JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2007,** *55,* 9685–9697 **9685**

Nonenzymatic *C*-Glycosylation of Flavan-3-ols by Oligo- and Polysaccharides

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Model reactions between the polysaccharide amylose and the polyphenol (−)-epicatechin followed by partial enzymatic hydrolysis of the reaction products formed led to the detection of mono- and oligo-*C*-glucosylated flavan-3-ols by means of LC-MS/MS experiments. To confirm the structure of these putative flavan-3-ol/oligosaccharide conjugates, (−)-epicatechin was reacted with maltose and maltotriose, respectively, giving rise to a series of previously unreported flavan-3-ol/maltose and flavan-3-ol/maltotriose conjugates, namely, (−)-epicatechin-8-*C*-*β*-D-glucopyranosyl-(4→1)-*O*-α-D-glucopyranoside, (−)-catechin-8-*C*-*β*-D-glucopyranosyl-(4→1)-*O*-α-D-glucopyranoside, (−)-catechin-6-*C*-*β*-D-glucopyranosyl-(4→1)-*O*-α-D-glucopyranoside, (−)-catechin-8-*C*-*β*-D-glucopyranosyl-(4→1)-*O*-α-D-glucopyranosyl-(4→1)- *O*-α-D-glucopyranoside, (−)-catechin-6-*C*-*β*-D-glucopyranosyl-(4→1)-*O*-α-D-glucopyranosyl-(4→1)-*O*-α-D-glucopyranoside, and (−)-epicatechin-6/8-*C*-*β*-D-glucopyranosyl-(4→1)-*O*-α-D-glucopyranosyl-(4→1)-*O*-α-D-glucopyranoside. Furthermore, quantitative analysis of flavan-3-ol-*C*-glucosides in an enzymatic total hydrolysate using a newly developed stable isotope dilution assay (SIDA) enabled a first insight into the yield of the formation of polyphenol/polysaccharide cross-links, for example, an amount of 14.0, 9.0, and 0.15 μmol of flavan-3-ol-6-*C*-*β*-D-glucopyranoside, flavan-3-ol-8-*C*-*β*-D-glucopyranoside, and flavan-3-ol-6-*C*,8-*C*-*β*-D-glucopyranoside were per mmol (−)-epicatechin when reacted with amylose.

KEYWORDS: Flavan-3-ol; epicatechin; catechin; *C*-glycosylation; starch; polysaccharides; cross-links; (−)-catechin-8-*C*-*β*-D-maltopyranoside

INTRODUCTION

Over the last decade, scientists and food manufacturers have become increasingly interested in polyphenols due to their great abundance in our diet, their various biological functions, and their probable role in the prevention of diseases associated with oxidative stress, such as cardiovascular and neurodegenerative diseases (*1*). However, polyphenols are highly reactive compounds and suitable substrates for numerous enzymatic and chemical reactions during postharvest food processing and storage such as condensation reactions between flavanols and anthocyanins (*2*), aldehydes (*3*), and ellagitannins (*4*), respectively, as well as redox reactions (*5*). Although the occurrence of such reactions and their contribution to food quality are well documented, the chemical structures of the reaction products formed by postharvest polyphenol transformation are still poorly understood.

Very recently, application of a molecular sensory science approach on alkalized and nonalkalized cocoa powder revealed the detection of a velvety, smoothly astringent-tasting polyphenol fraction that was predominantly present in the alkalized sample (*6*). LC-MS/MS analysis, 1D/2D-NMR, and CD spectroscopy, as well as model reactions led to the discovery and unequivocal identification of these sensory active molecules in cocoa as a series of flavan-3-ol-*C*-*β*-D-glycopyranosides (**Figure 1**), namely, (−)-catechin-8-*C*-*β*-D-glucopyranoside (**1**), (−)-catechin-6-*C*,8-*C*-*β*-D-diglucopyranoside (**2**), (−)-catechin-6-*C*-*β*-D-glucopyranoside (**3**), (−)-epicatechin-8-*C*-*β*-D-glucopyranoside (**4**), (−)-epicatechin-6-*C*,8-*C*-*β*-D-diglucopyranoside (**5**), and (−)-epicatechin-6-*C*-*β*-D-glucopyranoside (**6**) (*6*). Besides exhibiting a velvety astringent taste, these flavan-3-ol-*C*-glycosides were demonstrated to modify the bitter taste profile and decrease the bitter taste intensity of cocoa beverages as well as theobromine solutions. Most surprisingly, model experiments revealed that these polyphenol *C*-glycoconjugates are formed upon cocoa alkalization via a novel "non-enzymatic *C*-glycosylation" of flavan-3-ols (*6*).

Although flavan-3-ols were shown to react nonenzymatically with glucose (*6*), it is unclear whether these polyphenols are able to react covalently with the reducing terminus of polysaccharides such as amylose, thus giving rise to polysaccharide/polyphenol conjugates. Therefore, the purpose of the present

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10.1021/jf0719508 CCC: $37.00 © 2007 American Chemical Society
Published on Web 10/12/2007

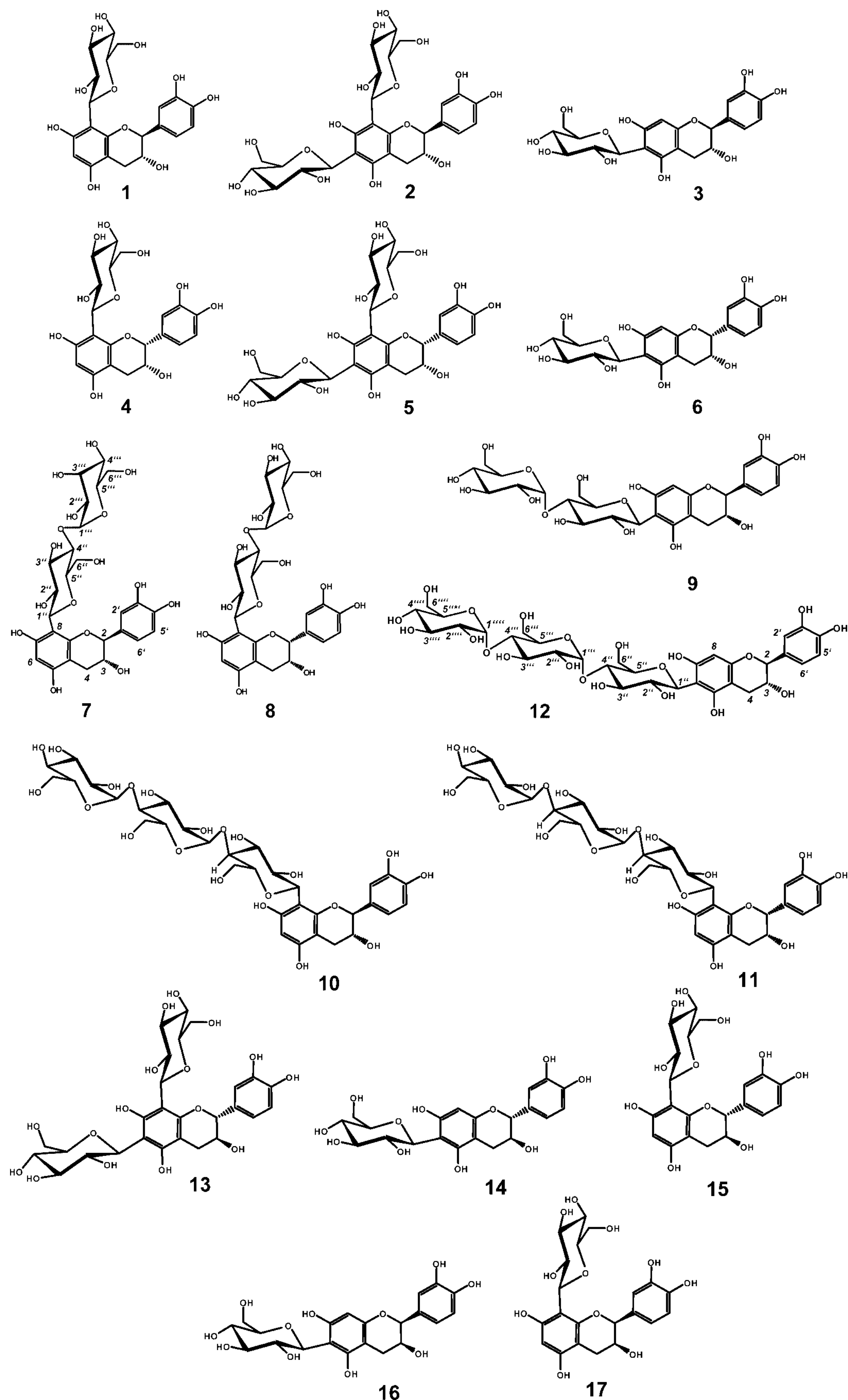

Figure 1. Chemical structures of flavan-3-ol-*C*-glycosides **1**–**17**.

study was to answer the question as to whether reducing oligo- and polysaccharides such as amylose can also bind covalently to plant flavan-3-ols as has been shown for monohexoses.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: (−)-epicatechin, (+)-catechin (Sigma, Steinheim,

Germany); potassium carbonate, D-glucose, D-maltose, D-maltotriose (Merck, Darmstadt, Germany); amylose from maize (Roth, Karlsruhe, Germany). Amyloglucosidase (from *Aspergillus niger* 119.7 U) (Fluka, Taufkirchen, Germany) showed an activity of 120 U/mg, where 1 U is the amount of enzyme necessary to break down 1 μmol of glucose per min at pH 4.8 and 60 °C. The thermostable exo-α-amylase Termamyl 120L (from *Bacillus licheniformis*) (Novo Nordisk, Denmark) showed an activity of 120 KNU/g, where 1 KNU is the amount of enzyme necessary to break down 5.26 g of starch/h under standard conditions (Termamyl Product Data Sheet, Novo Nordisk, 1995). Solvents were HPLC grade (Merck, Darmstadt). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). Bottled water (Evian) adjusted to pH 6.0 with aqueous formic acid (1%) was used for sensory evaluation. Flavan-3-ol -*C*-β-D-glucopyranosides **1–6** (**Figure 1**) were synthesized and purified following the procedure reported recently (*6*). Throughout the paper, D-glucopyranoside is abbreviated as D-glc *p*.

Sensory Analyses. *Training of the Sensory Panel.* Twelve subjects (seven women and five men, age 25–38 years), who gave their informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, were trained to evaluate the taste of aqueous solutions (3 mL each) of the following standard taste compounds by using a triangle test as described in the literature (*7*): sucrose (12.5 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, and sodium glutamate (3 mmol/L) for umami taste. For the puckering astringency and the velvety astringent, mouth-drying oral sensation, the panel was trained with aqueous solutions of gallotannic acid (0.05%) and quercetin-3-*O*-β-D-glc *p* (0.002 mmol/L), respectively, using the half-tongue test (*8*). Sensory analyses were performed in a sensory panel room at 22–25 °C in three different sessions.

Half-Tongue Test. To overcome carry-over effects of astringent compounds, threshold concentrations of astringent compounds were determined in bottled water by means of the recently developed half-tongue test (*8*). Serial 1:1 dilutions of the samples were presented in order of increasing concentrations to a trained panel of 12 persons in three different sessions, using the sip-and-spit method. At the start of the session and before each trial, the subject rinsed with water and expectorated. An aliquot (1 mL) of the aqueous solution containing the astringent compound was randomly applied with a pipette on one side of the tongue, whereas pure water was applied on the other side of the tongue as the control. The sensory panelists were then asked to move their tongue forward and backward towards the palate for 15 s and to identify the place of astringent sensation by comparison of both sides. After indicating which part of the tongue showed the typical astringent sensation induced by the tastant, the participant rinsed with water and, after 10 min, received another set of one blank and one taste-active sample. To prevent excessive fatigue, tasting began at a concentration level two steps below the threshold concentration that had been determined in a preliminary taste experiment. Whenever the panelist selected incorrectly, the next trial took place at the next higher concentration step. When the panelist selected correctly, the same concentration was presented again besides one blank as a proof for the correctness of the data. The geometric mean of the last and the second last concentration was calculated and taken as the individual recognition threshold. Values between individuals and three separate sessions differed not more than plus or minus one dilution step; that is, a threshold value of 1.4 μmol/L for (−)-epicatechin-8-*C*-β-D-glc*p*-(4→1)-*O*-α-D-glc*p*(4→1)-*O*-α-D-glc *p* represents a range from 0.7 to 2.8 μmol/L.

Preparation and Characterization of Flavan-3-ol *C*-Polyglucopyranosides from Flavan-3-ols and Amylose. *Preparation of Flavan-3-ol-C-Polyglucopyranosides.* (−)-Epicatechin (1 mmol) was mixed with amylose (5 g), K_2CO_3 (5 mmol), and water (30 mL), and then thermally treated for 10 min at 80 °C while stirring. After the sample was cooled, the reaction was stopped by the addition of acetic acid until a pH of 7.0 was reached.

Analysis of Flavan-3-ol-C-Polyglucopyranosides. After addition of the Termamyl preparation (1 mL), the reacted epicatechin/amylose mixture (pH 7.0) was kept in a boiling water bath for 15 min and then cooled to room temperature. The pH of the reaction mixture was adjusted to 4.6 by the addition of acetic acid, amyloglucosidase (2 mL, 10 mg/mL) was added, and the solution was incubated for 30 min at 60 °C. After cooling, the mixture was centrifuged (3000 rpm), the supernatant was collected and concentrated in a vacuum (aqueous fraction), and the insoluble residue was extracted four times with aqueous ethanol (40%, 30 mL each) for 30 min at room temperature upon stirring. After centrifugation, the ethanolic extract was freed from solvent under reduced pressure at 40 °C and combined with the water fraction to give a crude extract of the reaction mixture (10 mL). Aliquots (2 mL) of the crude extract were applied onto the top of a Strata Gigatube C18 cartridge (5g) (Phenomenex, Germany) preconditioned with methanol, followed by water. Fractionation was performed by flushing the cartridge with water (50 mL, fraction S1), followed by methanol (50 mL, fraction S2). Solvent was removed from fraction S2 in vacuum, the residue was taken up in a methanol/water mixture (1/9, v/v; 1 mL) adjusted to pH 2.5 with aqueous formic acid (0.1% in water), and then aliquots (5 μL) were analyzed by means of HPLC-MS/MS, which was equipped with a 150 × 2 mm i.d., 5 μm, RP Phenylhexyl column (Phenomenex, Germany) operated with a flow rate of 0.2 mL/min. Chromatography was performed starting isocratically with aqueous formic acid (0.1%, pH 2.5) for 10 min, then the methanol content was increased to 40% within 40 min, increased to 100% within another 10 min, and, finally, held at 100% for 10 min.

Preparation of Flavan-3-ol -*C*-Oligoglucopyranosides (7–12, Figure 1). *Analytical Scale.* A mixture of (−)-epicatechin (0.04 mmol), maltose, or maltotriose (0.04, 0.08, 0.2, 0.4, 0.8, or 4 mmol, respectively), K_2CO_3 (0.21 mmol), and water (1 mL) was heated at 80 °C while stirring. After 10, 20, 40, and 60 min, the reaction was stopped by adjusting the pH to 5.0 using aqueous hydrochloric acid (1 mol/L) and, after membrane filtration, aliquots (10–50 μL) of the mixture were analyzed by means of RP–HPLC–DAD.

Preparative Scale. (−)-Epicatechin (2 mmol) was mixed with maltose or maltotriose (40 mmol, each), K_2CO_3 (10.4 mmol), and water (40 mL). After homogenization, the mixture was heated for 10 min at 80 °C while stirring. After cooling, the reaction was stopped by the addition of aqueous hydrochloric acid (1 mol/L) until a pH of 5.0 was reached. Thereafter, the reaction mixture was concentrated under reduced pressure at 40 °C to about 10 mL and was then applied onto the top of a water-cooled 140 × 40 mm RP-18 column, LiChroprep, 25–40 μm (Merck, Darmstadt, Germany) conditioned with aqueous formic acid (0.1% in water; pH 2.5). Chromatography was performed using aqueous formic acid (0.1% in water; pH 2.5) as the effluent, followed by aqueous formic acid (0.1% in water;

pH 2.5) containing increasing amounts of methanol. Monitoring the effluent at 270 nm, the fractions containing the title compounds were freed from solvent in vacuum to give 10 subfractions (f1–f10), freeze-dried, and finally purified by means of HPLC (Jasco, Groβ-Umstadt, Germany) consisting of a HPLC-pump system PU 2087, a high-pressure gradient unit, and a PU-2075 UV-detector using a preparative 21.2 × 250 mm, 5 μm, RP-18 column, HyperClone micro ODS (C18) (Phenomenex, Aschaffenburg, Germany) as the stationary phase. Subfractions f3–f6 were dissolved in a mixture (30/70, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5), and after membrane filtration aliquots (0.5–2.0 mL) were fractionated by RP-HPLC. Monitoring the effluent at 280 nm, chromatography was performed with a mixture (5/95, v/v) of methanol and aqueous formic acid (0.1% in water, pH 2.5) for 10 min, increasing the methanol content to 40% over 30 min, and then to 100% over 5 min, thereafter, eluting with methanol for 10 min at a flow rate of 18.0 mL/min. After the solvent was removed in vacuum, the title compounds were suspended in water (10 mL), and freeze-dried two times to afford the corresponding flavan-3-ol-*C*-oligoglucopyranosides **7–12** (**Figure 1**) as white, amorphous powders in high purities of more than 99%.

(−)-Catechin-8-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p*, **7** (**Figure 1**): UV/vis (MeOH/0.1% HCOOH, 3/7, v/v, pH 2,5) λ_{max} = 218, 231, 280 nm; exact mass: *m/z* 637.1730 (calc. for $[C_{27}H_{34}O_{16} + Na]^+$ = 637.1739), *m/z* 613.1778 (calc. for $[C_{27}H_{33}O_{16}]^-$ = 613.1763); MS (ESI$^-$): *m/z* 613 (100% $[M - 1]^-$), 451 (40% $[M - 163]^-$), 433 (30% $[M - 181]^-$), 289 (20% $[M - 325]^-$), 331 (12% $[M - 283]^-$); MS/MS (ESI$^-$) (−50 V): *m/z* 331 (100), 123 (55), 109 (40), 373 (8), 613 (1); ^{1}H NMR (400 MHz, MeOD, COSY): δ 2.55 [dd, 1H, *J* = 7.0, 16.4 Hz, H-C(4α)], 2.74 [dd, 1H, *J* = 5.2, 16.4 Hz, H-C(4β)], 3.27 [pt, 1H, *J* = 9.2 Hz, H-C(4‴)], 3.42–3.46 [m, 2H, *J* = 3.6, 4.0, 9.2, 9.6 Hz, H-C(5″, 2″)], 3.59–3.71 [m, 5H, *J* = 8.0, 8.4, 10.0, 10.4 Hz, H-C(3″, 3‴, 4″, 5‴, 6α″)], 3.81–3.89 [m, 3H, *J* = 2.0, 12.0 Hz, H-C(6β″, 6α‴, 6β‴)], 3.99 [ddd, 1H, *J* = 5.6, 6.4, 6.6 Hz, H-C(3)], 4.10 [dd, 1H, *J* = 8.8, 9.2 Hz, H-C(2″)], 4.76 [d, 1H, *J* = 6.4 Hz, H-C(2)], 4.84 [d, 1H, *J* = 9.2 Hz, H-C(1″)], 5.16 [d, 1H, *J* = 3.6 Hz, H-C(1‴)], 6.01 [s, 1H, H-C(6)], 6.71 [dd, 1H, *J* = 1.6, 8.0 Hz, H-C(6′)], 6.75 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.83 [d, 1H, *J* = 1.6 Hz, H-C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC): δ 27.0 [C(4)], 62.3 [C(6″/6‴)], 62.7 [C(6‴/6″)], 68.5 [C(3)], 71.5 [C(4‴)], 73.0 [C(2″)], 74.3 [C(2‴)], 74.7 [C(5‴/3‴)], 75.1 [C(5‴/3‴)], 76.4 [C(1″)], 79.8 [C(3″)], 80.9 [C(5″)], 81.6 [C(4″)], 82.4 [C(2)], 97.1 [C(6)], 101.0 [C(4a)], 103.0 [C(1‴)], 104.1 [C(8)], 114.7 [C(2′)], 116.2 [C(5′)], 119.5 [C(6′)], 132.4 [C(1′)], 146.1 [C(3′/4′)], 146.2 [C(4′/3′)], 155.2 [C(8a)], 157.1 [C(5/7)], 157.6 [C(7/5)].

(−)-Epicatechin-8-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p*, **8** (**Figure 1**): UV/vis (MeOH/0.1% HCOOH, 3/7, v/v, pH 2,5) λ_{max} = 218, 231, 280 nm; exact mass: *m/z* 637.1730 (calc. for $[C_{27}H_{34}O_{16} + Na^+]$ = 637.1739), *m/z* 613.1778 (calc. for $[C_{27}H_{33}O_{16}]^-$ = 613.1763); MS (ESI$^-$): *m/z* 613 (100% $[M - 1]^-$), 451 (37% $[M - 163]^-$), 433 (31% $[M - 181]^-$), 289 (18% $[M - 325]^-$), 331 (17% $[M - 283]^-$); MS/MS (ESI$^-$) (−50 V): *m/z* 331 (100), 123 (55), 109 (40), 373 (8), 613 (1); ^{1}H NMR (400 MHz, MeOD, COSY): δ 2.83 [dd, 1H, *J* = 1.6, 17.2 Hz, H-C(4α)], 2.88 [dd, 2H, *J* = 4.0, 16.8 Hz, H-C(4β)], 3.29 [pt, 1H, *J* = 9.2 Hz, H-C(4‴)], 3.45–3.48 [m, 2H, *J* = 3.6, 9.6 Hz, H-C(5″, 2‴)], 3.63–3.74 [m, 6H, *J* = 8.4, 9.2, 9.6 Hz, H-C(2″, 3″, 3‴, 4″, 5‴, 6α″)], 3.83–3.95 [m, 3H, *J* = 2.8, 3.6, 11.6, 12.0, 12.4 Hz, H-C(6β″, 6α‴, 6β‴)], 4.16 [br s, 1H, H-C(3)], 4.89 [m, 2H, *J* = 9.2 Hz, H-C(2, 1″)], 5.19 [d, 1H, *J* = 3.2 Hz, H-C(1‴)], 6.02 [s, 1H, H-C(6)], 6.78 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.83 [d, 1H, *J* = 8.0 Hz, H-C(6′)], 7.08 [s, 1H, H-C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC): δ 29.8 [C(4)], 62.2 [C(6″/6‴)], 62.7 [C(6‴/6″)], 67.4 [C(3)], 71.5 [C(4‴)], 74.3 [C(2‴)], 74.8 [C(2″, 4″)], 75.1 [C(5″)], 76.5 [C(1″)], 79.8 [C(2)], 79.9 [C(3″)], 80.7 [C(5″)], 81.8 [C(4″)], 97.1 [C(6)], 100.0 [C(4a)], 103.3 [C(1‴)], 104.2 [C(8)], 115.1 [C(2′)], 116.1 [C(5′)], 118.8 [C(6′)], 132.5 [C(1′)], 145.6 [C(3′/4′)], 146.1 [C(4′/3′)], 157.1 [C(8a)], 158.2 [C(5, 7)].

(−)-Catechin-6-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p*, **9** (**Figure 1**): UV/vis (MeOH/0.1% HCOOH, 3/7, v/v pH 2,5) λ_{max} = 218, 231, 280 nm; exact mass: *m/z* 637.1730 (calc. for $[C_{27}H_{34}O_{16} + Na^+]$ = 637.1739), *m/z* 613.1778 (calc. for $[C_{27}H_{33}O_{16}]^-$ = 613.1763); MS (ESI$^-$): *m/z* 613 (100% $[M - 1]^-$), 451 (41% $[M - 163]^-$), 433 (28% $[M - 181]^-$), 289 (15% $[M - 325]^-$), 331 (10% $[M - 283]^-$); MS/MS (ESI$^-$) (−50 V): *m/z* 331 (100), 123 (55), 109 (40), 373 (8), 613 (1); ^{1}H NMR (400 MHz, MeOD, COSY): δ 2.51 [dd, 1H, *J* = 8.0, 16.0 Hz, H-C(4α)], 2.87 [dd, 1H, *J* = 5.2, 16.4 Hz, H-C(4β)], 3.29 [pt, 1H, *J* = 9.2 Hz, H-C(4‴)], 3.47 [dd, 1H, *J* = 3.6, 9.6 Hz, H-C(2‴)], 3.51 [m, 1H, H-C(5″)], 3.63–3.75 [m, 6H, *J* = 8.8, 10.0 Hz, H-C(2″, 3″, 3‴, 4″, 5‴, 6α″)], 3.84–3.95 [m, 3H, *J* = 3.2, 4.8, 10.4 Hz, H-C(6β″, 6α‴, 6β‴)], 3.98 [ddd, 1H, *J* = 5.6, 6.8, 8.0 Hz, H-C(3)], 4.57 [d, 1H, *J* = 7.2 Hz, H-C(2)], 4.88 [d, 1H, *J* = 8.6 Hz, H-C(1″)], 5.24 [d, 1H, *J* = 3.6 Hz, H-C(1‴)], 5.96 [s, 1H, H-C(8)], 6.71 [dd, 1H, *J* = 1.2, 8.0 Hz, H-C(6′)], 6.76 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.82 [d, 1H, *J* = 1.2 Hz, H-C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC): δ 28.5 [C(4)], 61.7 [C(6″/6‴)], 62.8 [C(6‴/6″)], 68.7 [C(3)], 71.6 [C(4‴)], 74.1 [C(2″)], 74.3 [C(2‴)], 74.9 [C(3‴/5‴)], 75.2 [C(5‴/3‴)], 77.3 [C(1″)], 79.4 [C(3″)], 81.0 [C(4″, 5″)], 82.8 [C(2)], 96.3 [C(8)], 102.2 [C(4a)], 103.0 [C(1‴)], 105.3 [C(6)], 115.2 [C(2′)], 116.1 [C(5′)], 120.0 [C(6′)], 132.1 [C(1′)], 146.28 [C(3′/4′)], 146.30 [C(4′/3′)], 156.11 [C(5/7)], 156.13 [C(7/5)], 156.4 [C(8a)].

(−)-Catechin-8-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p*, **10** (**Figure 1**): UV/vis (MeOH/0.1% HCOOH, 3/7, v/v, pH 2.5) λ_{max} = 218, 231, 280 nm; exact mass: *m/z* 775.2314 (calc. for $[C_{33}H_{43}O_{21}]^-$ = 775.2291); MS (ESI$^-$): *m/z* 775 (100% $[M - 1]^-$), 331 (33% $[M - 445]^-$), 289 (20% $[M - 487]^-$), 451 (12% $[M - 325]^-$); MS/MS (ESI$^-$) (−50 V): *m/z* 331 (100), 109 (40), 123 (27), 203 (26); ^{1}H NMR (400 MHz, D_2O, COSY): δ 2.61 [dd, 1H, *J* = 8.4, 16.0 Hz, H-C(4α)], 2.96 [dd, 1H, *J* = 5.2, 16.0 Hz, H-C(4β)], 3.47 [pt, 1H, *J* = 9.2 Hz, H-C(4⁗)], 3.53–3.55 [m, 2H, H-C(5″, 5‴)], 3.60–3.96 [m, 14H, *J* = 2.8, 3.6, 4.0, 8.4, 8.8, 9.2, 10.8 Hz, H-C(2‴, 2⁗, 3″, 3‴, 3⁗, 4″, 4‴, 5⁗, 6α″, 6α‴, 6α⁗, 6β″, 6β‴, 6β⁗)], 4.10 [ddd, 1H, *J* = 5.6, 6.8, 7.2 Hz, H-C(3)], 4.21 [pt, 1H, *J* = 8.8 Hz, H-C(2″)], 4.84 [d, 1H, *J* = 7.2 Hz, H-C(2)], 4.95 [d, 1H, *J* = 8.8 Hz, H-C(1″)], 5.31 [d, 1H, *J* = 4.0 Hz, H-C(1‴/1⁗)], 5.41 [d, 1H, *J* = 4.0 Hz, H-C(1⁗/1‴)], 6.18 [s, 1H, H-C(6)], 6.89 [s, 2H, H-C(6′, 5′)], 6.97 [s, 1H, H-C(2′)];^{13}C NMR (100 MHz, D_2O, HMQC, HMBC): δ 27.0 [C(4)], 60.3 [C(6″/6‴/6⁗)], 60.4 [C(6″/6‴/6⁗)], 67.0 [C(3)], 69.3 [C(4⁗)], 71.0 [C(2″)], 71.4, 71.6, 72.9, 73.0, 73.4 [7C, C(3″, 3‴, 3⁗, 4‴, 5⁗, 2‴, 2⁗)], 74.2 [C(1″)], 76.8 [C(5‴)], 78.0 [C(4″)], 78.7 [C(5″)], 80.8 [C(2)], 96.2 [C(6)], 99.9 [C(1‴/1⁗)], 100.2 [C(1⁗/1‴)], 100.7 [C(4a)], 102.4 [C(8)], 114.3 [C(2′)], 115.9 [C(5′)], 119.8 [C(6′)], 130.8 [C(1′)], 144.0 [C(3′, 4′)], 154.0 [C(8a)], 154.9 [C(5, 7)].

(−)-Epicatechin-8-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)- *O*-α-D-glc *p*, **11** (**Figure 1**): UV/vis (MeOH/0.1% HCOOH, 3/7, v/v, pH 2.5) λ_{max} = 218, 231, 280 nm; exact mass: *m/z* 775.2314

(calc. for $[C_{33}H_{43}O_{21}]^-$ = 775.2291); MS (ESI⁻): *m/z* 775 (100% [M – 1]⁻), 331 (35% [M – 445]⁻), 289 (23% [M – 487]⁻), 451 (13% [M – 325]⁻); MS/MS (ESI⁻) (−50 V): *m/z* 331 (100), 123 (80), 109 (51); ¹H NMR (400 MHz, MeOD, COSY): δ 2.82 [dd, 1H, *J* = 2.0, 17.6 Hz, H-C(4α)], 2.88 [dd, 1H, *J*=4.0, 16.7 Hz, H-C(4β)], 3.27 [pt, 1H, *J* = 9.6 Hz, H-C(4′′′′)], 3.43–3.92 [m, 17H, *J* = 1.2, 2.8, 3.6, 8.8, 9.2, 9.6, 12.4, 12.8 Hz, H-C(2′′, 2′′′, 2′′′′, 3′′, 3′′′, 3′′′′, 4′′, 4′′′, 5′′, 5′′′,5′′′′, 6α′′, 6α′′′, 6α′′′′, 6β′′, 6β′′′, 6β′′′′)], 4.16 [s, 1H, H-C(3)], 4.89 [d, 1H, *J*=9.6 Hz, H-C(1′′)], 4.90 [s, 1H, H-C(2)], 5.16 [d, 1H, *J* = 4.0 Hz, H-C(1′′′/1′′′′)], 5.20 [d, 1H, *J* = 3.6 Hz, H-C(1′′′/ 1′′′′)], 6.02 [s, 1H, H-C(6)], 6.78 [d, 1H, *J* = 8.2 Hz, H-C(5′)], 6.84 [dd, 1H, *J*=2.0, 8.2 Hz, H-C(6′)], 7.09 [s, 1H, H-C(2′)].

(−)-Catechin-6-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p*, **12** (**Figure 1**): UV/vis (MeOH/0.1% HCOOH, 3/7, v/v pH 2.5) λ_{max} = 218, 231, 280 nm; exact mass: *m/z* 775.2314 (calc. for $[C_{33}H_{43}O_{21}]^-$ = 775.2291); MS (ESI⁻): *m/z* 775 (100% [M – 1]⁻), 331 (29% [M – 445]⁻), 289 (21% [M – 487]⁻), 451 (8% [M – 325]⁻); MS/MS (ESI⁻) (−50 V): *m/z* 331 (100), 109 (43), 123 (24), 203 (20); ¹H NMR (400 MHz, D_2O, COSY): δ 2.59 [dd, 1H, *J* = 8.0, 16.0 Hz, H-C(4α)], 2.95 [dd, 1H, *J* = 5.6, 16.0 Hz, H-C(4β)], 3.46 [dd, 1H, *J* = 9.2, 9.6 Hz, H-C(4′′′′)], 3.62 [dd, 1H, *J* = 4.0, 10.0 Hz, H-C(2′′′′)], 3.66–3.71 [m, 2H, *J* = 4.8, 5.2, 9.6 Hz, H-C(5′′, 5′′′)], 3.72 [dd, 1H, *J* = 4.0, 9.6 Hz, H-C(2′′′)], 3.75–4.09 [m, 12H, *J* = 2.0, 4.8, 8.4, 8.8, 9.2, 10.4, 12.4 Hz, H-C(3′′, 3′′′, 3′′′′, 4′′, 4′′′, 5′′′′, 6α′′, 6α′′′, 6α′′′′, 6β′′, 6β′′′, 6β′′′′)], 4.03 [dd, 1H, *J* = 9.2, 9.6 Hz, H-C(2′′)], 4.20 [ddd, 1H, *J* = 5.6, 6.8, 7.6 Hz, H-C(3)], 4.77 [d, 1H, *J* = 6.8 Hz, H-C(2)], 4.93 [d, 1H, *J* = 8.8 Hz, H-C(1′′)], 5.43 [d, 1H, *J*=4.0 Hz, H-C(1′′′′)], 5.46 [d, 1H, *J* = 4.0 Hz, H-C(1′′′)], 6.12 [s, 1H, H-C(8)], 6.89 [d, 1H, *J* = 8.0 Hz, H-C(6′)], 6.97 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.98 [s, 1H, H-C(2′)]; ¹³C NMR (100 MHz, D_2O, HMQC, HMBC): δ 26.5 [C(4)], 60.4 [C(6′′, 6′′′, 6′′′′)], 66.5 [C(3)], 69.3 [C(4′′′′)], 71.1 [C(3′′′)], 71.4 [C(2′′, 2′′′)], 71.6 [C(2′′′′)], 72.6, 72.9, 73.2 [4C, C(3′′, 3′′′′, 4′′′, 5′′′′)], 74.8 [C(1′′)], 77.1 [C(5′′′)], 77.6 [C(4′′)], 78.7 [C(5′′)], 80.8 [C(2)], 95.8 [C(8)], 99.8 [C(1′′′, 1′′′′)], 101.8 [C(4a)], 104.2 [C(6)], 114.9 [C(2′)], 115.7 [C(5′)], 119.9 [C(6′)], 130.4 [C(1′)], 144.2 [C(3′/4′)], 144.4 [C(4′/3′)], 154.0 [C(5, 7)], 154.8 [C(8a)].

Preparation of (+)-Flavan-3-ol-*C*-glucopyranosides 13–17 (Figure 4). Compounds **13–17** were prepared by reacting (+)-catechin and D-glucose in aqueous K_2CO_3 solution for 10 min at 80 °C following exactly the procedure reported recently for the (−)-epicatechin (*6*).

(+)-Catechin-6-*C*,8-*C*-β-D-diglcp, **13** (**Figure 1**): UV/vis (MeOH) λ_{max} = 220, 241, 273 nm; MS (ESI⁻): *m/z* 613 (100% [M – 1]⁻), 373 (59% [M – 241]⁻), 493 (35% [M – 121]⁻), 331 (10% [M – 283]⁻); MS/MS (ESI⁻): 373 (100% [M – 124]⁻), 109 (96% [M-505]⁻), 123 (82% [M – 491]⁻), 129 (63% [M – 465]⁻), 331 (10% [M – 283]⁻); exact mass: *m/z* 613.1784 (calculated for $C_{27}H_{33}O_{16}^-$ 613.1773); ¹H NMR (400 MHz, MeOD, COSY): δ 2.58 [dd, 1H, *J* = 7.2, 16.4 Hz, H-C(4α)], 2.77 [dd, 1H, *J* = 5.2, 16.4 Hz, H-C(4β)], 3.31 [m, 1H, H-C(5′′/ 5′′′)], 3.43 [m, 3H, *J* = 9.2 H-C(4′′/4′′′, 3′′/3′′′, 5′′/5′′′)], 3.52 [m, 2H, *J* = 9.2 Hz, H-C(4′′/4′′′, 3′′/3′′′)], 3.63 [dd, 1H, *J* = 9.0, 9.6 Hz, H-C(2′′)], 3.74 [dd, 1H, *J* = 4.8, 12.0 Hz, H-C(6α′′)], 3.81 [m, 3H, *J* = 2.0, 2.4, 4.4, Hz, H-C(6β′′/6α′′′/ 6β′′′)], 3.91 [dd, 1H, *J* = 9.6 Hz, H-C(2′′′)], 4.00 [ddd, 1H, *J* = 5.2, 6.4, 6.8 Hz, H-C(3)], 4.72 [dd, 1H, *J* = 6.8 Hz, H-C(2)], 4.87 [d, 1H, *J* = 9.6 Hz, H-C(1′′′)], 4.90 [d, 1H, *J* = 9.6 Hz, H-C(1′′)], 6.74 [d, 1H, *J* = 8.4 Hz, H-C(5′)], 6.80 [dd, 1H, *J* = 1.8, 8.4 Hz, H-C(6′)], 6.96 [d, 1H, *J* = 1.8 Hz, H-C(2′)] ¹³C NMR (100 MHz, MeOD, HMQC, HMBC): δ 27.6 [C(4)], 61.7 [C(6′′/ 6′′′)], 62.7 [C(6′′/ 6′′′)], 68.2 [C(3)], 70.8 [C(4′′/ 4′′′)], 71.4 [C(4′′/ 4′′′)], 74.6 [C(2′′, 2′′′)], 76.0 [C(1′′/1′′′)], 77.4 [C(1′′/ 1′′′)], 79.2 [C(3′′/3′′′)], 82.1 [C(5′′/5′′′)], 82.3 [C(2)], 82.4 [C(5′′/ 5′′′)], 100.8 [C(4a)], 103.5 [C(6/8)], 104.1 [C(6/8)], 114.9 [C(2′)], 115.9 [C(5′)], 119.4 [C(6′)], 130.7 [C(1′)], 144.6 [C(3′,4′)], 152.8 [C(8a)], 153.4 [C(5)], 154.0 [C(7)].

(+)-Catechin-6-*C*-β-D-glcp, **14** (**Figure 1**): UV/vis (MeOH) λ_{max} = 220, 241, 273 nm; MS (ESI⁻): *m/z* 331 (100% [M – 121]⁻), 451 (38% [M – 1]⁻); MS/MS (ESI⁻): 123 (100% [M – 329]⁻), 331 (99% [M – 121]⁻), 109 (67% [M – 343]⁻), 149 (34% [M – 303]⁻); exact mass: *m/z* 451.1243 (calculated for $C_{21}H_{23}O_{11}^-$ 451.1246); ¹H NMR (400 MHz, MeOD, COSY): δ 2.55 [dd, 1H, *J* = 7.2, 7.6, 16.0 Hz, H-C(4α)], 2.79 [dd, 1H, *J* = 5.2, 16.0 Hz, H-C(4β)], 3.42 [m, 1H, *J* = 2.0, 4.0 Hz, H-C(5′′)], 3.48 [dd, 1H, *J* = 8.4, 8.8 Hz, H-C(4′′)], 3.52 [dd, 1H, *J* = 8.8, 9.2 Hz, H-C(3′′)], 3.66 [dd, 1H, *J* = 8.8, 9.6 Hz, H-C(2′′)], 3.79 [dd, 1H, *J* = 4.4, 12.0 Hz, H-C(6α′′)], 3.86 [dd, 1H, *J* = 2.4, 12.0 Hz, H-C(6β′′)], 4.00 [ddd, 1H, *J* = 5.2, 5.6, 7.2, 7.6 Hz, H-C(3)], 4.62 [d, 1H, *J* = 7.2 Hz, H-C(2)], 4.87 [d, 1H, *J* = 9.6 Hz, H-C(1′′)], 5.96 [s, 1H, H-C(8)], 6.69 [dd, 1H, *J* = 2.0, 8.0 Hz, H-C(6′)], 6.74 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.80 [d, 1H, *J* = 2.0 Hz, H-C(2′)]; ¹³C NMR (100 MHz, MeOD, HMQC, HMBC): δ 27.4 [C(4)], 61.7 [C(6′′)], 68.2 [C(3)], 70.8 [C(4′′)], 74.2 [C(2′′)], 77.1 [C(1′′)], 79.2 [C(3′′)], 82.1 [C(5′′)], 82.3 [C(2)], 96.0 [C(8)], 101.9 [C(4a)], 105.4 [C(6)], 114.8 [C(2′)], 115.6 [C(5′)], 119.5 [C(6′)], 132.0 [C(1′)], 145.0 [C(3′/ 4′)], 146.0 [C(4′/3′)], 156.0 [C(8a)], 155.8 [C(5, 7)].

(+)-Catechin-8-*C*-β-D-glcp, **15** (**Figure 1**): UV/vis (MeOH) λ_{max} = 220, 241, 273 nm; MS (ESI⁻): *m/z* 331 (100% [M – 121]⁻), 451 (72% [M – 1]⁻); MS/MS (ESI⁻): 331 (100% [M – 121]⁻), 123 (71% [M – 329]⁻), 109 (70% [M – 343]⁻); exact mass: *m/z* 451.1243 (calculated for $C_{21}H_{23}O_{11}^-$ 451.1246); ¹H NMR (400 MHz, MeOD, COSY): δ 2.55 [dd, 1H, *J* = 7.6, 16.0 Hz, H-C(4α)], 2.79 [dd, 1H, *J* = 5.2, 16.0 Hz, H-C(4β)], 3.35 [m, 1H, *J* = 2.8, 3.6 Hz, H-C(5′′)], 3.39 [m, 2H, *J* = 8.4, 9.2 Hz, H-C(3′′,4′′)], 3.72 [dd, 1H, *J* = 5.2, 12.0 Hz, H-C(6α′′)], 3.86 [dd, 1H, *J* = 2.0, 12.0 Hz, H-C(6β′′)], 4.00 [ddd, 1H, *J* = 5.6, 7.2 Hz, H-C(3)], 4.12 [dd, 1H, *J* = 8.8, 9.0 Hz, H-C(2′′)], 4.72 [d, 1H, *J* = 6.8 Hz, H-C(2)], 4.82 [d, 1H, *J*= 9.6 Hz, H-C(1′′)], 6.01 [s, 1H, H-C(6)], 6.76 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.80 [d, 1H, *J* = 1.6, 8.0 Hz, H-C(6′)], 7.01 [d, 1H, *J* = 1.6 Hz, H-C(2′)]; ¹³C NMR (100 MHz, MeOD, HMQC, HMBC): δ 27.4 [C(4)], 62.5 [C(6′′)], 68.2 [C(3)], 71.7 [C(4′′)], 72.9 [C(2′′)], 76.3 [C(1′′)], 79.7 [C(3′′)], 81.8 [C(5′′)], 81.9 [C(2)], 96.5 [C(6)], 99.4 [C(4a)], 103.8 [C(8)], 114.9 [C(2′)], 115.9 [C(5′)], 119.3 [C(6′)], 131.2 [C(1′)], 144.6 [C(3′,4′)], 154.8 [C(8a)], 155.9 [C(5, 7)].

(+)-Epicatechin-6-*C*-β-D-glcp, **16** (**Figure 1**): UV/vis (MeOH) λ_{max} = 220, 241, 273 nm; MS (ESI⁻): *m/z* 331 (100% [M – 121]⁻), 451 (75% [M – 1]⁻); MS/MS (ESI⁻): 331 (100% [M – 121]⁻), 123 (75% [M – 329]⁻), 109 (64% [M – 343]⁻), 149 (30% [M – 303]⁻); exact mass: *m/z* 451.1243 (calculated for $C_{21}H_{23}O_{11}^-$ 451.1246); ¹H NMR (400 MHz, MeOD, COSY): δ 2.76 [dd, 1H, *J* = 2.4, 16.4 Hz, H-C(4α)], 2.86 [dd, 1H, *J* = 4.4, 16.8 Hz, H-C(4β)], 3.39 [m, 3H, *J* = 2.4, 4.0 Hz, H-C(5′′)], 3.51 [m, 2H, *J* = 8.8, 9.2, 9.6 Hz, H-C(3′′,5′′)], 3.67 [dd, 1H, *J* = 8.8, 9.6 Hz, H-C(2′′)], 3.80 [dd, 1H, *J* = 4.4, 12.0 Hz, H-C(6α′′)], 3.85 [dd, 1H, *J* = 2.4, 12.0 Hz, H-C(6β′′)], 4.18 [m, 1H, *J* = 2.4, 4.4 Hz, H-C(3)], 4.80 [s, 1H, H-C(2)], 4.86 [d, 1H, *J* = 10.2 Hz, H-C(1′′)], 6.00 [s, 1H, H-C(8)], 6.75 [d, 1H, *J* = 8.4 Hz, H-C(5′)], 6.80 [dd, 1H, *J* = 2.0, 8.4 Hz, H-C(6′)], 6.97 [d, 1H, *J* = 2.0 Hz, H-C(2′)]; ¹³C NMR (100 MHz, MeOD, HMQC): δ 29.4 [C(4)], 62.0 [C(6′′)], 67.3 [C(3)], 71.3 [C(4′′)], 74.3 [C(2′′)], 77.3 [C(1′′)], 79.6 [C(3′′)], 79.7

[C(2)], 82.0 [C(5″)], 96.6 [C(8)], 115.2 [C(2′)], 116.0 [C(5′)], 119.2 [C(6′)].

(+)-Epicatechin-8-*C*-*β*-D-glcp, **17** (**Figure 1**): UV/vis (MeOH) λ_{max} = 220, 241, 273 nm; MS (ESI$^-$): *m/z* 451 (100% [M – 1]$^-$), 331 (95% [M – 121]$^-$); MS/MS (ESI$^-$): 331 (100% [M – 121]$^-$), 123 (67% [M – 329]$^-$), 109 (63% [M – 343]$^-$), 149 (31% [M – 303]$^-$); exact mass: *m/z* 451.1243 (calculated for $C_{21}H_{23}O_{11}^-$ 451.1246); ^{1}H NMR (400 MHz, MeOD, COSY): δ 2.78 [dd, 1H, *J* = 1.6, 16.8 Hz, H-C(4α)], 2.89 [dd, 1H, *J* = 4.4, 16.8 Hz, H-C(4*β*)], 3.40 [m, 3H, *J* = 8.4, 9.2 Hz, H-C(3″,4″,5″)], 3.65 [dd, 1H, *J* = 4.8, 12.0 Hz, H-C(6α″)], 3.85 [dd, 1H, *J* = 1.6, 12.0 Hz, H-C(6*β*″)], 4.23 [m, 1H, *J* = 1.6, 4.4 Hz, H-C(3)], 4.27 [br m, 1H, H-C(2″)], 4.85 [d, 1H, *J* = 9.2 Hz, H-C(1″)], 4.88 [s, 1H, H-C(2)], 6.02 [s, 1H, H-C(6)], 6.78 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.82 [dd, 1H, *J* = 1.6, 8.0 Hz, H-C(6′], 7.04 [d, 1H, *J* = 2.0 Hz, H-C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC): δ 29.4 [C(4)], 62.7 [C(6″)], 66.7 [C(3)], 71.8 [C(4″)], 72.4 [C(2″)], 76.0 [C(1″)], 79.6 [C(2)], 79.6 [C(3″)], 82.0 [C(5″)], 96.4 [C(6)], 99.2 [C(4a)], 102.6 [C(8)], 115.0 [C(2′)], 115.9 [C(5′)], 119.4 [C(6′)], 130.8 [C(1′)], 144.2 [C(4′/3′)], 144.6 [C(3′/4′)],154.2 [C(8a)], 155.2 [C(5)], 156.9 [C(7)].

Preparation of [$^{13}C_6$]-Labeled Flavan-3-ol-*C*-glucopyranosides 18–20. A mixture of (−)-epicatechin (0.55 mmol), [$^{13}C_6$]-D-glucose (5.55 mmol), K_2CO_3 (3.0 mmol), and water (7.1 g) were heated for 10 min at 80 °C while stirring. After cooling, the title compounds (−)-catechin-8-*C*-[$^{13}C_6$]-*β*-D-glucopyranoside (**18**), (−)-catechin-6,8-*C*-di-[$^{13}C_6$]-*β*-D-glucopyranoside (**19**), and (−)-catechin-6-*C*-[$^{13}C_6$]-*β*-D-glucopyranoside (**20**) were isolated and purified as detailed above for the corresponding nonlabelled analogues.

(−)-Catechin-8-*C*-[$^{13}C_6$]-*β*-D-glcp, **18**. UV/vis (MeOH/0.1% HCOOH, pH 2.5; 2/8, v/v) λ_{max} = 218, 231, 280 nm; MS (ESI$^-$): *m/z* 457 (100%, [M – 1]$^-$), 333 (45%, [M – 125]$^-$); MS/MS (ESI$^-$) (−50 V): *m/z* 333 (100), 123 (57), 109 (27), 457 (2); ^{13}C NMR (100 MHz, MeOD) δ 62.8 [d, *J* = 42.7 Hz, C-6″], 71.7 [dd, *J* = 38.9, 40.5 Hz, C-4″], 73.5 [dd, *J* = 37.9, 39.4 Hz, C-2″], 76.6 [d, *J* = 39.6 Hz, C-1″], 80.0 [dd, *J* = 38.4, 38.7 Hz, C-3″], 82.3 [dd, *J* = 41.4, 41.8 Hz, C-5″].

(−)-Catechin-6-*C*,8-*C*-di-[$^{13}C_6$]-*β*-D-glcp, **19**. UV/vis (MeOH/0.1% HCOOH, pH 2.5; 2/8, v/v) λ_{max} = 218, 231, 280 nm; MS (ESI$^-$): *m/z* 625 (100%, [M – 1]$^-$), 501 (16%, [M – 125]$^-$); MS/MS (ESI$^-$) (−50 V): *m/z* 377 (100), 123 (70), 109 (48), 408 (12), 333 (12), 625 (1); ^{13}C NMR (100 MHz, MeOD) δ 62.1 [d, *J* = 38.1 Hz, C-6″/C-6‴], 62.4 [d, *J* = 37.9 Hz, C-6″/C-6‴], 71.2 [dd, *J* = 40.2, 40.7 Hz, C-4″/C-4‴], 71.5 [dd, *J* = 38.6, 40.3 Hz, C-4″/C-4‴], 73.4 [dd, *J* = 38.3, 38.9 Hz, C-2″/C-2‴], 74.8 [dd, *J* = 37.7, 37.9 Hz, C-2″/C-2‴], 76.9 [d, *J* = 39.7 Hz, C-1″/C-1‴], 77.3 [d, *J* = 39.9 Hz, C-1″/C-1‴], 79.3 [dd, *J* = 37.0, 37.9 Hz, C-3″/C-3‴], 79.5 [dd, *J* = 37.7, 37.9 Hz, C-3″/C-3‴], 82.5 [dd, *J* = 41.6, 41.7 Hz, C-5″/C-5‴], 82.6 [dd, *J* = 41.2, 42.1 Hz, C-5″/C-5‴].

(−)-Catechin-6-*C*-[$^{13}C_6$]-*β*-D-glcp, **20**. UV/vis (MeOH/0.1% HCOOH, pH 2.5; 2/8, v/v) λ_{max} = 218, 231, 280 nm; MS (ESI$^-$): *m/z* 457 (100%, [M – 1]$^-$), 333 (22%, [M – 125]$^-$); MS/MS (ESI$^-$) (−50 V): *m/z* 333 (100), 123 (57), 109 (27), 457 (2). ^{13}C NMR (100 MHz, MeOD/D_2O) δ 61.9 [d, *J* = 42.5 Hz, C-6″], 70.9 [dd, *J* = 39.2, 40.0 Hz, C-4″], 74.2 [dd, *J* = 38.2, 38.9 Hz, C-2″], 77.1 [d, *J* = 39.7 Hz, C-1″], 79.3 [dd, *J* = 38.4, 38.2 Hz, C-3″], 82.1 [dd, *J* = 41.6, 41.3 Hz, C-5″].

Quantitative Analyses of the Degree of Flavan-3-ol Glycosylation. A mixture of (−)-epicatechin (0.1 mmol), amylose (0.5 g), K_2CO_3 (0.5 mmol), and water (5 mL) was treated for 10 min at 80 °C while stirring. After cooling, the reaction mixture was adjusted to pH 7.0 by the addition of aqueous acetic acid. To measure the catechin- and epicatechin-*C*-glycosides **1–6** as the flavan-3-ol/carbohydrate linkages, the (−)-epicatechin/amylose reaction mixture was spiked with solutions (20 μL, each) of the labeled internal standards **18–20**, dissolved in methanol (1.0 mg/mL), then vortexed and equilibrated for 30 min at room temperature. After addition of Termamyl (100 μL), the mixture was kept in a boiling water bath for 60 min and was then cooled to room temperature. The pH of the solution was adjusted to 4.6 by adding aqueous acetic acid, amyloglucosidase (0.3 mL, 10 mg/mL) was added, and the solution was maintained for 3 h at 60 °C while stirring. After centrifugation, the liquid supernatant was collected (water fraction), and the insoluble residue was extracted four times with aqueous ethanol (40%, 10 mL each) for 30 min at room temperature with stirring. The ethanolic extract was freed from solvent under reduced pressure at 40 °C, combined with the water fraction, and freeze-dried to give the ethanolic/water extract. Aliquots (~50 mg) of the ethanol/water extract were taken up in a methanol/water mixture (1/9, v/v; 1 mL), which was acidified to pH 2.5 with aqueous formic acid (0.1% in water). After membrane filtration, aliquots (5 μL) were analyzed by means of LC-MS/MS using the same conditions as described above. The labeled compounds were used as the internal standards for the quantitative analysis of the corresponding analytes. In addition, compound **4** was quantified via **18**, compound **5** via **19**, and compound **6** via **20** as the internal standard. The amounts of the individual flavan-3-ol-*C*-glycosides were calculated using the response factors (given in parentheses) for the individual compounds **1** (0.97), **2** (0.96), **3** (0.98), **4** (0.91), **5** (0.92), and **6** (0.94), which had been determined by analysis of solutions containing defined amounts of the internal standards and the target compounds in five mass ratios from 0.2 to 5.0.

High Performance Liquid Chromatography (HPLC). The HPLC apparatus (Kontron, Eching, Germany) consisted of low pressure gradient system 525 HPLC pump, a M800 gradient mixer, a type 560 auto sampler, and a DAD type 540+ diode array detector. Chromatography was performed on 250 × 4.6 mm stainless-steel columns packed with RP-Phenylhexyl material, 5 μm (Phenomenex, Germany) operated with a flow rate of 0.8 mL/min.

Liquid Chromatography–Mass Spectrometry (LC-MS/MS). LC-MS/MS analysis was performed using an Agilent 1100 HPLC-system connected to the API 3200 LC-MS/MS (Applied Biosystems, Darmstadt, Germany) running in the negative electrospray ionization (ESI$^-$) mode. Zero grade air served as nebulizer gas (35 psi), and as turbo gas (300 °C) for solvent drying (45 psi). Nitrogen served as curtain (20 psi) and collision gas (4.5×10^{-5} torr). Both quadrupoles were set at unit resolution. By means of the multiple reaction monitoring (MRM) mode, the individual flavan-3-ol-*C*-glycosides **1**, **3**, **4**, **6**, and **14–17** (*m/z* 451.2 → 331.0), **18** and **20** (*m/z* 457.1 → 333.0), **2**, **5**, and **13** (*m/z* 613.1 → 373.0), **19** (*m/z* 625.1 → 377.0), **7–9** (*m/z* 613.5 → 331.0), and **10–12** (*m/z* 775.5 → 331.0), as well as flavan-3-ol-*C*-maltotetraoside (*m/z* 937.8 → 331.0), flavan-3-ol-*C*-maltopentaoside (*m/z* 1099.9 → 331.0), flavan-3-ol-*C*-maltohexaoside (*m/z* 1261.0 → 331.0), flavan-3-ol-*C*-maltoheptaoside (*m/z* 1423.0 → 331.0), and flavan-3-ol-*C*-maltooctaoside (*m/z* 1585.0 → 331.0) were analyzed using the mass transitions (given in brackets) monitored for a duration of 75 ms. In addition, the mass transitions of the corresponding pseudomolecular ions of the *C*-glycosides to the daughter fragment ions *m/z* 123.0 and 109.0 were recorded. ESI$^-$ mass and product ion spectra were acquired with direct flow infusion.

For ESI$^-$, the ion spray voltage was set at −4500 V in the negative mode. The MS/MS parameters were tuned for each individual compound, detecting the fragmentation of the [M – H]$^-$ molecular ions into specific product ions after collision with nitrogen (4.0×10^{-5} torr).

LC/Time-of-Flight Mass Spectrometry (LC/TOF-MS). Mass spectra of the target compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer with flow injection referenced on sodium formiate and polyethylene glycol (PEG) 600, respectively. The compounds were dissolved in 1 mL of MeOH and 10 μL of a saturated solution of $NaBF_4$ in MeOH was added to measure the exact mass of the sodium adducts.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, COSY, HMQC, HMBC, ^{13}C and DEPT-135 NMR measurements were performed on a DMX 400 spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts were referenced to the solvent signal. Data processing was performed by using XWin-NMR software (version 3.5; Bruker) as well as Mestre-C software (Mestrelab Research, Santiago de Compostela, Spain).

RESULTS AND DISCUSSION

Very recent studies demonstrated that a series of velvety astringent and bitterness supressing (−)-catechin- and (−)-epicatechin-*C*-glucopyranosides (**1–6**, **Figure 1**) are formed during the alkalization process of cocoa powder manufacturing via a novel "nonenzymatic *C*-glycosylation" of flavan-3-ols with D-glucose. On the basis of that finding, the question arose as to whether this nonenzymatic *C*-glycosylation also takes place between flavan-3-ols and polysaccharides such as amylose. Therefore, binary mixtures of (−)-epicatechin and amylose were thermally treated in an aqueous K_2CO_3 solution for 10 min at 80 °C to generate polyphenol/amylose conjugates such as exemplified for the flavan-3-ol-6-*C*-polyglucopyranosides outlined in **Figure 2**. To confirm the formation of such flavan-3-ol/amylose linkages, the reaction products formed were treated with Termamyl, followed by amyloglucosidase to break-down step-wise the *O*-glucosidic linkages in the polysacharide backbone of the conjugates and to release *C*-oligoglycosylated flavan-3-ols. After RP18 cartridge clean-up, the partially hydrolyzed reaction mixture was screened for the presence of flavan-3-ol-*C*-mono- and oligoglycopyranosides by means of LC-MS/MS(ESI$^-$). To achieve this, the mass transitions for various conjugates were calculated based on the fragmentation pattern reported recently by us for the *C*-monoglucopyranosides **1–6** (*6*). Assuming that several glucose monomers in an oligoglucopyranoside chain will give the same characteristic main daughter ion fragment with *m/z* 331, which is typical for the *C*-glycosides **1–6** (*6*), the mass transition of *m/z* 775.5 → 331.0 was selected for a flavan-3-ol *C*-oligoglucopyranoside containing three glucose moieties. For each homologous *C*-glucosylated flavan-3-ol with an additional glucose moiety, the putative mass transitions were calculated by addition of another 162 mass units.

As shown in **Figure 3**, recording of the mass transition *m/z* 451.2→ 331.0 revealed that (−)-epicatechin-8-*C*-β-D-glc *p* (**4**), (−)-catechin-8-*C*-β-D-glc *p* (**1**), (−)-catechin-6-*C*-β-D-glc *p* (**3**), and (−)-epicatechin-6-*C*-β-D-glc *p* (**6**) were present in the partial hydrolysate of the reaction products formed. In addition, we screened for flavan-3-ol-*C*-oligoglucopyranosides by recording the mass transitions expected for flavan-3-ol-*C*-maltosides (*m/z* 613.5 → 331.0), *C*-maltotriosides (*m/z* 775.5 → 331.0), *C*-maltotetraosides (*m/z* 937.8 → 331.0), and *C*-maltopentaosides (*m/z* 1099.9 → 331.0) as given in parentheses. As shown in **Figure 3**, *C*-glucosylated flavan-3-ols containing up to five glucopyranose moieties were detected in the partial hydrolysate. On the basis of these data, it can be suggested that the reaction between (−)-epicatechin and amylose leads to the formation of conjugates by covalently attaching the flavan-3-ol to the anomeric carbon atom of the terminal carbohydrate moiety at the reducing end of the polysaccharide.

To unequivocally confirm the presence of such flavan-3-ol-*C*-oligoglucopyranosides in the partially hydrolyzed flavan-3-ol/amylose reaction mixture, reference compounds needed to be synthesized. Closely following the procedure reported recently for the preparation of the flavan-3-ol-*C*-glucopyranosides **1–6** (*6*), the corresponding flavan-3-ol-*C*-maltosides and maltotriosides were prepared by reacting (−)-epicatechin either with D-maltose, or with D-maltotriose, respectively, under alkaline conditions. After prefractionation by means of RP-18 column chromatography and final purification by means of RP-HPLC, the target compounds (−)-catechin-8-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**7**), (−)-epicatechin-8-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**8**), (−)-catechin-6- *C*-β-D-glc *p*-(4 → 1)- *O*-α-D-glc *p*(1 → 4) (**9**), (−)-catechin-8- *C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p* (**10**), (−)-epicatechin-8-*C*-β-D-glc *p*-(4→1)- *O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p* (**11**), and (−)-catechin-6-*C*-β-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p* (**12**) (**Figure 1**) were obtained as white amorphous powders in a purity of about 99%. The structures of these previously unreported flavan-3-ol/oligosaccharide conjugates were unequivocally determined by means of UV/vis, LC-MS/MS, and 1D/2D-NMR spectroscopy.

Exemplified for (−)-catechin-8-*C*-β-D-glc *p*-(4 → 1)-*O*-α-D-glc *p* (**7**), this compound showed the typical UV/vis absorption maxima expected for flavan-3-ols, and showed a pseudomolecular ion [M – 1]$^-$ ion with *m/z* 613 as well as the fragment ions *m/z* 331, 451, and 433 in the MS-ESI$^-$ spectrum (**A** in **Figure 4**). High resolution LC-MS(ESI$^-$) analysis confirmed compound **7** to have the molecular formula $C_{27}H_{34}O_{16}$. The ^{1}H NMR spectrum showed an aromatic singlet for H-C(6) at 6.01 ppm, three aromatic protons H-C(6′), H-C(5′), and H-C(2′) resonating at 6.71, 6.75, and 6.83 ppm and showing an ABX coupling system. In addition, the four aliphatic protons H-C(4α), H-C(4β), H-C(3), as well as the proton H-C(2) were detectable at 2.55, 2.74, 3.99, and 4.76 ppm coupling with each other and confirming the flavan-3-ol aglycone. Besides the signals of the polyphenol, the ^{1}H NMR spectrum also exhibited 14 aliphatic protons resonating at 3.27 ppm [H-C(4‴)], 3.42–3.46 ppm [H-C(5″, 2‴)], 3.59–3.71 ppm [H-C(3″, 3‴, 4″, 5‴, 6α″)], 3.81–3.89 ppm [H-C(6β″, 6α, β‴)], 4.10 ppm [H-C(2″)], 4.84 ppm [H-C(1″)], and 5.16 ppm [H-C(1‴)] as expected for two hexose moieties. Considering all the coupling constants of the sugar moieties in the molecule, and, in particular, the coupling constants of 9.2 and 3.6 Hz measured for H-C(1″) and H-C(1‴) with the corresponding protons H-C(2″) and H-C(2‴), respectively, and comparing these values with the H-C(1)/H-C(2) coupling constants reported for β-D-glucopyranosides and α-D-glucopyranosides (*9, 10*), the two sugar moieties were unequivocally identified as one β-D- and one α-D-glucopyranose.

A comparison of the ^{13}C NMR spectrum showing 27 signals with the results of the DEPT-135 experiment exhibiting 19 signals, revealed eight signals corresponding to quarternary carbon atoms. Unequivocal assignment of these quarternary carbon atoms and the hydrogen-substituted carbon atoms, respectively, could be successfully achieved by means of heteronuclear multiple bond correlation spectroscopy (HMBC) optimized for $^2J_{C,H}$ and $^3J_{C,H}$ coupling constants and heteronuclear multiple-quantum correlation spectroscopy (HMQC)

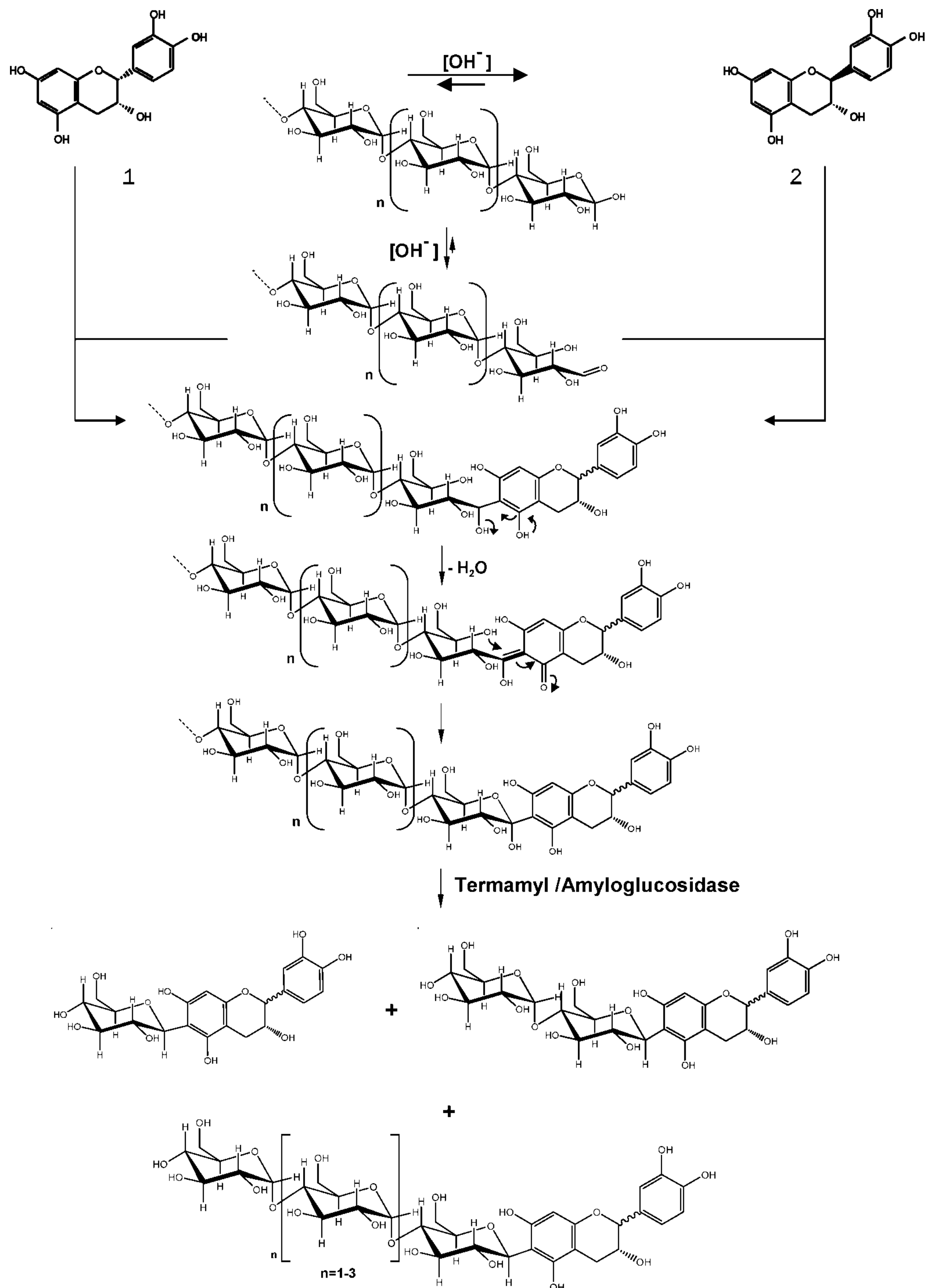

Figure 2. Formation of (−)-epicatechin/amylose conjugates and partial enzymatic hydrolysis.

optimized for ${}^{1}J_{C,H}$ coupling constants, respectively. Additionally, the HMBC experiment revealed a correlation between the sugar proton H-C(1″) resonating at 4.84 ppm and neighbouring carbon atoms C(7), C(8), and, in particular C(8a), thus demonstrating clearly the intramolecular 8-*C*-linkage of the *β*-D-glucopyranose to the aglycone. In addition, a heteronuclear ${}^{3}J$ coupling between the sugar proton H-C(1‴) resonating at 5.16 ppm and the carbon atom C(4″) of the *β*-D-glucopyranose could be detected, thus demonstrating the intramolecular *O*-linkage of the *β*-D-glucopyranose with the α-D-glucopyranose. In addition, the chemical shifts of the carbon atoms C(1″) and C(1‴) resonating at 76.4 and 103.0 ppm confirmed the *C*-linkage to the *β*-D-glucopyranose moiety to the aglycone and the *O*-linkage of the α-D-glucopyranose to the other sugar moiety.

As described recently (*6*), comparison of the chemical shifts and the coupling constants of the protons H-C(4α), H-C(4*β*), H-C(3), H-C(2) and the sequence of increasing chemical shifts of the protons H-C(5′), H-C(6′), and H-C(2′) of (−)-epicatechin, (+)-catechin, as well as their corresponding epimers, with the isolated *C*-glycoside **7**, allowed its configuration at carbon atom C(2) and at C(3) to be deduced. The coupling constants and the chemical shifts of the protons H-C(2), H-C(3), H-C(4α*β*), H-C(2′), H-C(5′), and H-C(6′), as well as the chemical shifts of their corresponding C-atoms, indicated the 2(*S*),3(*R*)-configuration of the (−)-catechin aglycone of compound **7**. Taking all these spectroscopic data into consideration, the structure of compound **7** prepared from (−)-epicatechin and maltose could be unequivocally identified as (−)-catechin-

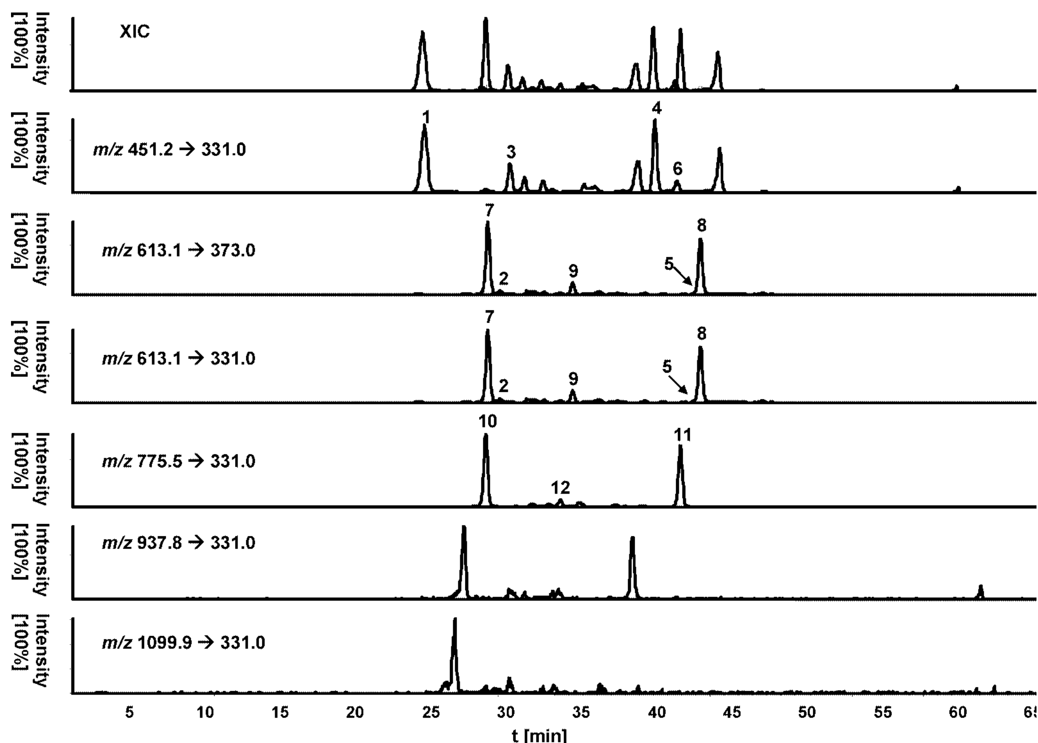

Figure 3. HPLC-MS/MS(ESI$^-$)-MRM analysis of the (−)-epicatechin/amylose reaction mixture after partial enzymatic hydrolysis using Termamyl (15 min) and amyloglucosidase (30 min).

8- *C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**Figure 1**). Using the same analytical strategy, (−)-catechin-6-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**9**) and (−)-epicatechin-8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**8**) were isolated as main reaction products formed from (−)-epicatechin and D-maltose (**Figure 1**). To the best of our knowledge, the compounds **7–9** have not previously been reported.

Reaction of (−)-epicatechin and D-maltotriose revealed the previously unreported, corresponding flavan-3-ol-*C*-maltotriosides, identified as (−)-catechin-8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p* (**10**), (−)-catechin-6-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p* (**12**), and (−)-epicatechin-6/8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)- *O*-α-D-glc *p* (**11**) (**Figure 4**) by means of LC-MS/MS and 1D/2D-NMR experiments. Exemplified for compound **12**, the mass spectrum (ESI$^-$) of these compounds showed a pseudomolecular ion [M – 1]$^-$ ion with *m/z* 775 as well as the characteristic fragment ions *m/z* 331, 451, and 289 (**Figure 4B**).

Identification of Flavan-3-ol-*C*-glycosides 1–12 in the Partial Hydrolysate of the (−)-Epicatechin/Amylose Reaction Mixture. To unequivocally verify the covalent binding of flavan-3-ols to the anomeric carbon atom of the reducing terminus of polysacharides and to confirm the presence of the proposed flavan-3-ol-*C*-glycosides **1–12** in the partial hydrolysate, the mass spectrometer was tuned for the mass transitions of the target compounds **1–12** and then the enzymatically treated reaction mixture was analyzed by RP–HPLC–MS/MS(ESI$^-$) running in the multiple reaction monitoring mode. The mass transition *m/z* 451.2 → 331.0 was recorded for the flavan-3-ol-*C*-glucosides **1**, **3**, **4**, and **6**, the mass transition *m/z* 613.5 → 331.0 was monitored for the flavan-3-ol-*C*-maltopyranosides **7–9**, and the mass transition *m/z* 775.5 → 331.0 was recorded for the flavan-3-ol- *C*-glucosides **10–12**, respectively. Besides the previously reported (−)-catechin- and (−)-epicatechin-*C*-glucopyranosides **1–6**, the presence of the *C*-maltosides and *C*-maltotriosides **7–12** was confirmed in the partial hydrolysate of the (−)-epicatechin/amylose reaction mixture by comparing the retention times and the mass transitions with those obtained for the corresponding reference compounds and, finally, by co-chromatography (data not shown).

The flavan-3-ol-6-*C*,8-*C*-*β*-D-diglc *p* **2** and **5** have very similar retention times when compared to their corresponding flavan-3-ol-8-C-*β*-D-maltosides **7** and **8**, whereas (−)-epicatechin-6-*C*,8-*C*-*β*-D-diglucopyranoside (**5**) and (−)-epicatechin-8-C-*β*-D-maltoside (**8**) were found to coelute. By comparing the MS/MS data obtained at a collision energy of − 50V and, in particular, the intensities of the fragment ions *m/z* 331, 373, and 403 measured for the *C*-diglucopyranosides **2** and **5** with those observed for the corresponding *C*-maltosides **7** and **8** (**Figure 5**), it was possible to clearly distinguish between a flavan-3-ol-6-*C*,8-*C*-digluco- or a -6- *C*- and -8-*C*-maltopyranoside. In the case of the *C*-diglucopyranosides **2** and **5** the ion *m/z* 373 is observed as the main fragment, whereas the daughter ion *m/z* 331 showed only an intensity of about 10% (**Figure 5A**). In comparison, the *C*-maltopyranosides **7** and **8** showed the highest intensity for the fragment ion *m/z* 331 and only 8% for the ion *m/z* 373 (**Figure 5B**). In addition, the *C*-diglucopyranosides **2** and **5** exclusively showed a fragment ion with *m/z* 403. As shown in **Figure 6**, the differences in the mass transitions can be used to clearly distinguish between *C*-maltopyranosides (*m/z* 613.1 → 373/331) and *C*-diglucopyranosides (*m/z* 613.1 → 403) showing the same pseudomolecular ion of 613 [M – 1]$^-$.

In the traces showing the mass transitions *m/z* 937.8 → 331.0 and *m/z* 1099.9 → 331.0 calculated for flavan-3-ol-*C*-maltotetraosides and maltopentaosides, respectively, either two or only one peak was detectable (**Figure 3**). As shown for all the various reference compounds **1–12** the flavan-3-ol-8-*C*-glycosides eluted earlier than the corresponding 6-*C*-glycosides, and the catechin-*C*-glycosides eluted earlier than the corresponding epicatechin-*C*-glycosides (*6*), so that the compounds detected with the mass transitions of *m/z* 937.8 → 331.0 (**Figure 3**) were tentatively

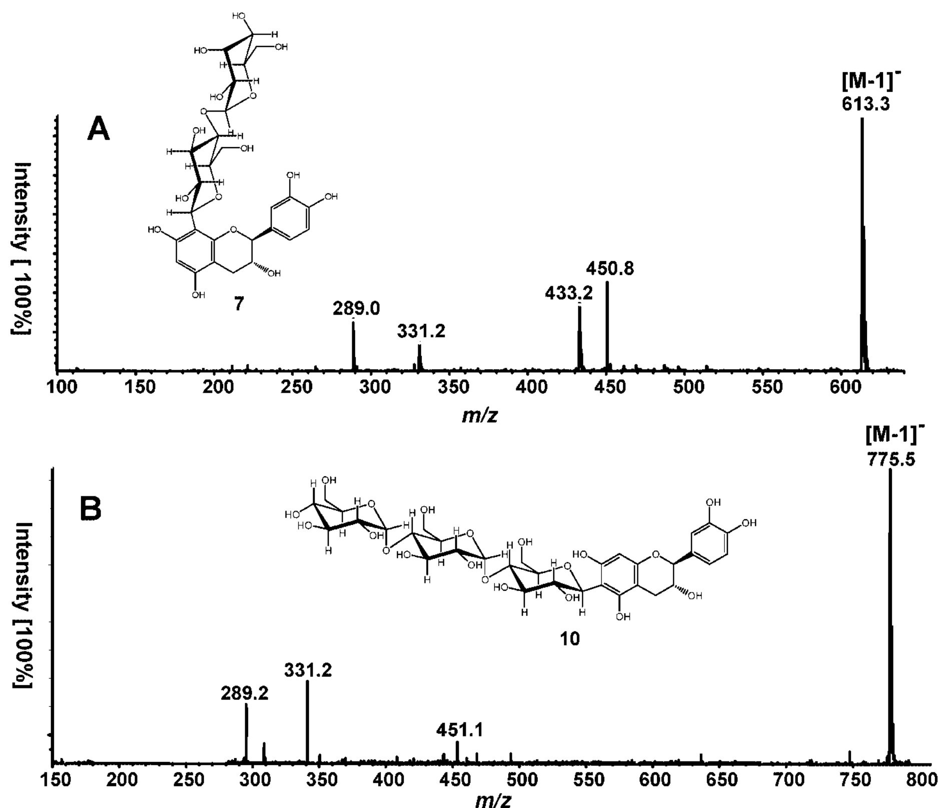

Figure 4. MS (ESI⁻) spectrum of (**A**) (−)-catechin-8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**7**) and (**B**) (−)-catechin-6-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p*- (4→1)-*O*-α-D-glc *p* (**12**).

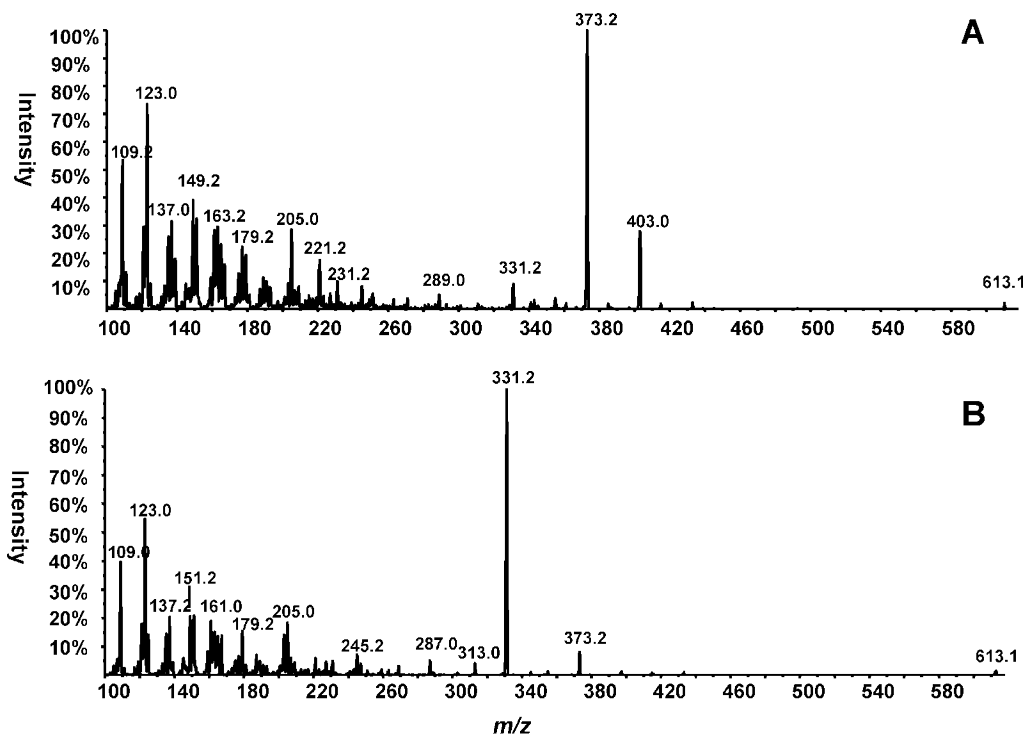

Figure 5. LC-MS/MS spectrum (ESI⁻, collision energy −50V) of (**A**) (−)-catechin-6-*C*,8-*C*-*β*-D-diglc *p* (**2**) and (**B**) (−)-catechin-8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**7**).

identified as (−)-catechin-8-*C*- and (−)-catechin-6-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p*, respectively. In addition, the compound detected with mass transitions of *m/z* 1099.9 → 331.0 might be suggested to be (−)-catechin-8-*C*-*β*-D-maltopentaoside.

To answer the question as to whether also (+)-catechin is susceptible to nonenzymatic glycosylation, (+)-catechin and D-glucose were heated in aqueous K_2CO_3 solution for 10 min at 80 °C while stirring. Closely following the procedure described recently for a (−)-epicatechin/D-glucose reaction mixture (*6*), five reaction products (**13–17**), showing the typical absorption maxima expected for flavan-3-ols, were isolated and purified by means of semipreparative RP-HPLC and their structures were determined by means of LC-MS/

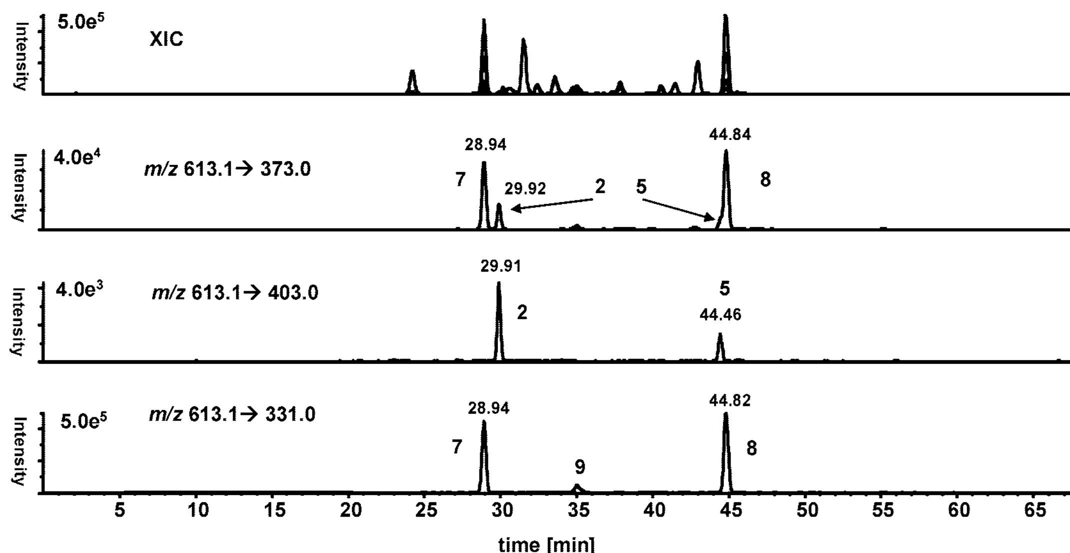

Figure 6. HPLC-MS/MS(ESI$^-$)-MRM analysis of the (−)-epicatechin/amylose reaction mixture after enzymatic hydrolysis using Termamyl (30 min) and amyloglucosidase (60 min).

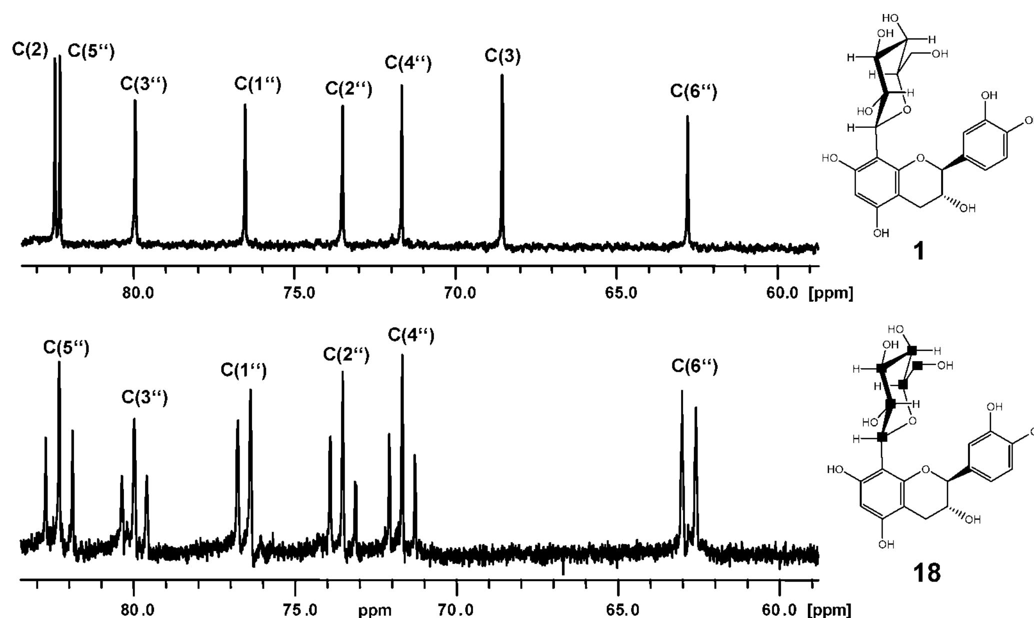

Figure 7. Excerpt of the ^{13}C NMR spectra (100 MHz, MeOD-d_3) of (**A**) nonlabelled (**1**) and (**B**) [$^{13}C_6$]-labelled (−)-catechin-8-*C*-β-D-glc *p* (**18**), respectively.

MS and NMR spectroscopy. High resolution LC-MS(ESI$^-$) analysis revealed compound **13** to have the molecular formula $C_{27}H_{34}O_{16}$, whereas the molecular formula $C_{21}H_{24}O_{11}$ was found for compounds **14**–**17**. By means of LC-MS(ESI$^-$), the reaction products **14**–**17** showed an $[M - 1]^-$ ion with *m*/*z* 451 as well as the fragment ions *m*/*z* 331, 123 and 109, compound **13** showed an $[M - 1]^-$ ion with *m*/*z* 613 as well as the fragment ions *m*/*z* 373, 123, 109, 331, and 403. 1D- and 2D-NMR experiments led to the identification of the five reaction products as (+)-catechin-6-*C*,8-*C*-β-D-diglc *p* (**13**), (+)-catechin-6-*C*-β-D-glc *p* (**14**), (+)-catechin-8-*C*-β-D-glc *p* (**15**), (+)-epicatechin-6-*C*-β-D-glc *p* (**16**), and (+)-epicatechin-8-*C*-β-D-glc *p* (**17**), the structures of which are given in **Figure 1**. In addition, these compounds were identified in a Termamyl/amyloglucosidase treated (+)-catechin/amylose reaction mixture by means of HPLC-MS/MS, thus demonstrating the formation of flavan-3-ol/amylose conjugates (data not shown). To the best of our knowledge, compounds **13**, **16**, and **17** have not previously been reported in the literature. Although compounds **14** and **15**have been reported earlier as phytochemicals in rhubarb (*11*), the formation of such (+)-catechin- *C*-glycopyranosides by nonenzymatic *C*-glycosylation of the corresponding flavan-3-ol has not been previously described.

To accurately determine the conversion rate of (−)-epicatechin with amylose, a stable isotope dilution analysis (SIDA) was developed. To achieve this, first, corresponding labelled internal standards needed to be synthesized.

Synthesis of [^{13}C]-Labelled Flavan-3-ol-*C*-glucosides. As the use of stable isotopologues of analytes is known to enable the correction of compound discrimination during extraction, cleanup, chromatographic separation, and MS detection, a versatile and reliable stable isotope dilution assay (SIDA) was developed to determine the rate of product formation in flavan-3-ol/polysaccharide model reactions. Aimed at synthesizing [^{13}C]-isotopologues of the flavan-3-ol *C*-glucosides, an aqueous solution of (−)-epicatechin and [$^{13}C_6$]-D-glucose were thermally treated in the presence of K_2CO_3 for 10 min at 80 °C. The target

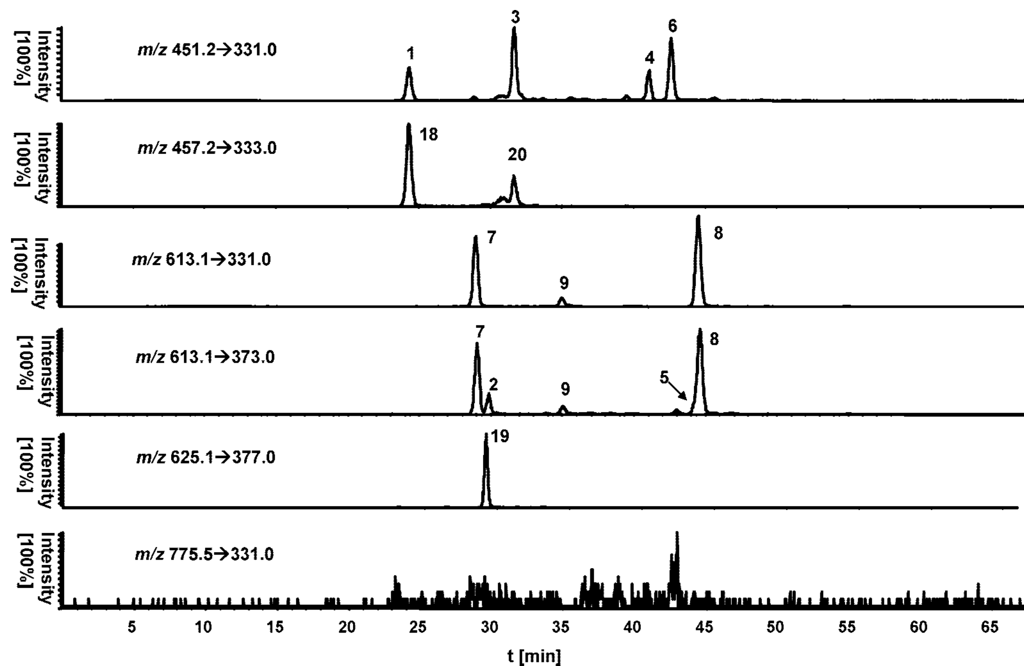

Figure 8. MS/MS chromatograms for the quantitative analysis of flavan-3-ol-*C*-glucopyranosides in a Termamyl/amyloglucosidase-treated (−)-epicatechin/amylose reaction mixture via the internal standards **18**–**20** by using the multiple reaction monitoring (MRM) mode.

Table 1. Human Recognition Taste Thresholds for the Oral Sensation Induced by Flavan-3-ols and Flavan-3-ol-*C*-glycosides, Respectively

compound	threshold conc [μmol/L][a] for astringency	threshold conc [μmol/L][a] for bitterness
(−)-epicatechin	800.0[b]	800.0
(−)-catechin	600.0[b]	1000.0
(−)-catechin-6- *C*-β-D-glc *p* (**3**)	63.6[c]	n.d.[d]
(−)-catechin-8- *C*-β-D-glc *p* (**1**)	16.2[c]	n.d.
(−)-epicatechin-6- *C*-β-D-glc *p* (**6**)	10.0[c]	n.d.
(−)-epicatechin-8- *C*-β-D-glc *p* (**4**)	3.1[c]	n.d.
(−)-epicatechin-6- *C*,8- *C*-β-D-diglc *p* (**5**)	2.5[c]	n.d.
(−)-catechin-6- *C*,8- *C*-β-D-diglc *p* (**2**)	1.1[c]	n.d.
(−)-catechin-8- *C*-β-D-glc *p*-(4 → 1)- *O*-α-D-glc *p* (**7**)	18.0[c]	n.d.
(−)-catechin-6- *C*-β-D-glc *p*-(4 → 1)- *O*-α-D-glc *p* (**9**)	14.0[c]	n.d.
(−)-epicatechin-8- *C*-β-D-glc *p*-(4 → 1)- *O*-α-D-glc *p* (**8**)	2.2[c]	n.d.
(−)-catechin-6- *C*-β-D-glc *p*-(4 → 1)- *O*-α-D-glc *p*(4 → 1)- *O*-α-D-glc *p* (**12**)	3.0[c]	n.d.
(−)-catechin-8- *C*-β-D-glc *p*-(4 → 1)- *O*-α-D-glc *p*(4 → 1)- *O*-α-D-glc *p* (**10**)	2.5[c]	n.d.
(−)-epicatechin-8- *C*-β-D-glc *p*-(4 → 1)- *O*-α-D-glc *p*(4 → 1)- *O*-α-D-glc *p* (**11**)	1.4[c]	n.d.

[a] Taste threshold concentrations were determined by means of the half-tongue test (*4*). [b] The astringent sensation was described a rough and extremely puckering. [c] The astringent sensation was described as a very smooth and velvety mouthcoating. [d] n.d.: not detectable.

compounds were isolated by column chromatography on RP-18 material, followed by a final HPLC purification yielding (−)-catechin-8-*C*-[$^{13}C_6$]-β-D-glucopyranoside (**18**), (−)-catechin-6-*C*,8-*C*-[$^{13}C_{12}$]-β-D-diglucopyranoside (**19**), and (−)-catechin-6-*C*-[$^{13}C_6$]-β-D-glucopyranoside (**20**) each as a white, amorphous powder in high purities of >98%.

When compared to the nonlabelled analogue **2**, the mass spectrum of (−)-catechin-6-*C*,8-*C*-β-D-[$^{13}C_{12}$]-diglucopyranoside (**19**), obtained in the ESI$^-$ mode, revealed an increase of the pseudomolecular ion by 12 units, thus reflecting the incorporation of two molecules of [$^{13}C_6$]-glucose into the molecule. In addition, the incorporation of the ^{13}C-atoms into the target molecules was confirmed by means of ^{1}H broad-band-decoupled ^{13}C NMR spectroscopy. For example, the 60–85 ppm region of the ^{13}C NMR spectrum obtained from (−)-catechin-8-*C*-β-D-glucopyranoside (**1**) showed eight carbon signals resonating as singlets (**Figure 7A**), whereas the same spectrum segment obtained from the isotopologue **18** showed six coupled resonance signals for the carbon atoms of the carbohydrate moiety (**Figure 7B**) due to homonuclear ^{13}C-^{13}C connectivities. As expected, the signals of the carbon atoms C(2) and C(3) were not detectable in the NMR spectrum obtained from **18** due to their low ^{13}C abundance.

Development of a Stable Isotope Dilution Assay (SIDA) for the Quantitative Analysis of Flavan-3-ol-*C*-Glucosides. To convert the measured ion intensities into the mass ratios of labelled and nonlabelled flavan-3-ol-*C*-glycosates, a graph was calculated from calibration mixtures of known mass ratios and the corresponding peak area ratios obtained by means of LC-MS/MS. Good linearity was found for mass ratios ranging from 0.2 to 5.0.

To quantify the degree of flavan-3-ol glycosylation in a flavan-3-ol/polysaccharide reaction, the epicatechin/amylose reaction mixture was spiked with defined amounts of the [^{13}C]-labelled internal standards **18**, **19**, **20**, followed by homogenisation, equilibration at room temperature, and enzymatic depolymerization by means of Termamyl and amyloglucosidase treatment. In order to ensure a total enzymatic hydrolysis of the flavan-3-ol- *C*-oligoglucosides, aliquots of the reaction mixture were treated with Termamyl for 2h, followed by an amyloglucosidase treatment for an additional 4h. Thereafter, the

hydrolysates obtained were analyzed by RP-HPLC-MS/MS (**Figure 8**). Only trace amounts of flavan-3-ol-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* were detectable and neither flavan-3-ol-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p*-(4→1)-*O*-α-D-glc *p*, nor higher *C*-oligomers could be observed, thus demonstrating a nearly quantitative hydrolytic break-down of the *O*-glucoside linkages in the flavan-3-ol- *C*-oligoglucosides. On the basis of this finding, the yield of flavan-3-ol-6-*C*-*β*-D-glc *p* (**3**), flavan-3-ol-8-*C*-*β*-D-glc *p* (**1**), and flavan-3-ol-6-*C*,8-*C*-*β*-D-glc *p* termini (**2**) in the (−)-epicatechin/amylose reaction was determined (**Figure 8**). The SIDA revealed an average amount of 14.0 (±2.2), 9.0 (±1.4), and 0.15 (±0.3) μmol per mmol (−)-epicatechin for compound **3**, **1**, and **2**, respectively. These data clearly demonstrate that the reducing terminus of the polysaccharide is reacting preferentially with the 6- and 8-position of flavan-3-ols. To a much smaller extent, flavan-3-ols seem also to be able to cross-link the reducing termini of two polysaccharide moieties via the 6- and 8-position.

Sensory Activity of Flavan-3-ol-*C*-glycosides. As recent studies decribed the velvetly astringency of the glucosides **1–6** (*6*), the taste thresholds of the flavan-3-ol-*C*-glycosides **7–12** was evaluated next. Prior to sensory analysis, the purity of all compounds was checked by LC-MS as well as ^{1}H NMR spectroscopy. To study the sensory activity of **7–12**, the human sensory recognition thresholds were determined in bottled water (pH 6.0) using the half-mouth test (*5, 8*) (**Table 1**).

Compared to the aglycones (−)-epicatechin and (−)-catechin, exhibiting a puckering astringent sensation as well as bitter taste at threshold concentrations between 600 and 1000 μmol/L, the flavan-3-ol-*C*-glucosides **1**, **3**, **4**, and **6** induced a smooth astringent and velvety mouth-coating sensation at very low threshold concentrations ranging from 3.1 to 63.6 μmol/L without exhibiting any bitter taste (**Table 1**). In comparison, the flavan-3-ol-*C*-diglucosides (**2**, **5**) showed the same sensory attributes at even lower thresholds of 1.1 and 2.5 μmol/L, respectively. Sensory analysis of the flavan-3-ol-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* isomers **10** and **12** as well as the flavan-3-ol-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4 → 1)-*O*-α-D-glc *p* (**11**) revealed somewhat lower thresholds when compared to those found for the flavan-3-ol-*C*-glucosides. In particular, the threshold concentration of 1.4 and 2.2 μmol/L determined for (−)-epicatechin-8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p*(4→1)-*O*-α-D-glc *p* and (−)-epicatechin-8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* were found to be extraordinarily low. These data clearly demonstrate that the elongation of the carbohydrate chain up to three glucose units favors the sensory impact of these flavan-3-ol glycoconjugates.

In line with our previous observations (*6*), the aglycone moiety has a significant influence on the perception of astringency. Comparing (−)-epicatechin-8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**8**) with the corresponding (−)-catechin-8-*C*-*β*-D-glc *p*-(4→1)-*O*-α-D-glc *p* (**7**) showed that the aglycone strongly influences the taste intensity of these compounds; for example, compound **7** exhibited a threshold concentration of 18.0 μmol/L, which is 8.2 times above the threshold concentration determined for the corresponding (−)-epicatechin derivative.

In conclusion, individual flavan-3-ol-*C*-*β*-D-glycopyranosides could be identified as the cross-linker molecules linking the reducing terminus of a polysaccharide to the A-ring of flavan-3-ols. These data allow first structural insights into the chemistry of the formation of covalent polyphenol/polysaccharides conjugates generated upon food processing.

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Received for review June 30, 2007. Revised manuscript received August 28, 2007. Accepted August 30, 2007. This work was supported by the International Graduate Research School Münster, Germany / Nagoya, Japan (IRGT MS-NG 1143): "Complex Functional Systems in Chemistry: Design, Development and Applications" funded by the Deutsche Forschungsgemeinschaft (DFG) and the Japan Society for the Promotion of Science (JSPS).

JF0719508