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Chemical Synthesis and Characterization of Epicatechin Glucuronides and Sulfates: Bioanalytical Standards for Epicatechin Metabolite Identification

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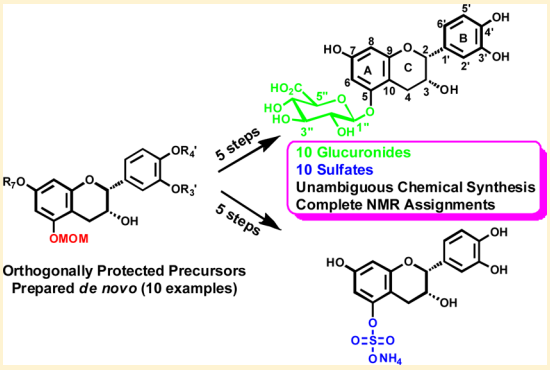
Supporting Information

ABSTRACT: The monoglucuronides and sulfates of epicatechin, 3′-*O*-methylepicatechin, and 4′-*O*-methylepicatechin, respectively, were synthesized as authentic bioanalytical standards. Reversed-phase HPLC methods capable of baseline separation of the glucuronides and sulfates have been developed. Both the epicatechin glucuronides and sulfates were stable in the solid state when stored under ambient conditions and in aqueous solution when stored refrigerated. These results should prove invaluable to the research community as analytical standards as well as in future studies of the biological and pharmacological effects of epicatechin in humans.



Epicatechin and its C-2 epimer catechin are members of the flavan-3-ol family.[1] Together with their oligomeric derivatives, the procyanidins, flavan-3-ols are found in many plants that are part of the human diet.[2] There is accumulating scientific evidence that diets rich in flavan-3-ols may provide important health benefits, including a reduced risk of cardiovascular disease.[3−10] Contrary to a widespread assumption, procyanidins do not contribute directly to the systemic pool of flavan-3-ols in humans.[11] Furthermore, the absorption, metabolism, and biological activity of dietary flavan-3-ols are strongly influenced by stereochemistry, and the predominant effects are attributed to epicatechin and its metabolites.[12] Indeed, a recent study has shown that oral administration of epicatechin to human subjects resulted in vascular effects similar to those observed with flavan-3-ol-rich cocoa, thus providing evidence for a causality chain directly linking epicatechin to the reported vascular effects observed after consumption of flavan-3-ols.[9]

Consequently, there has been a great deal of interest in elucidating the full metabolic profile of epicatechin after human ingestion.[13−15] It has been documented that ingested epicatechin undergoes rapid phase-II metabolism to form a wide range of metabolites including structurally related epicatechin metabolites (SREM) containing an intact C-ring and *seco*-metabolites from the breakdown of epicatechin by the gut microbiome.[14,16,17] It is further established that ingested epicatechin is extensively metabolized into SREM by *O*-methylation, sulfation, *O*-glucuronidation, and combinations thereof.[14,18−21]

In order to accurately assess the systemic levels of epicatechin in humans, the chemical structures of the metabolites must be elucidated and their individual systemic exposures determined. Furthermore, some of the metabolites may themselves be biologically active and, thus, responsible for some of the benefits observed after ingestion of epicatechin.[22,23] In this context, authentic standards of various epicatechin metabolites prepared in a chemically unambiguous fashion are essential. In the past, and due to the absence of such authentic standards, the structure elucidation of epicatechin metabolites has mainly relied on various NMR techniques such as COSY, HSQC, HMBC, and NOE. However, since the structurally related epicatechin metabolites are mainly regioisomers, NMR spectroscopic techniques alone do not always yield unequivocal elucidation of their structures due to weak correlations.[24] In addition, the availability of authentic flavan-3-ol metabolite standards would greatly enhance the development of sample preparation and analytical methods.[14] Various attempts have been made to synthesize authentic standards of some of the metabolites either enzymatically[25] or chemically[26,27] from epicatechin. However, due to the nonregioselective nature of these earlier syntheses, mixtures of regioisomers are unavoidably obtained, which further requires labor-intensive chromatographic separations, an approach that is intrinsically ambiguous, especially when considering the absence of authentic standards for method validation in the first place. Ironically, so-called

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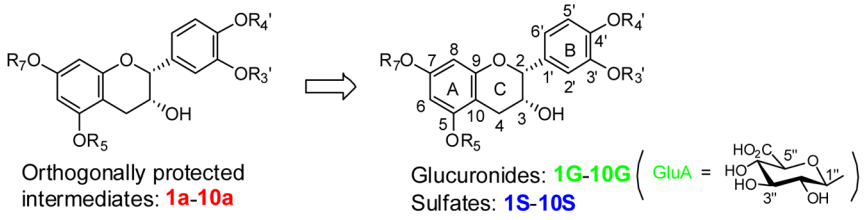
Chart 1. Structures of Orthogonally Protected Epicatechin Intermediates 1a−10a (red), the Corresponding *O*-β-D-Glucuronides 1G−10G (green), and Sulfates 1S−10S (blue)



Compounds	R_3'	R_4'	R_5	R_7
1a	MOM	Bn	Bn	Bn
1G	GluA	H	H	H
1S	SO_3NH_4	H	H	H
2a	Bn	PMB	Bn	Bn
2G	H	GluA	H	H
2S	H	SO_3NH_4	H	H
3a	Bn	Bn	MOM	Bn
3G	H	H	GluA	H
3S	H	H	SO_3NH_4	H
4a	Bn	Bn	Bn	MOM
4G	H	H	H	GluA
4S	H	H	H	SO_3NH_4
5a	Me	MOM	Bn	Bn
5G	Me	GluA	H	H
5S	Me	SO_3NH_4	H	H
6a	Me	Bn	MOM	Bn
6G	Me	H	GluA	H
6S	Me	H	SO_3NH_4	H
7a	Me	Bn	Bn	MOM
7G	Me	H	H	GluA
7S	Me	H	H	SO_3NH_4
8a	MOM	Me	Bn	Bn
8G	GluA	Me	H	H
8S	SO_3NH_4	Me	H	H
9a	Bn	Me	MOM	Bn
9G	H	Me	GluA	H
9S	H	Me	SO_3NH_4	H
10a	Bn	Me	Bn	MOM
10G	H	Me	H	GluA
10S	H	Me	H	SO_3NH_4

"authentic standards" obtained in this fashion are further prone to structural ambiguities, because the site(s) of the methylation, glucuronidation, and sulfation cannot always be readily ascertained.

Recently, we have focused our attention on the chemical synthesis of all theoretically possible monoglucuronides and sulfates[28] of epicatechin, 3′-*O*-methyl-, and 4′-*O*-methylepicatechin as authentic bioanalytical standards (see also ref 29). The advantage of this approach is that not only are we making NMR assignments to a predetermined structure, but we are also able to compare assignments directly with all of the other possible isomers and show that they are distinct and that the data are internally consistent. The key to our synthetic strategy is the availability of a set of orthogonally protected epicatechin intermediates, **1a−10a** (Chart 1), prepared *de novo*. In this approach, the specific phenolic hydroxy group to be glucuronidated or converted to the sulfate group is selectively protected with a methoxymethyl (MOM) or *p*-methoxybenzyl (PMB) group (highlighted in red in Chart 1), while all of the remaining hydroxy groups are protected as benzyl ethers. This strategy allows the regiospecific glucuronidation/sulfation of a single hydroxy group while the rest remain protected. With this synthetic approach, the site of glucuronidation/sulfation of epicatechin is unambiguous. Using these chemically unambiguous authentic standards of epicatechin glucuronides and sulfates, a method to quantify epicatechin metabolites in human plasma after ingestion of epicatechin has been successfully developed.[14]

In a recent preliminary communication, some of us have reported the preparation of the orthogonally protected epicatechin intermediates (**4a**, **7a−10a**) and the final synthesis of five *O*-β-D-glucuronides (**4G**, **7G−10G**, Chart 1) derived from epicatechin, 3′-*O*-methylepicatechin, and 4′-*O*-methylepicatechin, respectively.[30] In the present paper, we advance on previous work and describe the chemical synthesis of the remaining five monoglucuronides, **1G−3G**, **5G**, and **6G**, and all 10 monosulfates **1S−10S** (Chart 1) from the orthogonally protected epicatechin intermediates **1a−10a**. Also presented in

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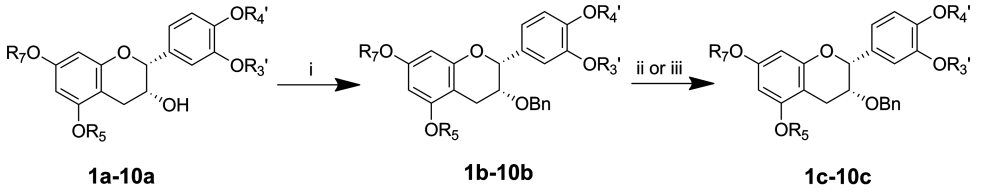
Scheme 1. Synthesis of Intermediates 1c−10c[a]



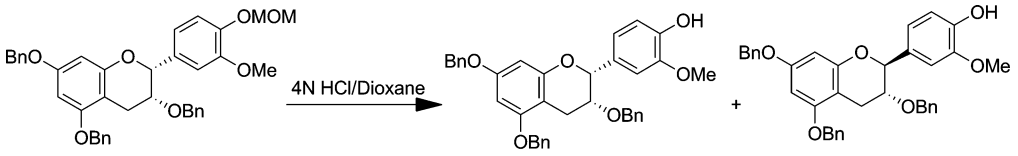
1a-10a **1b-10b** **1c-10c**

1a, **1b**: R_3'=MOM, R_4'=R_5=R_7=Bn
2a, **2b**: R_4'=PMB, R_3'=R_5=R_7=Bn
3a, **3b**: R_5=MOM, R_3'=R_4'=R_7=Bn
4a, **4b**: R_7= MOM, R_3'=R_4'=R_5=Bn
5a, **5b**: R_4'=MOM, R_3'=Me, R_5=R_7=Bn
6a, **6b**: R_5=MOM, R_3'=Me,R_4'=R_7=Bn
7a, **7b**: R_7=MOM, R_3'=Me, R_4'=R_5=Bn
8a, **8b**: R_3'=MOM, R_4'=Me,R_5=R_7=Bn
9a, **9b**: R_5=MOM, R_4'=Me, R_3'=R_7=Bn
10a, **10b**: R_7=MOM,R_4'=Me, R_3'=R_5=Bn

1c: R_3'=H, R_4'=R_5=R_7=Bn
2c: R_4'=H, R_3'=R_5=R_7=Bn
3c: R_5=H, R_3'=R_4'=R_7=Bn
4c: R_7= H, R_3'=R_4'=R_5=Bn
5c: R_4'=H, R_3'=Me, R_5=R_7=Bn
6c: R_5=H, R_3'=Me,R_4'=R_7=Bn
7c: R_7=H, R_3'=Me, R_4'=R_5=Bn
8c: R_3'=H, R_4'=Me,R_5=R_7=Bn
9c: R_5=H, R_4'=Me, R_3'=R_7=Bn
10c: R_7=H,R_4'=Me, R_3'=R_5=Bn

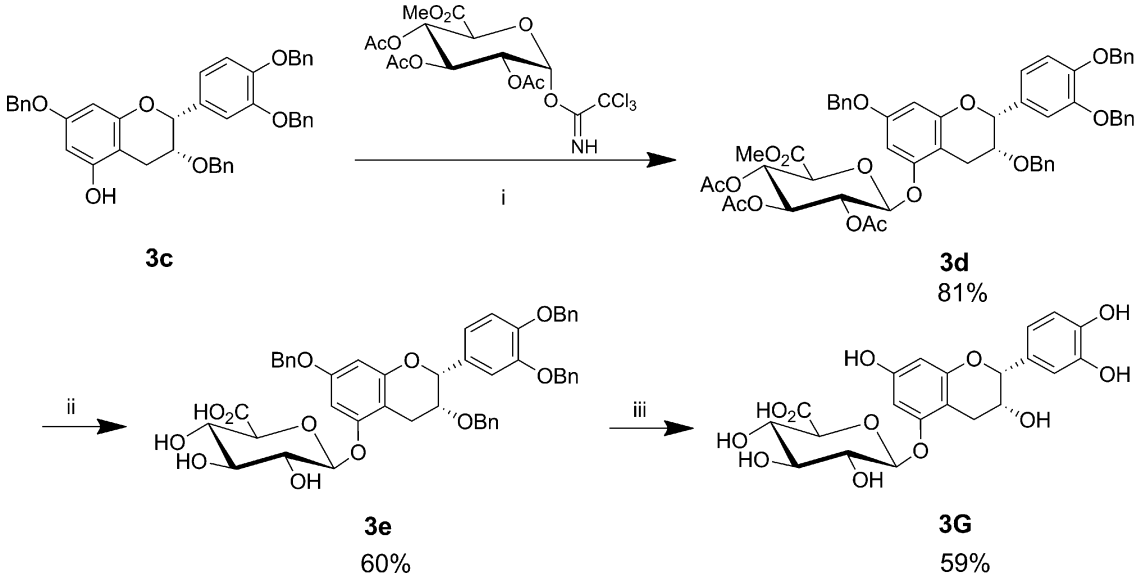
[a]Reagents and conditions: (i) NaH, BnBr, DMF, 0 °C; (ii) 4 N HCl in dioxane, DCM/MeOH, room temperature for **1c** and **3c−10c**; (iii) pyridinium *p*-toluenesulfonate, 2-propanol/toluene, reflux for **2c**.

Scheme 2. Epimerization at C-2 during the Removal of the MOM Protective Group in 5b



5b **5c** **5c'**

Scheme 3. Synthesis of Epicatechin-5-*O*-*β*-D-glucuronide (3G)[a]



3c **3d** 81% **3e** 60% **3G** 59%

[a]Reagents and conditions: (i) BF_3·OEt_2, DCM, 0 °C; (ii) 1 N NaOH, THF/MeOH, 0 °C; (iii) $Pd(OH)_2$/C, H_2, MeOH.

this paper are complete assignments of ^{1}H and ^{13}C NMR signals of **1G−10G** and **1S−10S**. HPLC methods capable of baseline separation of **1G−10G** and **1S−10S** are also reported, together with assessments of their stability in both the solid state and aqueous solution.

RESULTS AND DISCUSSION

Epicatechin-*O*-*β*-D-glucuronides. *Chemical Synthesis.* The synthesis shown in Scheme 1 relies on a series of orthogonally protected epicatechin intermediates, **1a−10a** (Chart 1), described elsewhere.[31] Protection of the 3-OH group by benzylation afforded the orthogonally protected epicatechin intermediates **1b−10b**. The phenolic OH group to be glucuronidated was then selectively unmasked by the removal of the MOM or the PMB group. The MOM group was removed by treatment with 4 N HCl/dioxane in DCM at room temperature. Under these conditions, intermediate **5b** afforded a 1:1 mixture of epicatechin **5c** and *ent*-catechin **5c′** (Scheme 2), while the rest of the intermediates bearing an MOM group

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Table 1. NMR Spectroscopic Data for Epicatechin Glucuronides 1G−4G in Acetone-d_6 Unless Specified Otherwise

	3′-glucuronide **1G**		4′-glucuronide **2G**		5-glucuronide **3G**		7-glucuronide **4G**	
position	δ_C[a]	δ_H (J in Hz)[b]	δ_C	δ_H (J in Hz)	δ_C[a]	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)[c]
2	78.9	5.04, s	79.2	4.94, s	79.2	4.84, s	79.6	4.90, s
3	66.4	4.32, m	66.8	4.25, br, s	66.3	4.19, br, s	66.7	4.22, m
4	28.7	2.92, dd (16.8, 4.4)	29.1	2.88, d (16.5)	28.6	2.88, d (16.8)	29.1	2.89, dd (16.8, 4.6)
		2.72, dd (17.0, 2.8)		2.75, d (16.5)		2.81, d (16.8)		2.76, d (16.8, 3.1)
5	157.4		157.6		157.5		157.5	
6	96.2	6.10, d (2.4)	96.3	6.03, s	96.3	6.25, s	97.5	6.21, d (2.3)
7	157.2		157.6		156.5		158.0	
8	95.4	6.08, d (2.4)	95.8	5.94, s	98.0	6.07, s	96.9	6.16, d (2.3)
9	156.6		157.0		157.2		157.1	
10	99.5		99.8		102.1		102.9	
1′	131.9		137.2		131.6		132.1	
2′	117.8	7.26, d (1.9)	116.0	7.10, s	115.0	7.00, s	115.3	7.06, d (1.8)
3′	145.4		148.6		145.0		145.4	
4′	147.5		145.3		145.2		145.4	
5′	116.4	6.99, d (8.3)	119.3	7.13, d (8.0)	115.5	6.75, d (8.6)	115.5	6.79, d (8.1)
6′	123.3	7.11, dd (8.6, 1.9)	119.0	6.94, d (8.0)	119.0	6.79, d (8.6)	119.5	6.84, dd (8.1, 1.8)
1″	103.7	5.11, d (7.4)	104.8	4.89, d (7.3)	101.6	4.99, d (7.2)	102.0	5.01, d (7.7)
2″	73.8	3.62, m	74.4	3.58, m	73.8	3.54, t (8.6)	74.4	3.48, t (7.9)
3″	76.3	3.63, m	76.8	3.62, m	76.6	3.58, t (8.7)	77.2	3.58, t (9.0)
4″	72.3	3.67, m	72.6	3.72, t (9.2)	72.2	3.66, t (8.6)	72.7	3.70, t (9.0)
5″	76.3	3.96, d (9.6)	76.2	4.08. d (9.6)	75.9	4.04[d]	76.1	4.07, d (9.7)
COOH	170.9[e]		170.2		171.1		170.2	

[a]In acetone-d_6 containing 10% D_2O. [b]In D_2O. [c]The two phenolic OHs were visible as broad singlets (δ 7.8 and 8.4). [d]Overlapping with H_2O peak. [e]Observed in H−C HMBC spectrum (correlation with H-5″).

Table 2. NMR Spectroscopic Data for 3′-*O*-Methylepicatechin Glucuronides 5G−7G in Acetone-d_6 Unless Specified Otherwise

	3′-OMe-4′-glucuronide **5G**		3′-OMe-5-glucuronide **6G**		3′-OMe-7-glucuronide **7G**	
position	δ_C[a]	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)[b]
2	78.0	4.83, s	79.6	4.92, s	79.7	4.95, s
3	65.7	4.27, br, s	66.8	4.21, br, s	66.8	4.25, m
4	27.4	2.85, dd (17.1, 4.3)	29.2	2.93, d (16.8)	29.3	2.91, dd (16.8, 4.5)
		2.73, d (17.1)		2.85, d (16.8)		2.80, dd (16.8, 2.8)
5	155.6		158.0		157.5	
6	96.0	6.08, d (2.3)	96.6	6.30, s	97.6	6.22, d (2.3)
7	155.3		157.6		158.0	
8	95.5	6.07, d (2.3)	98.3	6.09, s	97.1	6.18, d (2.3)
9	155.1		156.8		157.1	
10	99.7		102.5		102.9	
1′	134.0		131.9		131.9	
2′	110.9	7.08, s	111.8	7.17, s	111.9	7.19, d (1.8)
3′	148.6		147.9		147.9	
4′	144.9		146.9		146.9	
5′	116.1	7.13, d (8.4)	115.3	6.80, d (7.8)	115.2	6.81, d (8.1)
6′	119.1	6.99, d (8.4)	120.6	6.96, d (7.8)	120.6	6.97, dd (8.1, 1.8)
1″	100.6	5.09, d (7.1)	102.2	5.01, d (7.4)	102.1	5.01, d (7.7)
2″	72.6	3.64, m	74.4	3.56, t (8.5)	74.4	3.48, t (7.8)
3″	75.2	3.64, m	77.3	3.60, t (8.8)	77.2	3.58, t (9.0)
4″	71.3	3.64, m	72.7	3.72, t (9.2)	72.7	3.70, t (9.0)
5″		4.04, d (8.3)	76.1	4.07, d (9.6)	76.1	
COOH			170.2		170.2	
OMe	55.9	3.83, s	56.3	3.83, s	56.3	3.84, s

[a]In D_2O. [b]The two phenolic OHs were visible as broad singlets (δ 7.5 and 8.4).

(**1a**, **3a**, **4a**, and **6a**−**10a**) afforded the epicatechin products without significant *ent*-catechin byproducts. The formation of the *ent*-catechin byproduct **5c**′ was the result of the 4′-OH-induced C-2 epimerization of epicatechin.[32] In order to avoid a similar acid-catalyzed epimerization in the synthesis of epicatechin-4′-*O*-β-D-glucuronide (**2G**), removal of the PMB group in **2b** was achieved by treatment with pyridinium *p*-toluenesulfonate in 2-propanol/toluene at reflux. Under these conditions, a smaller amount (ca. 17%) of the *ent*-catechin was detected by ^{1}H NMR. The *ent*-catechin byproducts are readily

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Table 3. NMR Spectroscopic Data for 4′-*O*-Methylepicatechin Glucuronides 8G−10G in Acetone-d_6

	4′-OMe-3′-glucuronide **8G**		4′-OMe-5-glucuronide **9G**		4′-OMe-7-glucuronide **10G**	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
2	79.2	4.94, s	79.4	4.92, s	79.5	4.94, s
3	66.6	4.25, br, s	66.7	4.21, br, s	66.7	4.25, br, s
4	29.1	2.86, dd (16.5, 4.1)	29.0	2.88, m[a]	29.2	2.91, d (16.8)
		2.76, d (16.5)		2.88, m[a]		2.78, d (16.8)
5	157.5		157.9		157.5	
6	96.2	6.03, d (2.2)	96.5	6.29, s	97.6	6.21, s
7	157.5		157.6		158.0	
8	95.8	5.93, d (2.2)	98.1	6.09, s	96.9	6.18, s
9	157.0		156.8		157.0	
10	99.7		102.5		102.9	
1′	133.4		133.5		133.5	
2′	118.1	7.39, s	114.9	7.06, s	115.1	7.07, s
3′	147.0		146.9		147.0	
4′	150.5		147.7		147.8	
5′	113.3	6.99, d (8.2)	112.0	6.94, d (8.5)	112.0	6.95, d (8.2)
6′	122.8	7.20, d (8.5)	118.9	6.91, d (8.1)	119.0	6.91, d (8.0)
1″	102.9	5.09, d (7.3)	102.1	5.02, d (7.2)	102.0	5.02, d (7.3)
2″	74.5	3.54, t (8.8)	74.3	3.55, t (8.7)	74.4	3.49, t (7.9)
3″	77.1	3.57, t (9.3)	77.2	3.59, t (9.2)	77.2	3.59, t (8.8)
4″	72.5	3.71, t (9.2)	72.6	3.72, t (8.5)	72.7	3.70, t (9.0)
5″	76.1	3.99, d (9.5)	76.1[b]	4.08, d (9.6)	76.1[b]	4.07, d (9.5)
COOH	170.1		170.1[b]		170.2[b,c]	
OMe	56.6	3.84, s	56.3	3.84, s	56.4	3.84, s

[a]Overlapping signals. [b]Not observable in ^{13}C NMR when ca.10% D_2O was present. [c]Observed in H−C HMBC spectrum (correlation with H-5″) in acetone-d_6 with 10% D_2O.

distinguishable from the corresponding epicatechin precursors by ^1H NMR based on the $^3J_{2,3}$ coupling constants of 8.3 Hz.

The phenolic intermediates **1c−10c** were glucuronidated by coupling with methyl 2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-α-D-glucuronate. For clarity, only the synthesis of epicatechin-5-*O*-β-D-glucuronide (**3G**) is shown in Scheme 3. The glucuronidation of the 5-OH group of epicatechin **3c** was carried out under known reaction conditions (BF_3 etherate, 4 Å molecular sieves, DCM, 0 °C).[33,34] BF_3 etherate was dissolved in anhydrous DCM and added to the reaction slowly. Although this coupling reaction ran smoothly most of the time, the manner in which the Lewis acid was added is important.[35]

The coupling product **3d** was purified by flash chromatography (silica gel, heptane/DCM/EtOAc, 5:4:1, v/v/v). Alkaline ester hydrolysis/*O*-deacetylation followed by hydrogenolysis to remove the benzyl protecting groups afforded the crude glucuronide, which was purified by preparative reversed-phase HPLC to give epicatechin-5-*O*-β-D-glucuronide (**3G**). Compound **3G** was obtained as a white powder in 59% yield after lyophilization.

The synthesis of **5G** was carried out with the mixture of **5c** and **5c′** obtained from the removal of the MOM protective group shown in Scheme 2 in the same fashion as described in Scheme 3. The crude product was isolated as a 1:1 mixture of 3′-*O*-methylepicatechin-4′-*O*-β-D-glucuronide (**5G**) and 3′-*O*-methyl-*ent*-catechin-4′-*O*-β-D-glucuronide (**5G′**) after unmasking all the OH groups. Pure **5G** and the byproduct **5G′**[36] were obtained after separation by preparative reversed-phase HPLC.

Compounds **1G**, **2G**, **4G**, and **6G−10G** were prepared in a similar fashion to that described for **3G** starting from the corresponding orthogonally protected epicatechin intermediates **1a**, **2a**, **4a**, and **6a−10a**, respectively.

NMR Characterization. The ^1H and ^{13}C NMR spectra of the epicatechin glucuronides **1G−10G** were unambiguously assigned using COSY, HSQC, and HMBC 2D NMR methods, and the data are collated in Tables 1−3.

^1H NMR Assignments. The ^1H NMR spectra of the epicatechin glucuronides exhibit three key features: (a) H-2 appears as a singlet ca. δ 4.9, indicating that it is in the *syn* relationship with H-3 (δ 4.3). H-3 is coupled to H-4a and in some cases to H-4b as well with very small coupling constants (ca. 3−4 Hz), thus appearing as partially resolved multiplets or broad singlets; (b) the anomeric H-1″ of the glucuronides appears at ca. δ 5.0 as a doublet with a coupling constant around 7 Hz, characteristic of the β-anomer;[33,34] (c) the two C-4 protons H-4a and H-4b are diastereotopic and generally appear as AB quartets around 2.7−2.9 ppm with J_{AB} between 16 and 17 Hz and $\Delta\nu_{AB}$ between 25 and 50 Hz (reported as two distinct doublets in Tables 1−3 for convenience). There are two exceptions however. In epicatechin-3′-*O*-β-D-glucuronide (**1G**), H-4a and H-4b exhibit an AX splitting pattern with a similar coupling constant (ca. 17 Hz); in 4′-*O*-methylepicatechin-5-*O*-β-D-glucuronide (**9G**), H-4a and H-4b appear as an unresolved multiplet centered around δ 2.88. Complete assignments of the protons of the B-ring, C-ring, and the glucuronic acid unit were accomplished using 2D-COSY. The two A-ring protons H-6 and H-8 were assigned on the basis of analogy with those of epicatechin, which have been assigned unambiguously according to NOE difference spectroscopy.[37,38]

When one of the epicatechin phenolic hydroxy groups is glucuronidated, significant chemical shift changes are observed only for those protons in the *ortho*- and *para*-positions. Little change in chemical shift is observed for protons in the *meta*-position or those on the non-glucuronidated aromatic ring (Table 4). The effects are generally larger on the *ortho* protons

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Table 4. Changes in Chemical Shifts ($\Delta\delta$, ppm) of the A- and B-Ring Protons of Epicatechin Glucuronides 1G−4G Relative to Epicatechin[a]

	H-6	H-8	H-2′	H-5′	H-6′
3′-glucuronide **1G**[b]	0.00	0.01	0.34	0.06	0.29
4′-glucuronide **2G**[c]	0.01	0.02	0.050	0.34	0.10
5-glucuronide **3G**[b]	0.24	0.16	−0.01	0.00	0.00
7-glucuronide **4G**[c]	0.19	0.24	0.01	0.00	0.00

[a]Chemical shift changes are calculated using ^{1}H chemical shifts of epicatechin in the same solvent. [b]In acetone-d_6 containing 10% D_2O. [c]In acetone-d_6.

than the *para* ones. For example, when comparing epicatechin-3′-*O*-*β*-D-glucuronide (**1G**) to the parent, epicatechin, H-2′ and H-6′ were downfield shifted by 0.34 and 0.29 ppm, respectively, while the chemical shifts of the other aromatic protons remained virtually unchanged. For epicatechin-4′-*O*-*β*-D-glucuronide (**2G**), H-5′ showed a large downfield shift of 0.34 ppm. For epicatechin-5-*O*-*β*-D-glucuronide (**3G**), H-6 showed a downfield shift of 0.24 ppm. The proton H-8 was also downfield shifted, but to a lesser degree (0.16 ppm). For epicatechin-7-*O*-*β*-D-glucuronide (**4G**), H-6 and H-8 underwent a downfield shift by a similar degree (0.19 and 0.24 ppm, respectively). No significant changes were observed for the three B-ring protons.

^{13}C NMR Assignments. The assignments of ^{13}C NMR spectra for proton-bearing carbons were achieved using HSQC. The anomeric carbon of the glucuronic acid motif appears around δ 102 ppm, consistent with literature data.[33,34] The assignments of the A- and B-ring *ipso* carbons C-5, C-7, C-9, C-10, C-1′, C-3′, and C-4′ were accomplished using HMBC spectroscopy. The *ipso* carbon to which the OGluA unit is attached was assigned on the basis of its three-bond long-range coupling with the anomeric H-1″. The B-ring *ipso* carbons C-1′ and C-3′ were assigned on the basis of their three-bond coupling with H-5′, and C-4′ was assigned on the basis of its three-bond coupling with H-2′ and H-6′. When attached to an OGluA unit, C-3′ and C-4′ also showed three-bond coupling with the anomeric H-1″. The A-ring *ipso* carbons C-5, C-7, and C-9 were assigned on the basis of their two-bond correlations with H-6 and H-8. The *ipso* carbon C-10 showed three-bond correlations with H-6 and H-8 and in some cases two-bond correlations with H-4a and H-4b. For the methylated epicatechin glucuronides, the *ipso* carbon to which the OMe group is attached showed strong three-bond coupling with the methyl protons. Thus, by the combination of HSQC and HMBC, all carbons could be assigned unambiguously.

LC/MS Analysis. The four epicatechin glucuronides **1G**−**4G**, in the negative ionization mode, exhibited a molecular ion of 465 as the base peak resulting from the loss of a proton [M − H]. The *m*/*z* 289 ion resulting from the loss of the glucuronic acid unit was also detected. Peaks from dimerization of the glucuronides (*m*/*z* 931) were detected in the negative ionization mode as well. The six methylated analogues **5G**−**10G** showed a molecular ion of 479 [M − H]. Like the nonmethylated epicatechin glucuronides, a fragment from the loss of the glucuronic acid unit (*m*/*z* 303) was detected for all methylated analogues. Dimers (*m*/*z* 959) were also observed. The MS/MS spectra of **1G**−**3G**, **5G**, **6G**, and **10G** appear in the Supporting Information.

HPLC Analysis. The 10 epicatechin glucuronides **1G**−**10G** were analyzed by reversed-phase HPLC on an Agilent Eclipse XDB-Phenyl analytical column. The Eclipse XDB-Phenyl column was chosen because of its ability to handle mobile phases with aqueous content as high as 100%. With a mobile phase consisting of 25 mM Na_3PO_4 buffer pH 2.5 and MeCN, the epicatechin glucuronides exhibited adequate retention on the Eclipse XDB-Phenyl column. Shown in Figure 1 are HPLC

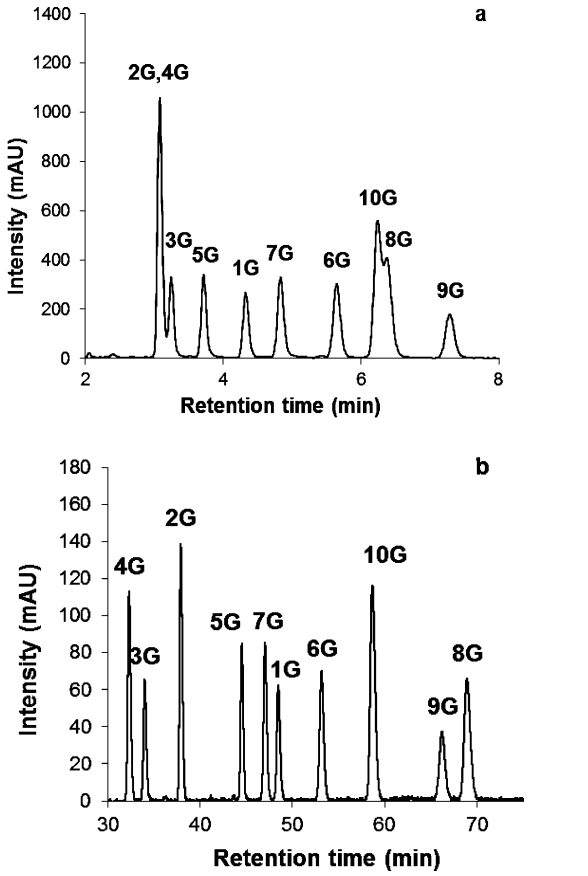

Figure 1. HPLC traces of epicatechin glucuronides **1G**−**10G** on an Agilent Eclipse XDB-Phenyl column (3.5 μm, 4.6 × 150 mm). Flow rate: 1 mL/min. UV detection: 230 nm. Temperature: ambient. Mobile phase: solvent A, 25 mM Na_3PO_4 buffer pH 2.5; solvent B, MeCN. (a) Isocratic conditions (85% A/15% B). (b) Gradient conditions (0−8% B in 40 min).

traces of the glucuronides **1G**−**10G**. A mixture of **1G**−**10G** was artificially created by mixing aqueous solutions of the individual components. The peaks were assigned by comparing the retention times to those of the individual components under the same HPLC conditions. Under isocratic conditions (85% of 25 mM Na_3PO_4 buffer/15% MeCN), baseline separation was achieved for nearly all the compounds, except **2G**, **4G**, **8G**, and **10G**, in less than 10 min run time (Figure 1a). Epicatechin itself co-eluted with compound **8G** under these conditions. Baseline separation of all 10 glucuronides was achieved using gradient conditions by slowly increasing MeCN content from 0 to 8% in 40 min (Figure 1b). Under these conditions, epicatechin co-eluted with epicatechin-3′-*O*-*β*-D-glucuronide (**1G**).

Stability Assessment. The stability of epicatechin glucuronides under storage conditions was assessed by appearance, ^{1}H NMR, and HPLC. All the glucuronides were white solids after lyophilization. No change in appearance was observed after 30 days at ambient temperature. ^{1}H NMR spectra were

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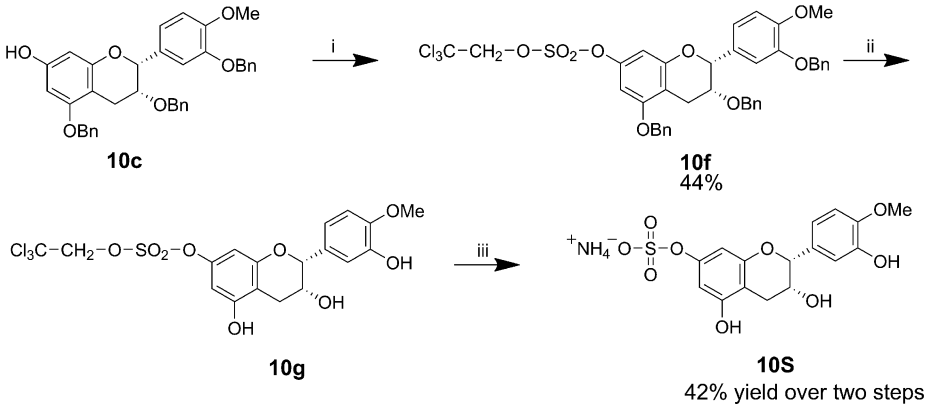
Scheme 4. Synthesis of 4′-*O*-Methylepicatechin-7-sulfate Ammonium Salt (10S)[a]



[a]Reagents and conditions: (i) DMAP (1 equiv), Et_3N (2 equiv), $Cl_3CCH_2OSO_2Cl$ (2 equiv), THF; (ii) $Pd(OH)_2/C$, H_2, EtOAc; (iii) HCO_2NH_4, Zn dust, MeOH.

Table 5. NMR Spectroscopic Data for Epicatechin Sulfate Ammonium Salts 1S−4S (DMSO-d_6)

	3′-sulfate **1S**		4′-sulfate **2S**		5-sulfate **3S**		7-sulfate **4S**	
position	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
2	77.7	4.79, s	77.8	4.81, s	78.2	4.73, s	78.2	4.75, s
3	64.7	4.03, br, s	64.7	4.04, br, s	64.8	4.00, br, s	64.7	4.03, br, s
4	28.3	2.69, dd (16.8, 4.6) 2.50[b]	28.2	2.70, dd (16.4, 3.8) 2.49, d (16.4)[b]	28.7	2.80, dd (16.8, 4.2) 2.59, dd (16.8, 2.5)	28.5	2.72, dd (16.8, 3.8) 2.55, d (17.0)
5	156.6		156.6		152.7		156.1	
6	95.2	5.89, d (2.2)	95.2	5.91, s	101.1	6.54, s	99.8	6.32, s
7	156.3		156.3		155.7		152.2	
8	94.1	5.73, d (2.2)	94.0	5.74, s	97.8	5.95, s	99.3	6.12, s
9	155.6		155.5		155.1		155.0	
10	98.4		98.4		103.5		102.7	
1′	131.0		136.5		130.5		130.4	
2′	122.3	7.17, d (1.5)	116.1	6.93, s	115.1	6.89, s	115.1	6.89, s
3′	140.1		148.5		144.6		144.85	
4′	148.5		139.9		144.6		144.90	
5′	116.5	6.78, d (8.4)	122.3	7.07, d (8.3)	114.9	6.66 (s)	115.1	6.66, s
6′	123.9	7.05, d (8.4)	117.9	6.79, d (8.2)	117.9	6.66 (s)	117.8	6.66, s

[a]The 3-OH proton was also visible at δ 4.70 as a broad singlet. [b]Partially overlapping with the DMSO peak.

identical to those obtained prior to storage. In aqueous solution, the compounds were stable when stored in a refrigerator for 2 days, as no change in HPLC peak area was detected for any of the compounds from their initial values. However, when its aqueous solution was stored at ambient temperature, compound **3G** turned yellow overnight. The solution stability of the ammonium salt of 4′-*O*-methylepicatechin-7-*O*-β-D-glucuronide (**10G**) in D_2O was also assessed by ^{1}H NMR after 4 weeks of storage at ambient temperature. There was no change in ^{1}H NMR except disappearance of H-6 and H-8 due to H−D exchange.[39] These observations suggest that epicatechin glucuronides are stable in the solid state at ambient conditions and aqueous solutions when refrigerated.

Epicatechin Sulfates. *Chemical Synthesis.* The epicatechin sulfates **1S−10S** were synthesized from the epicatechin intermediates **1c−10c**. The synthesis of 4′-*O*-methylepicatechin-7-sulfate (**10S**) is shown in Scheme 4. Reaction of the phenolic compound **10c** with chlorosulfuric acid 2,2,2-trichloroethyl (TCE) ester in the presence of Et_3N and DMAP in THF afforded the TCE-protected epicatechin-7-sulfate intermediate **10f** in 44% isolated yield. Debenzylation via catalytic hydrogenolysis cleanly afforded the TCE-protected 4′-*O*-methylepicatechin-7-sulfate **10g**. Removal of the TCE moiety was accomplished using Zn-ammonium formate in MeOH[40] to give 4′-*O*-methylepicatechin-7-sulfate ammonium salt (**10S**). The crude was purified by preparative reversed-phase HPLC. The pure **10S** was obtained as an off-white powder in 42% yield over two steps after lyophilization. Epicatechin sulfates **1S−9S** were prepared and isolated in the same fashion as described for **10S** starting from the corresponding epicatechin intermediates **1c−9c**.

NMR Characterization. The ^{1}H and ^{13}C NMR spectra of the epicatechin sulfates **1S−10S** were unambiguously assigned using COSY, HSQC, and HMBC 2D NMR methods, and the data are collated in Tables 5−7.

^{1}H NMR Assignments. The ^{1}H NMR spectra of epicatechin sulfates **1S−10S** in DMSO-d_6 exhibit three key features: (a) H-2 appears as a singlet (ca. δ 4.8), indicating its *syn* relationship (J < 1 Hz) with H-3; (b) H-3 (ca. δ 4) is coupled to H-4a and in some cases to H-4b as well with small coupling constants (ca. 3−4 Hz), thus appearing as partially resolved multiplets or broad singlets; (c) H-4a and H-4b are diastereotopic, exhibiting an AX splitting pattern with coupling constants in the range 16−17 Hz. The assignments of H-6 and H-8 of epicatechin

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Table 6. NMR Spectroscopic Data for 3′-*O*-Methylepicatechin Sulfate Ammonium Salts 5S−7S (DMSO-d_6)

	3′-OMe-4′-sulfate **5S**		3′-OMe-5-sulfate **6S**		3′-OMe-7-sulfate **7S**	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)
2	78.0	4.84, s	78.2	4.79, s	78.1	4.83, s
3	64.7	4.07, br, s	64.7	4.03, br, s	64.7	4.07, br, s
4	28.0	2.71, dd (16.6, 4.3)	28.7	2.80, d (16.4)	28.6	2.74, dd (17.4, 3.9)
		2.53, d (16.6)[b]		2.62, d (17.2)		2.58, d (16.7)
5	156.6		152.8		155.8	
6	95.2	5.91, s	101.1	6.55, s	99.9	6.32, s
7	156.3		155.6		152.3	
8	94.1	5.75, s	97.8	5.95, s	99.4	6.15, s
9	155.6		155.0		155.0	
10	98.4		103.5		102.5	
1′	134.9		130.5		130.6	
2′	111.9	7.06, s	111.7	7.03, s	111.5	7.06, s
3′	149.9		146.9		146.9	
4′	141.9		145.7		145.7	
5′	120.8	7.40, d (8.4)	114.7	6.72, d (7.8)	114.7	6.73, d (7.9)
6′	118.6	6.90, d (8.4)	119.6	6.83, d (8.1)	119.5	6.85, d (8.2)
OMe	55.7	3.73, s	55.6	3.74, s	55.6	3.76, s

[a]The 3-OH proton was visible at δ 4.70 as a broad singlet. [b]Overlapping with the DMSO peak.

Table 7. NMR Spectroscopic Data for 4′-*O*-Methylepicatechin Sulfate Ammonium Salts 8S−10S (DMSO-d_6)

	4′-OMe-3′-sulfate **8S**		4′-OMe-5-sulfate **9S**		4′-OMe-7-sulfate **10S**	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)
2	77.9	4.78, s	78.0	4.78, s	78.0	4.81, s
3	64.7	4.03, m	64.8	4.01, m	64.7	4.05, br, s
4	28.4	2.72, dd (16.6, 4.1)	28.6	2.80, dd (16.8, 4.5)	28.4	2.74, dd (16.8, 4.1)
		2,53, d (16.6)[b]		2.58, dd (16.8, 3.0)		2.56, d (16.2)
5	156.7		152.7		156.1	
6	95.2	5.91, s	101.1	6.55, d (2.0)	99.8	6.32, s
7	156.3		155.7		152.3	
8	94.1	5.73, s	97.8	5.94, d (2.0)	99.3	6.12, s
9	155.6		155.0		154.9	
10	98.4		103.4		102.7	
1′	131.6		132.3		132.4	
2′	120.4	7.51, s	114.8	6.93, s	114.7	6.96, s
3′	142.0		145.9		146.0	
4′	150.0		146.8		146.9	
5′	112.1	6.92, d (8.4)	111.7	6.85, d (8.2)	111.8	6.86, d (8.4)
6′	122.1	7.10, d (8.2)	117.7	6.78, d (8.3)	117.5	6.79, d (8.4)
OMe	55.8	3.73, s	55.7	3.74,s	55.7	3.75, s

[a]The 3-OH proton was visible at δ 4.67 as a doublet (J = 3.6 Hz). [b]Overlapping with the DMSO peak.

Table 8. Changes in A- and B-Ring Proton Chemical Shifts of Epicatechin Sulfates 1S−4S Relative to Epicatechin in DMSO-d_6[a]

	H-6	H-8	H-2′	H-5′	H-6′
epicatechin-3′-sulfate (**1S**)	0.00	0.02	0.28	0.12	0.39
epicatechin-4′-sulfate (**2S**)	0.02	0.03	0.04	0.41	0.13
epicatechin-5-sulfate (**3S**)	0.65	0.24	0.00	0.00	0.00
epicatechin-7-sulfate (**4S**)	0.43	0.41	0.00	0.00	0.00

[a]^{1}H NMR data for epicatechin (DMSO-d_6, 400 MHz): δ 6.89 (H-2′), 6.66 (s, H-5′, H-6′), 5.89 (s, H-6), 5.71 (s, H-8), 4.73 (s, H-2), 4.00 (s, H-3), 2.68 (d, J = 16.4 Hz, H-4a), 2.47 (d, J = 16.4 Hz, H-4b).

sulfates **1S**−**10S** were made on the basis of analogy to those of epicatechin.[37,38] Complete assignments of all ^{1}H NMR signals were accomplished using 2D-COSY.

The sulfate group causes the *ortho* and *para* protons to shift downfield but has little effect on the chemical shifts of the *meta* protons relative to epicatechin (Table 8). Specifically, H-2′ and H-6′ of epicatechin-3′-sulfate (**1S**) underwent downfield shifts of 0.28 and 0.39 ppm, respectively, while the chemical shift of H-5′ remained relatively unaffected. For epicatechin-4′-sulfate (**2S**), H-5′ was shifted downfield by 0.41 ppm, while H-2′ and H-6′ remained relatively unaffected. For both **1S** and **2S**, the chemical shifts of the A-ring protons were unaffected. For epicatechin-5-sulfate (**3S**), H-6 underwent a downfield shift by as much as 0.65 ppm compared with a downfield shift of 0.24 ppm for the H-8. For epicatechin-7-sulfate (**4S**), both H-6 and H-8 underwent a similar downfield shift of ca.0.4 ppm. In both instances, the chemical shifts of the B-ring protons remained unchanged.

^{13}C NMR Assignments. The assignment of proton-bearing carbons was achieved using HSQC. The assignments of the A-ring and B-ring *ipso* carbons C-5, C-7, C-9, C-10, C-1′, C-3′, and C-4′ were accomplished using HMBC. Specifically, the B-ring *ipso* carbons C-1′ and C-3′ were assigned on the basis of their three-bond coupling with H-5′, and C-4′ was assigned on the basis of correlations with H-2′ and H-6′. The A-ring *ipso* carbons C-5, C-7, and C-9 were assigned on the basis of their two-bond correlations with H-6 and H-8. The nonoxygenated *ipso* carbon C-10 showed three-bond correlations with H-6 and H-8 and in some cases two-bond correlations with H-4a and H-4b. For the methylated epicatechin sulfates, the *ipso* carbon to which the OMe group was attached showed strong three-bond coupling with the methyl protons. Thus, by the combination of

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Table 9. Changes in ^{13}C NMR Chemical Shifts of Epicatechin Sulfates 1S−10S Relative to Epicatechin[a]

Position	1S	2S	3S	4S	5S	6S	7S	8S	9S	10S
2	-0.4	-0.2	0.2	0.2	-0.0	0.1	0.1	-0.2	-0.0	-0.1
3	-0.2	-0.2	-0.1	-0.2	-0.2	-0.2	-0.2	-0.2	-0.1	-0.2
4	0.1	0.0	0.5	0.3	-0.1	0.6	0.4	0.2	0.4	0.3
5	0.1	0.1	-3.8	-0.4	0.1	-3.7	-0.7	0.2	-3.8	-0.4
6	0.1	0.1	6.0	4.8	0.2	6.0	4.8	0.2	6.0	4.7
7	0.1	0.1	-0.5	-4.0	0.1	-0.6	-3.9	0.1	-0.5	-3.9
8	0.0	-0.0	3.8	5.2	0.1	3.8	5.4	0.0	3.7	5.2
9	-0.1	-0.2	-0.6	-0.7	-0.2	-0.7	-0.8	-0.2	-0.8	-0.8
10	-0.1	-0.1	5.0	4.2	-0.1	5.0	4.0	-0.1	5.0	4.2
1′	0.4	6.0	-0.1	-0.2	4.3	-0.0	-0.0	1.0	1.7	1.8
2′	7.5	1.2	0.2	0.3	-3.0	-3.2	-3.3	5.5	-0.1	-0.1
3′	-4.4	4.0	0.2	0.5	5.4	2.5	2.5	-2.5	1.5	1.6
4′	4.0	-4.6	0.2	0.4	-2.6	1.3	1.3	5.5	2.4	2.4
5′	1.8	7.5	0.2	0.4	6.1	-0.1	-0.0	-2.6	-3.1	-3.0
6′	6.0	0.0	0.0	-0.2	0.7	1.7	1.5	4.1	-0.3	-0.4

[a]^{13}C NMR of epicatechin (DMSO-d_6, 100 MHz): δ 78.0 (C-2), 64.9 (C-3), 28.2 (C-4), 156.5 (C- 5), 95.1 (C-6), 156.2 (C-7), 94.1 (C-8), 155.7 (C-9), 98.5 (C-10), 130.6 (C-1′), 114.9 (C-2′), 144.4 (C-3′), 144.5 (C-4′), 114.7 (C-5′), 117.9 (C-6′).

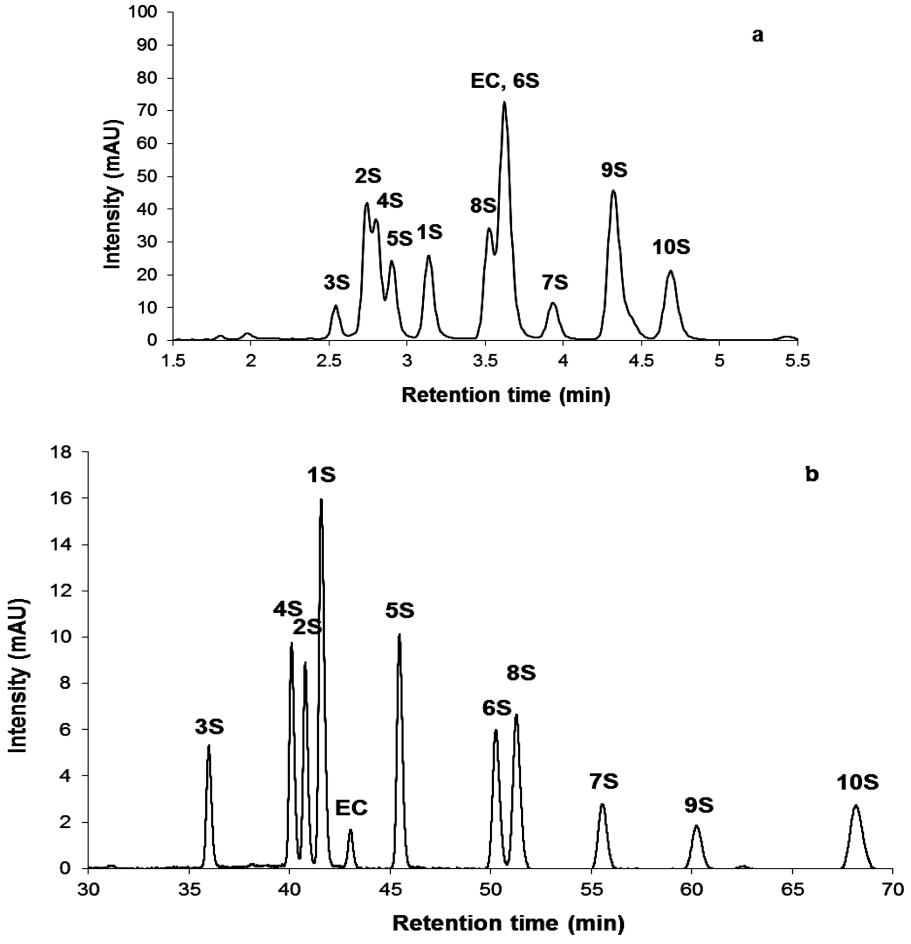

Figure 2. HPLC traces of epicatechin sulfates **1S−10S** and the parent epicatechin (EC) on an Agilent Eclipse XDB-Phenyl 3.5 μm, 4.6 × 150 mm column. Mobile phases: solvent A, 25 mM Na_3PO_4 buffer pH 2.5; solvent B, MeCN. Flow rate: 1 mL/min. UV detection: 230 nm. Temperature: ambient. (a) Isocratic conditions (25 mM Na_3PO_4 buffer pH 2.5/MeCN = 80:20). (b) Gradient conditions (0−10% B in 40 min).

HSQC and HMBC, all carbons could be assigned unambiguously.

Effects of the sulfate group on the ^{13}C NMR spectrum of epicatechin were also examined. Listed in Table 9 are changes in ^{13}C NMR chemical shifts of epicatechin sulfates relative to epicatechin. The comparison data in Table 9 reveal some interesting trends which are only apparent because of the unequivocal assignment of each signal.

As can be seen from the data, sulfation had significant effects on the *ortho* and *para* carbons in addition to the *ipso* carbon of the sulfated hydroxyl group, but little or no effect on the *meta* carbons. As might be expected, sulfation had virtually no effect on the ^{13}C chemical shifts of carbons on the other (nonsulfated) aromatic ring or on the aliphatic C-ring. Specifically, sulfation of an A- or B-ring OH group caused the *ipso* carbon to move upfield (yellow shading), whereas the

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ortho and *para* carbons moved downfield (blue shading). Similar effects of replacing a phenolic OH with a sulfate group on the ^{13}C NMR shifts of the aromatic ring have been previously reported.[41] For the six methylated epicatechin sulfates **5S**−**10S**, the unsubstituted *ortho* carbon next to the OMe group always showed an upfield shift of approximately 3 ppm (lavender shading). In addition, the *ipso* carbon bearing the OMe was deshielded relative to epicatechin as expected (green shading).

LC/MS Analysis. The epicatechin sulfate ammonium salts **1S**−**4S** exhibited a base peak of m/z 369, in the negative ionization mode, corresponding to a loss of NH_4, and a smaller peak at m/z 289 from the loss of SO_3NH_4. Under the same conditions, the methylated epicatechin sulfate ammonium salts **5S**−**10S** exhibited a base peak of m/z 383 $[M - NH_4]$ and a second peak at m/z 303 $[M - SO_3NH_4]$.

HPLC Analysis. The epicatechin sulfates **1S**−**10S** were analyzed by reversed-phase HPLC on an Agilent Eclipse XDB-Phenyl analytical column. Owing to the highly polar nature, the sulfates are not retained very well under typical reversed-phase conditions (aqueous content <50%). Using a mobile phase that consisted of 25 mM Na_3PO_4 buffer pH 2.5 and MeCN, the epicatechin sulfates exhibited adequate retention on the Eclipse XDB-Phenyl column. A mixture of sulfates **1S**−**10S** was artificially created by mixing aqueous solutions of the individual sulfates. Shown in Figure 2 are HPLC traces of the 10-sulfate mixture under isocratic conditions (80% of 25 mM Na_3PO_4 buffer pH 2.5/20% MeCN). In less than 5 min, all 10 sulfates were separated, with five having baseline separation (Figure 2a). Under gradient conditions (from 0 to 10% MeCN in 40 min) the same column provided baseline separation of all 10 sulfates plus epicatechin (Figure 2b). The HPLC peaks were assigned on the basis of retention times of the individual sulfates under the same HPLC conditions.

Stability Assessments. The chemical stability of the epicatechin sulfates in both solid state and aqueous solution was assessed by appearance, LC/MS, HPLC, and ^1H NMR. Solid samples of epicatechin sulfates **3S**, **5S**, **6S**, and **10S** stored in tightly closed vials in a refrigerator for more than 13 months did not exhibit any significant changes in appearance. LC/MS, ^1H NMR, and HPLC showed no major degradation products after the storage. In dilute aqueous solutions (<1 mg/mL), the epicatechin sulfates **1S**−**10S** are stable when kept refrigerated for at least three days, as determined by HPLC. For selected sulfates, aqueous HPLC samples kept in a refrigerator for more than a year were reinspected by visual appearance and reanalyzed by HPLC. 3′-*O*-Methylepicatechin-4′-sulfate (**5S**), 4′-*O*-methylepicatechin-3′-sulfate (**8S**), and 4′-*O*-methylepicatechin-5-sulfate (**9S**) showed no change in appearance and no decomposition products when reanalyzed by HPLC. Epicatechin-4′-sulfate (**2S**), epicatechin-5-sulfate (**3S**), 3′-*O*-methylepicatechin-5-sulfate (**6S**), and 3′-*O*-methylepicatechin-7-sulfate (**7S**) showed no change in appearance, suggesting no major oxidation of the phenols during storage. However, when reanalyzed by HPLC, the one-year-old aqueous samples of **3S**, **6S**, and **7S** all showed a new peak (11−35%) in addition to the main compound.[42]

In summary, we have synthesized all monoglucuronides and sulfates of epicatechin, 3′-*O*-methylepicatechin, and 4′-*O*-methylepicatechin, respectively, from the corresponding orthogonally protected epicatechin intermediates prepared *de novo*. Since only the phenolic hydroxy group to be glucuronidated/sulfated is unmasked while all the remaining hydroxy groups remain protected as benzyl ethers during the sulfate/glucuronide formation step, there is no ambiguity with regard to the site of glucuronidation/sulfation. The glucuronides and sulfates have been characterized by ^1H NMR, ^{13}C NMR, and LC/MS. HPLC methods capable of baseline separation of all the glucuronides and sulfates were developed. The epicatechin glucuronides and sulfates are stable in the solid state at ambient temperature and in aqueous solution when stored refrigerated. These results should prove invaluable to the research community as analytical standards as well as in future studies of the biological and pharmacological effects of epicatechin in humans.

EXPERIMENTAL SECTION

General Experimental Procedures. ^1H NMR, ^{13}C NMR, and all 2D NMR spectra were obtained on a Bruker Avance III 400 MHz spectrometer. ^1H NMR spectra were recorded at 400 MHz, and ^{13}C NMR spectra were recorded at 100 MHz. Samples were dissolved in $CDCl_3$, DMSO-d_6, acetone-d_6, and D_2O. Chemical shifts are expressed in ppm relative to TMS. Coupling constants are expressed in Hz. LC/MS data were obtained on a PE SCIEX API 100 LC/MS system equipped with a Perkin-Elmer 200 Series autosampler and LC pump. MS/MS analyses were performed on a PE SCIEX API 4000 QTrap system. The samples were introduced into the system by infusion. Preparative HPLC purifications were performed on a Gilson system consisting of a liquid handler, a syringe pump, an injection module, a binary pump, and a UV detector on a Phenomenex preparative column (Synergy 4 μ Polar-RP 80A, 250 × 21.20 mm, part no. 00G-4336-P0). Analytical HPLC was carried out on an HP 1100 series system equipped with a photodiode array detector on an Agilent Eclipse XDB-Phenyl 3.5 μm, 4.6 × 150 mm column (part no. 963967-912). The mobile phase for the preparative epicatechin glucuronide runs was a binary gradient system consisting of H_2O with 0.1% TFA by volume (solvent A) and MeCN with 0.1% TFA by volume (solvent B). The mobile phase for preparative epicatechin sulfate runs consisted of H_2O with 10 mM NH_4OAc (solvent A) and MeCN with 10 mM NH_4OAc (solvent B). The amount of solvent B was increased from 10% to 40% in 14 min or from 0 to 20% in 20 min. The flow rate was 20 mL/min. Detection by UV was set at 230 nm. The crude products were dissolved in H_2O. Injection volume was 2 mL. The mobile phases for the analytical runs were binary systems (isocratic or gradient) consisting of 25 mM Na_3PO_4 buffer pH 2.5 (solvent A) and MeCN (solvent B). Flow rate was 1 mL/min and UV detection was at 230 nm. The experiments were performed at ambient temperature.

Reagents and solvents were obtained from commercial sources and used without further purification. Specifically, methyl 2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-α-D-glucuronate was purchased from LC Scientific Inc. (Ontario, Canada). HPLC grade MeCN and H_2O were purchased from Burdick & Jackson (Honeywell). Orthogonally protected epicatechin intermediates **1a**−**10a** were prepared in house using a synthesis strategy outlined in a preliminary communication by Mull et al.[30] A full report will be published elsewhere.[31] All reactions were run under a nitrogen atmosphere at room temperature unless specified otherwise.

Representative Procedure for Benzylation of the 3-OH Group of Protected Epicatechin Intermediates 1a−**10a.** To a suspension of NaH 60% dispersion (280 mg, 7 mmol) in anhydrous DMF (10 mL) cooled in an ice-H_2O bath was added a solution of **3a** (3.0 g, 5 mmol) in anhydrous DMF (20 mL) with a syringe. After the addition, the cooling bath was removed and the reaction was stirred at ambient temperature for 30 min. The reaction was recooled in the ice-H_2O bath, and BnBr (1.2 g, 7 mmol) was added. The reaction was stirred at ambient temperature for 3 h, poured onto crushed ice (100 g), and extracted with EtOAc (150 mL × 3). The combined organic layers were washed with H_2O (100 mL) and brine (100 mL) and dried over anhydrous Na_2SO_4. Removal of solvent *in vacuo* afforded the crude product as an oil, which was purified by flash chromatography (silica gel, heptane then heptane/EtOAc, 3:1, v/v) to afford the

product **3b** as a white solid (2.5 g, 73% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.52−7.27 (15H, m), 7.22−7.17 (4H, m), 7.06−7.01 (2H, m), 6.95 (2H, s, H-5′, H-6′), 6.42 (1H, s, H-6), 6.32 (1H, s, H-8), 5.26−5.00 (8H, m, 3 × OCH_2Ph and OCH_2OCH$_3$), 4.97 (1H, s, H-2), 4.42 (2H, AB$_q$, J_{AB} = 12.4 Hz, $\Delta\nu_{AB}$ = 48.6 Hz, 3-OCH_2Ph), 3.95 (1H, m, H-3), 3.49 (3H, s, OCH_3), 2.99 (1H, dd, J = 17.2, 2.7 Hz, H-4a), 2.79 (1H, dd, J = 17.4, 4.5 Hz, H-4b); ^{13}C NMR ($CDCl_3$,100 MHz) δ 158.8, 156.7, 155.8, 149.0, 148.6, 138.3, 137.6, 137.5, 137.2, 132.4, 128.7, 128.6, 128.5, 128.3, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 120.0, 115.0, 114.1, 102.1, 96.0, 95.6, 94.7, 78.2, 72.7, 71.6, 71.4, 71.3, 70.2, 56.2, 24.7; LC/MS m/z 695 $[M + H]^+$, 717 $[M + Na]^+$.

Representative Procedure for Selective Removal of the MOM Protective Group. To a solution of **3b** (2.4g, 3.45 mmol) in a 1:1 mixture of MeOH and DCM (41 mL) was added 4 N HCl in dioxane (20.5 mL). The reaction was stirred at ambient temperature for 2 h and concentrated *in vacuo* without heating. The orange residue thus obtained was dissolved in EtOAc (200 mL) and washed with H_2O (50 mL × 3) and brine (50 mL) until the aqueous phase was close to neutral (pH 5−6). The organic layer was dried over anhydrous Na_2SO_4. Removal of solvent *in vacuo* afforded the crude product as a light brown oil, which was purified by flash chromatography (silica gel, heptane/EtOAc, 3:1, v/v) to afford the product **3c** as a pale pink solid (2.1 g, 94% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.55−7.30 (15H, m), 7.20 (4H, m), 7.07−7.01 (2H, m), 6.94 (2H, s, H-5′, H-6′), 6.27 (1H, d, J = 1.7 Hz, H-6), 6.10 (1H, d, J = 1.9 Hz, H-8), 5.26−4.95 (6H, m, 3 × OCH_2Ph), 4.73 (1H, s, H-2), 4.42 (2H, AB$_q$, J_{AB} = 12.4 Hz, $\Delta\nu_{AB}$ = 47.0 Hz, 3-OCH_2Ph), 3.97 (1H, m, H-3), 2.92 (1H, dd, J = 16.9, 3.0 Hz, H-4a), 2.78 (1H, dd, J = 16.7, 4.2 Hz, H-4b); ^{13}C NMR ($CDCl_3$,100 MHz) δ 158.7, 156.1, 155.1, 148.9, 148.5, 138.1, 137.5, 137.4, 137.2, 132.3, 128.7, 128.6, 128.5, 128.3, 128.0, 127.9, 127.8, 127.6, 127.5, 119.9, 115.0, 114.0, 100.0, 96.1, 95.5, 78.1, 72.5, 71.6, 71.4, 71.2, 70.1, 24.3; LC/MS m/z 651 $[M + H]^+$, 673 $[M + Na]^+$.

Procedure for Selective Removal of PMB Protective Group. Intermediate **2b** (0.5 g, 0.65 mmol) and pyridinium *p*-toluenesulfonate (0.082 g, 0.325 mmol) were heated in a mixture of 2-propanol (5 mL) and toluene (3 mL) at reflux for 24 h. The reaction was cooled and concentrated *in vacuo*. The residue was dissolved in EtOAc (10 mL) and washed with H_2O (5 mL). The aqueous layer was further extracted with EtOAc (5 mL). The combined EtOAc layers were washed with H_2O (5 mL × 2) and dried over anhydrous Na_2SO_4. Removal of solvent afforded the crude product as an oil, which was purified by flash chromatography (silica gel, heptane/EtOAc, 3:1, v/v) to obtain the epicatechin product **2c** as a waxy solid containing ca. 17% of the *ent*-catechin byproduct **2c′** by ^{1}H NMR (0.28 g, 66% yield). The mixture was inseparable by TLC. NMR and LC/MS data for **2c**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.39−6.79 (23H, m), 6.21 (1H, s, H-6), 6.19 (1H, s, H-8), 5.58 (1H, s, phenolic OH), 4.97−4.83 (7H, m, 3 × OCH_2Ph and H-2), 4.35 (2H, AB$_q$, J_{AB} = 12.4 Hz, $\Delta\nu_{AB}$ = 69.8 Hz, 3-OCH_2Ph), 3.88 (1H, m, H-3), 2.96 (1H, dd, J = 17.3, 2.5 Hz, H-4a), 2.72 (1H, dd, J = 17.2, 4.4 Hz, H-4b); ^{13}C NMR ($CDCl_3$,100 MHz) δ 158.8, 158.2, 155.8, 145.7, 145.6, 138.3, 137.3, 137.2, 136.5, 131.0, 128.8, 128.7, 128.5, 128.3, 128.1, 128.0, 127.8, 127.7, 127.6, 127.4, 120.3, 114.1, 111.5, 101.7, 95.0, 94.0, 78.4, 73.1, 71.4, 71.2, 70.3, 70.1, 24.7; LC/MS m/z 651 $[M + H]^+$, 673 $[M + Na]^+$.

Representative Procedure for Glucuronidation. The intermediate **3c** (325 mg, 0.5 mmol) and methyl 2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-α-D-glucuronate (275 mg, 0.575 mmol) were dissolved in anhydrous DCM (10 mL) in the presence of 4 Å molecular sieves. The reaction was stirred at ambient conditions for 30 min and then cooled in an ice-H_2O bath for 15 min. A solution of BF_3-etherate (35.5 mg, 0.25 mmol) in anhydrous DCM (2 mL) was added slowly with a syringe. The reaction was stirred for 1.5 h in the ice-H_2O bath and quenched by saturated $NaHCO_3$ solution (15 mL). The organic layer was separated, washed with brine (5 mL), and dried over anhydrous Na_2SO_4. The crude product, obtained after removal of solvent, was purified by flash chromatography (silica gel, heptane/DCM/EtOAc, 5:4:1, v/v/v) to afford the product **3d** as a white solid (390 mg, 81% yield). ^{1}H NMR data indicated the presence of ca. 10% *ent*-catechin **3d′**. NMR and LC/MS data for **3d**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.53−6.92 (23H, m), 6.38 (1H, s, H-6), 6.28 (1H, s, H-8), 5.38−5.32 (3H, m), 5.21 (2H, s, OCH_2Ph), 5.18 (1H, d, J = 7.5 Hz, H-1″), 5.08 (2H, s, OCH_2Ph), 5.02 (2H, s, OCH_2Ph), 4.92 (1H, s, H-2), 4.33 (2H, AB$_q$, J_{AB} = 12.3 Hz, $\Delta\nu_{AB}$ = 65.6 Hz, 3-OCH_2Ph), 4.20−4.14 (1H, m), 3.91 (1H, s, br, H-3), 3.75 (3H, s, OCH_3), 2.89 (1H, d, J = 17.2 Hz, H-4a), 2.79 (1H, dd, J = 17.1, 3.7 Hz, H-4b), 2.08 (9H, s, 3 × OC(═O)CH_3); ^{13}C NMR ($CDCl_3$,100 MHz) δ 170.1, 169.5, 169.3, 166.9, 158.6, 156.0, 149.0, 148.6, 138.0, 137.5, 136.9, 132.2, 128.8, 128.6, 128.5, 128.3, 128.2, 127.9, 127.7, 127.6, 127.5, 120.0, 115.0, 114.0, 103.0, 99.0, 97.4, 96.3, 78.4, 72.7, 72.3, 72.0, 71.6, 71.3, 71.2, 70.4, 69.3, 68.8, 68.7, 53.2, 53.1, 24.7, 20.8, 20.7, 20.6; LC/MS m/z 967 $[M + H]^+$, 989 $[M + Na]^+$.

Representative Procedure for Methyl Ester Hydrolysis and Removal of Acetyl Groups of the Glucuronic Acid Unit. The glucuronidation product **3d** (390 mg, 0.4 mmol) was dissolved in a mixture of THF (18 mL) and MeOH (3 mL). The mixture was cooled in an ice-H_2O bath. A solution of 1 N NaOH (6 mL) was added slowly with a syringe. The reaction was stirred for 2 h in an ice-H_2O bath and partially concentrated *in vacuo* (no heat) to remove the organic solvents. The aqueous residue (a white suspension) was acidified to pH 1 with 1 N HCl. The white solid formed was collected by filtration, rinsed with H_2O, and dried by suction to afford the product **3e** (200 mg, 60% yield): ^{1}H NMR (acetone-d_6, 400 MHz) δ 7.61−7.04 (23H, m), 6.51 (1H, s, H-6), 6.28 (1H, s, H-8), 5.24−5.02 (8H, m, 3 × OCH_2Ph plus H-1 and H-1″), 4.43 (2H, AB$_q$, J_{AB} = 11.9 Hz, $\Delta\nu_{AB}$ = 101.9 Hz, 3-OCH_2Ph), 3.82−3.70 (1H, m, H-4″), 4.09 (1H, m, H-3), 4.15 (1H, d, J = 9.7 Hz, H-4″), 3.66−3.55 (2H, m, H-2″, H-3″), 3.28 (1H, dd, J = 17.1, 2.6 Hz, H-4a), 2.84 (1H, dd, J = 16.9, 3.9 Hz, H-4b); ^{13}C NMR (acetone-d_6, 100 MHz) δ 170.3, 159.4, 158.0, 156.8, 149.6, 149.5, 139.6, 138.8, 138.7, 138.5, 133.7, 129.3, 129.2, 128.9, 128.6, 128.5, 128.4, 128.0, 121.0, 115.3, 115.2, 103.9, 102.7, 97.4, 97.0, 78.8, 77.3, 76.0, 74.4, 73.8, 72.6, 71.7, 71.6, 70.6, 24.9; LC/MS m/z 827 $[M + H]^+$, 849 $[M + Na]^+$.

Representative Procedure for the Removal of the Benzyl Groups by Hydrogenolysis to Afford the Final Glucuronides. A solution of intermediate **3e** (109 mg, 0.132 mmol) in MeOH (10 mL) containing 20% $Pd(OH)_2$/C (wet, 44 mg) was stirred under an atmosphere of H_2 for 1 h. The flask was purged with N_2, and the reaction mixture was filtered through a pad of Celite to remove catalyst. The Celite pad was rinsed with MeOH (20 mL). The combined filtrate was concentrated to dryness *in vacuo* (no heat) and purified by preparative HPLC in five injections. The purity of the product fractions was checked by analytical HPLC. Fractions with purity >95% were combined and partially evaporated *in vacuo* (no heat) to a volume of ca. 5 mL. The aqueous residue was lyophilized overnight to afford epicatechin-5-*O*-β-D-glucuronide (**3G**) as a white solid (36 mg, 59% yield).

Representative Procedure for the Preparation of the 2,2,2-Trichloroethyl-Protected Epicatechin Sulfates. The 7-OH epicatechin intermediate **10c** (780 mg, 1.36 mmol) and DMAP (166 mg, 1.36 mmol) were dissolved in anhydrous THF (15 mL) under N_2. To the reaction were added Et_3N (275 mg, 2.72 mmol) and chlorosulfuric acid 2,2,2-trichloroethyl ester (506 mg, 2.04 mmol) with a syringe. The mixture was stirred overnight at room temperature. The resulting white suspension was diluted with EtOAc (50 mL) and washed with H_2O (50 mL), 0.5 N HCl (50 mL), and 5% K_2CO_3 (50 mL), successively. The organic layer was further washed with brine (50 mL each) until the aqueous wash became neutral. The organic layer was separated and dried over anhydrous $MgSO_4$. Removal of solvent *in vacuo* afforded the crude as an oil, which was dissolved in a minimal amount of DCM and purified by flash column (silica gel, heptane/EtOAc, 3:1, v/v) to afford the epicatechin sulfate 2,2,2-trichloroethyl ester **10f** (0.47g, 44% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.60−7.01 (18H, m), 6.79 (1H, s, H-6), 6.70 (1H, s, H-8), 5.27−5.16 (4H, m, 2 × OCH_2Ph), 5.13 (1H, s, H-2), 4.94 (2H, s, OSO$_3$CH_2CCl$_3$), 4.51 (2H, AB$_q$, J_{AB} = 12.2 Hz, $\Delta\nu_{AB}$ = 54.4 Hz, 3-OCH_2Ph), 4.11 (1H, s, br, H-3), 4.07 (3H, s, 3′-OCH_3), 3.19 (1H, d, J = 17.6 Hz, H-4a), 2.96 (1H, dd, J = 17.8, 4.1 Hz, H-4b); ^{13}C NMR ($CDCl_3$,100 MHz) δ 158.1, 156.0, 149.6, 149.3, 148.2, 138.0, 137.3, 136.4, 130.8, 128.8, 128.6, 128.4, 128.3, 127.9, 127.8, 127.7, 127.5, 119.7, 113.1, 111.5,

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108.8, 102.9, 97.7, 92.6, 80.6, 78.5, 72.3, 71.6, 71.1, 70.6, 56.3, 25.1; LC/MS m/z 785 $[M + H]^+$, 807 $[M + Na]^+$.

Representative Procedure for Debenzylation of the 2,2,2-Trichloroethyl-Protected Epicatechin Sulfates. The epicatechin sulfate 2,2,2-trichloroethyl ester **10f** (470 mg, 0.60 mmol) was dissolved in EtOAc (25 mL) in the presence of $Pd(OH)_2/C$ (211 mg) and placed under an atmosphere of H_2 for 30 min. The catalyst was removed by filtration over a pad of Celite. The filtrate was concentrated *in vacuo* (with no heat) to obtain the crude 2,2,2-trichloroethyl-4′-*O*-methylepicatechin-7-sulfate (**10g**) as an off-white foam (0.29 g): ^{1}H NMR (DMSO-d_6, 400 MHz) δ 6.96 (1H, s, H-2′), 6.89 (1H, d, J = 8.3 Hz, H-5′), 6.81 (1H, d, J = 8.2 Hz, H-6′), 6.49 (1H, s, H-6), 6.48 (1H, s, H-8), 5.32 (2H, s, $OSO_3CH_2CCl_3$), 4.92 (1H, s, H-2), 4.09 (1H, br, s, H-3), 3.76 (3H, s, OCH_3), 2.81 (1H, dd, J = 17.0, 3.4 Hz, H-4a), 2.65 (1H, d, J = 16.8 Hz, H-4b); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 156.9, 155.9, 148.1, 147.0, 145.9, 131.7, 117.7, 114.7, 111.6, 107.8, 100.1, 99.7, 92.9, 80.0, 78.4, 64.0, 55.7, 28.6; LC/MS m/z 515 $[M + H]^+$, 537 $[M + Na]^+$. The product was used directly for next step.

Representative Procedure for the Removal of the 2,2,2-Trichloroethyl Group to Generate the Epicatechin Sulfates. The crude sulfate ester **10g** (270 mg, 0.52 mmol) was treated with ammonium formate (198 mg, 3.14 mmol) and Zn dust (67 mg, 1.02 mmol) in MeOH (10 mL) at room temperature. After completion of the reaction (TLC-silica gel, heptane/EtOAc, 1:2, v/v), the mixture was filtered through a pad of Celite. The filtrate was concentrated *in vacuo* (no heat). The oily residue, thus obtained, was mixed with H_2O (6 mL). The precipitated solid was removed by filtration, and the filtrate was purified by preparative HPLC in three injections using the conditions described earlier (*vide supra*). Fractions containing pure product were pooled and concentrated *in vacuo* to a volume of about 5 mL. The aqueous residue was lyophilized to obtain the 4′-*O*-methylepicatechin-7-sulfate ammonium salt (**10S**) as a white powder (101 mg, 42% over two steps).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Copies of ^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC spectra of **1G**−**10G** and **1S**−**10S** and MS/MS spectra of **1G**−**3G**, **5G**, **6G**, and **10G**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(36) Characterization data of 3′-*O*-methyl-*ent*-catechin-4′-*O*-*β*-D-glucuronide (**5G′**): ^{1}H NMR (acetone-d_6 with 10% D_2O, 400 MHz) *δ* 7.13 (1H, d, *J* = 8.2 Hz, H-5′), 7.08 (1H, s, H-2′), 6.96 (1H, d, *J* = 8.3 Hz, H-6′), 6.01 (1H, s, H-6), 5.86 (1H, s, H-8), 5.08 (1H, d, *J* = 7.0 Hz, H-1″), 4.58 (1H, d, *J* = 8.3 Hz, H-2), 4.06−3.93 (2H, m, H-5″, H-3), 3.84 (3H, s, OCH_3), 3.70−3.62 (1H, m, H-3″), 3.62−3.53 (2H, m, H-4″, H-2″), 2.93 (1H, dd, *J* = 16.1, 5.7 Hz, H-4a), 2.49 (1H, dd, *J* = 15.9, 9.0 Hz, H-4b); ^{13}C NMR (acetone-d_6 with 10% D_2O, 100 MHz) *δ* 157.5, 157.1, 156.4, 149.7, 146.8, 135, 121.1, 116.5, 112.6, 101.8, 100.4, 96.2, 95.1, 82.3, 76.5, 73.9, 72.3, 68.1, 56.4, 29.0; LC/MS (negative mode) *m*/*z* 479 $[M - H]^-$.
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