ^{1}H- and ^{13}C-signals are given in Table II.

Product I exhibited a quasi-molecular ion peak at m/z 369 $[M-H]^-$, corresponding to the molecular formula $C_{15}H_{14}O_9S$, in negative ion fast atom bombardment mass spectrum (FAB-MS). The ^{1}H- and ^{13}C-NMR spectra, analyzed with the aid of ^{1}H–^{1}H COSY and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) techniques, showed marked downfield shifts of ^{1}H- and ^{13}C-

TABLE II. ^{1}H- and ^{13}C-NMR Data for (+)-Catechin (**1**), Product I (**2**) and Product II (**3**) (Coupling Constants in Parentheses)

Position	1				2		3	
	δH[a]	δC[a,c]	δH[b]	δC[b,c]	δH[a]	δC[a,d]	δH[a]	δC[a,d]
2	4.57 d (7.5)	83.6 d	4.57 d (7)	83.1 d	4.61 d (7.6)	83.2 d	4.65 d (7.5)	83.5 d
3	3.98 ddd (8, 7.5, 5.5)	69.6 d	4.01 ddd (8, 7, 5)	68.8 d	3.97 ddd (8, 7.6, 5.5)	69.7 d	3.98 ddd (8, 7.5, 5.5)	69.5 d
4	2.50 dd (16, 8) 2.85 dd (16, 5.5)	29.2 t	2.54 dd (16.6, 8) 2.92 dd (16.6, 5)	29.2 t	2.50 dd (16, 8) 2.84 dd (16, 5.5)	29.3 t	2.71 dd (16.5, 8) 3.09 dd (16.5, 5.5)	29.8 t
5	—	158.3 s	—	157.6 s	—	158.3 s	—	153.5 s
6	5.93 d (2.5)	97.1 d	6.02 d (2.5)	96.6 d	5.92 d (2.2)	97.2 d	6.62 d (2)	104.1 d
7	—	158.6 s	—	158.1 s	—	158.6 s	—	158.3 s
8	5.86 d (2.5)	96.3 d	5.87 d (2.5)	95.9 d	5.86 d (2.2)	96.3 d	6.17 d (2)	101.5 d
9	—	157.7 s	—	157.4 s	—	157.5 s	—	157.2 s
10	—	101.6 s	—	101.1 s	—	101.6 s	—	107.5 s
1′	—	133.0 s	—	132.6 s	—	139.6 s	—	139.4 s
2′	6.84 d (2)	116.0 d	6.90 d (2)	115.7 d	6.95 d (2)	117.9 d	6.96 d (2)	117.9 d
3′	—	147.0 s	—	146.07 s	—	151.1 s	—	151.1 s
4′	—	147.0 s	—	146.13 s	—	141.8 s	—	141.8 s
5′	6.76 d (8)	116.9 d	6.80 d (8)	116.2 d	7.27 d (8)	124.6 d	7.29 d (8)	124.6 d
6′	6.72 dd (8, 2)	120.8 d	6.76 dd (8, 2)	120.5 d	6.85 dd (8,2)	120.5 d	6.87 dd (8, 2)	120.6 d

δ Values in ppm and coupling constants in Hz. Multiplicities of carbon signals were determined by means of the distortionless enchancement by polarization transfer method and were indicated as s, d, t and q. *a, b*) Measured in MeOH-d_4 and acetone-d_6, respectively. *c*) Signal assignments are based on the analyses of the ^{1}H–^{13}C and ^{1}H–^{13}C long-range COSY spectra. *d*) Signal assignments are based on the analyses of the HMQC and HMBC spectra.

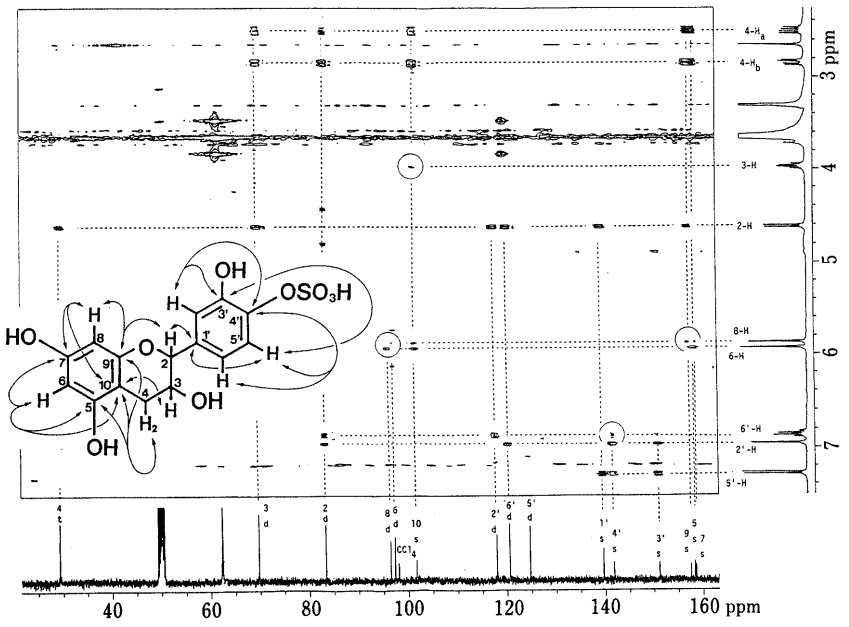

Fig. 5. HMBC Spectrum of Product I (**2**) in Methanol-d_4

8 mg, 40 h run, J_{CH}=6 Hz.

signals assignable to the 5′-methine group in the ring-B, suggesting that the sulfation took place at the 3′- or 4′-hydroxyl group.

Further, in the ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) spectrum (Fig. 5), the quaternary carbon at 139.6 (C-1′) showed long-range correlation with 2-H (δ 4.61, d, J=7.6 Hz) and 6′-H (δ 6.85, dd, J=8.0, 2.0 Hz), while the quaternary carbon at δ 141.8 (C-4′) showed long-range correlation with 2′-H (δ 6.95, d, J=2.0 Hz), 5′-H (δ7.27, d, J=8.0 Hz) and 6′-H (δ 6.85). Therefore, the former carbon was assigned to C-1′ and the latter to C-4′, unambiguously. On the other hand, the quaternary carbon at 151.1 (C-3′) showed long-range correlations with 2′-H (δ 6.95) and 5′-H (δ 7.27) and was

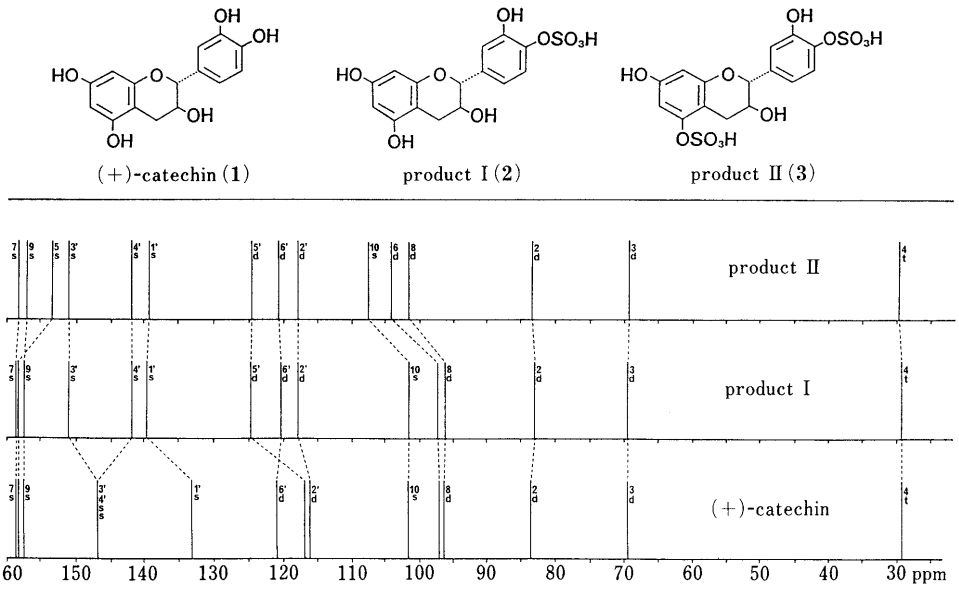

Fig. 6. Comparison of the ^{13}C-NMR Signals with (+)-Catechin (**1**), Product I (**2**) and Product II (**3**)

assigned to C-3′ unambiguously. In a similar manner, all quaternary carbons in ring-A were assigned as shown in Fig. 5.

Now, comparison of the ^{13}C-NMR date of product I with those of (+)-catechin disclosed an upfield shift of the signal due to C-4′ by 5.21 ppm, and downfield shifts of the signals due to C-3′, C-1′ and C-5′ by about 4—7 ppm[5] (Table II). This finding led us to conclude that the structure of product I is represented by formula **2** (Fig. 6).

Product II had the molecular formula $C_{15}H_{14}O_{12}S_2$ as proven by the negative ion FAB-MS, which revealed a quasi-molecular ion peak at *m/z* 449. The ^{1}H-NMR spectrum of product II showed a pattern similar to that of product I, but it was characterized by the downfield shifts of the ^{1}H-signals assignable to 6-H and 8-H in ring-A (Table II). The ^{13}C-NMR spectrum could also be analyzed completely by the use of HMBC and the results are compiled in Table II. Comparison of the ^{13}C-NMR data of product II with that of product I indicated that the signal due to C-5 shifted upfield by 4.81 ppm, while the signals due to C-6, C-8 and C-10 shifted downfield (6.83, 5.19 and 5.96, respectively). Thus, the structure of product II was determined to be **3** (Fig. 6).

Discussion

Tannins, as well as flavonoids, are widely distributed in the plant kingdom and are ingested daily as components of vegetable food. Human intestinal flora play an important role in the metabolism of such vegetable components, for examle, by β-glycosidase for glycosides,[18,19] and by C-ring cleavage to a flavonoid.[20] Through studies on the metabolism of oriental medicine components by human intestinal bacteria, we discovered a novel type of arylsulfotransferase, which catalyzes a specific trans-sulfation reaction between phenolic hydroxyl groups. We have already reported the sulfation of flavons, chalcones and xanthones by an enzyme from human intestinal bacterium[14] and established the chemical structure of sulfated quercetin.[9] The present study demonstrated that tannins and alkyl gallates were also effectively sulfated by the enzyme using PNS as a donor substrate, and the chemical structures of sulfated (+)-catechins, 4′-mono- and 5,4′-disulfated (+)-catechins were elucidated. Therefore, ingested polyphenols may be enzymatically sulfated in the intestine, and converted to be more soluble in water and less absorbable. Such sulfate conjugation in the intestine may represent one of the detoxication mechanisms of exogeneous polyphenols, though the sulfate donors have not yet been proven in the human intestinal tract. The position and number of sulfate groups incorporated to tannins have not yet been established, but will be clarified in the near future.

As alkyl gallate esters were better substrates than gallic acid (Table I), it seems that the presence of a carboxylic anion has an adverse effect on enzymatic sulfation. This result is similar to the fact that tyrosine methyl ester was sulfated more than tyrosine.[14] Since pedunculagin, corilagin and geraniin were less sulfated than tyramine, the presence of biphenyl also has an unfavorable influence on the sulfation.

Spectrometric analysis using an excess of PNS as a donor substrate showed that only one hydroxy group among many hydroxyl groups of pedunculagin, corilagin, geraniin, rosmarinic acid and penta-*O*-galloyl-β-D-glucose was sulfated (data not shown).

These findings suggest that some acidic amino acids and hydrophobic moieties of arylsulfotransferase might be present in the neighborhood of the active site of the enzyme, but we suggested previously that tyrosine and histidine residues are involved in the active site of the enzyme.[14] From the data on the chemical structure of sulfated catechins, a certain phenolic hydroxyl group(s) of polyphenols is specifically and rapidly sulfated. Therefore, the enzymatic trans-sulfation may be a useful method for the preparation of tannins sulfated at specific positions.

Acknowledgment This study was supported in part by a Grant-in-Aid (No. 01480491) from the Ministry of Education, Science and Culture of Japan. We wish to thank Mr. K. Tanaka of the National Research Institute of Police Science for the FAB-MS measurements, Miss M. Kamitani and Mr. K. Dohi for their technical assistance and Miss S. Takayanagi for her secretarial assistance.

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