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Application of a Molecular Sensory Science Approach to Alkalized Cocoa (*Theobroma cacao*): Structure Determination and Sensory Activity of Nonenzymatically C-Glycosylated Flavan-3-ols

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Application of comparative taste dilution analyses on nonalkalized and alkalized cocoa powder revealed the detection of a velvety, smoothly astringent tasting fraction, which was predominantly present in the alkalized sample. LC-MS/MS analysis, 1D- and 2D-NMR, and CD spectroscopy as well as model alkalization reactions led to the unequivocal identification of the velvety, smoothly astringent molecules as a series of catechin- and epicatechin-*C*-glycopyranosides. Besides the previously reported (−)-epicatechin-8-*C*-*β*-D-galactopyranoside, additional flavan-3-ol-*C*-glycosides, namely, (−)-epicatechin-8-*C*-*β*-D-glucopyranoside, (−)-catechin-8-*C*-*β*-D-glucopyranoside, (−)-catechin-6-*C*-*β*-D-glucopyranoside, (−)-epicatechin-6-*C*-*β*-D-glucopyranoside, (−)-catechin-8-*C*-*β*-D-galactopyranoside, (−)-catechin-6-*C*-*β*-D-galactopyranoside, (−)-catechin-6-*C*,8-*C*-*β*-D-diglucopyranoside, (−)-epicatechin-6-*C*,8-*C*-*β*-D-digalactopyranoside, (−)-catechin-6-*C*,8-*C*-*β*-D-digalactopyranoside, and epicatechin-6-*C*,8-*C*-*β*-D-diglucopyranoside, were identified for the first time in cocoa. Most surprisingly, these phenol glycoconjugates were demonstrated by model experiments to be formed via a novel nonenzymatic C-glycosylation of flavan-3-ols. Using the recently developed half-tongue test, human recognition thresholds for the astringent and mouth-drying oral sensation were determined to be between 1.1 and 99.5 *μ*mol/L (water) depending on the sugar and the intramolecular binding position as well as the aglycone.

KEYWORDS: Cocoa; C-glycosylation; alkalization; astringency; taste dilution analysis; half-tongue test; flavan-3-ol-*C*-glycosides

INTRODUCTION

Due to its pleasant aroma as well as its typical bitter and astringent taste, altogether imparting rich mouthfeel, complexity, and palatability, the fermented and roasted seeds of the cocoa tree, *Theobroma cacao*, are widely enjoyed by consumers as the desirable tasty ingredient in cocoa-based products.

Very recently, application of taste dilution analysis (TDA) on roasted cocoa nibs (*T. cacao*) revealed that, besides the alkaloids theobromine and caffeine, a series of bitter-tasting 2,5-diketopiperazines and monomeric and oligomeric flavan-3-ols were the key inducers of the bitter taste imparted upon consumption of roasted cocoa (*1*−*3*). In addition, the flavan-3-ols were found to contribute to the astringency of cocoa nibs. Moreover, a number of velvety mouth-coating *O*-*β*-D-glycopyranosides of quercetin, naringenin, luteolin, and apigenin as well as a series of puckering astringent *N*-phenylpropenoyl-L-amino acids have been identified among the key taste compounds of roasted cocoa (*1*−*4*).

To bridge the gap between pure structural chemistry and human taste perception, taste reconstitution and omission experiments were performed on the basis of quantitative data demonstrating the bitter-tasting alkaloids theobromine and caffeine, seven bitter-tasting diketopiperazines, seven bitter- and astringent-tasting flavan-3-ols, six puckering astringent *N*-phenylpropenoyl-L-amino acids, four velvety astringent flavonol glycosides, *γ*-aminobutyric acid, *β*-aminoisobutyric acid, and six organic acids as the key organoleptics of the roasted cocoa nibs (*3*, *5*). Sensory analyses of a cocktail of these taste compounds, each in "natural" concentration, revealed that the taste profile of this artificial mixture was very close to the taste profile of an aqueous suspension of roasted cocoa nibs (*5*).

For the manufacturing of cocoa powder used as the basis for cocoa-containing beverages, the roasted cocoa nibs are subjected to an alkalization process involving the use of potassium hydroxide or magnesium oxide. This alkalization process is needed to mellow the flavor, to partially neutralize free organic acids, to enhance the color of the product, and to improve dispersability and lengthen suspension-holding ability of cocoa, thus preventing rapid sedimentation of the cocoa drink. In a typical process, the cocoa nibs are treated with a dilute 2.0−

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2.5% alkali solution at an ambient temperature of 75−100 °C, then neutralized, and dried to a moisture content of about 2% in a vacuum dryer or by further kneading of the mass at a temperature above 100 °C. Finally, the alkalized cocoa material is ground using fine roller mills. Although the taste of alkalized cocoa is well-known to be less astringent and milder in bitterness compared to the sensory profile of a nonalkalized cocoa, there are no data available on the differences in the key organoleptics in both products.

Aimed at defining the influence of the alkalization process on the key taste compounds in cocoa powder, the objectives of the present investigation were, therefore, to screen the taste compounds in nonalkalized as well as alkalized cocoa powder by means of a "molecular sensory science" approach: after screening for the cocoa components inducing the most intense human taste response using a comparative TDA, the target compounds differing in both types of cocoa were isolated and their chemical structures determined. To evaluate their taste impact, the human oral recognition threshold concentrations of the sensory active compounds were determined.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: (−)-epicatechin, (+)-catechin (Sigma, Steinheim, Germany); potassium carbonate, D-glucose, D-galactose (Merck, Darmstadt, Germany); apigenin-8-*C*-*β*-D-glucopyranoside, apigenin-6-*C*-*β*-D-glucopyranoside (Roth, Karlsruhe, Germany). Solvents were of HPLC grade (Merck). 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. Alkalized cocoa (AC) and nonalkalized cocoa (NC) prepared from the same blend of cocoa were obtained from the German food industry. For the alkalization process, cocoa nibs were mixed with an aqueous solution of potassium carbonate, heated for 1 h at 80 °C, then dried for 24 h at 40 °C, and, finally, roasted.

Sensory Analyses. *Training of the Sensory Panel.* Twelve subjects (seven women and five men, 25−38 years of age), who gave informed consent to participate in the sensory tests of the present investigation and have 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 (*6*): 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 by using gallotannic acid (0.05%) and quercetin-3-*O*-*β*-D-glucopyranoside (0.002 mmol/L), respectively, using the half-tongue test (*7*). Sensory analyses were performed in a sensory panel room at 22−25 °C in three different sessions.

Pretreatment of Fractions. Prior to sensory analysis, the fractions or compounds isolated were suspended in water, and, after removal of the volatiles in high vacuum (<5 mPa), were freeze-dried twice. GC-MS and ion chromatographic analyses revealed that food fractions treated by that procedure are essentially free of the solvents and buffer compounds used.

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 (*7*). 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 applied with a pipet 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 toward 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 beside one blank as a proof for the correctness of the data. The geometric mean of the last and the second to last concentrations was calculated and taken as the individual recognition threshold. Values between individuals and three separate sessions differed by not more than plus or minus one dilution step; that is, a threshold value of 3.1 *μ*mol/L for (−)-epicatecin-8-*C*-*β*-D-glucopyranoside represents a range from 1.55 to 6.2 *μ*mol/L.

Table 1. Yield and Sensorial Evaluation of Fractions I–V Isolated from Nonalkalized (NC) and Alkalized Cocoa (AC) Powder

sample[b]	yield[c] (g/100 g)	intensity[a] perceived for bitterness	astringency	sourness
NC		4.7	3.0	2.2
fraction I	13.6	0	0	0
fraction II	2.0	3.0	0	0
fraction III	1.6	1.8	1.0	0.5
fraction IV	16.7	4.0	2.5	1.5
fraction V	66.1	<0.5	<0.5	<0.5
AC		3.1	2.1	1.1
fraction I	15.7	0	0	0
fraction II	1.5	2.0	0	0
fraction III	0.9	1.1	0.8	0.4
fraction IV	14.2	2.5	2.0	1.0
fraction V	67.7	<0.5	<0.5	<0.5

[a] The taste intensity of aqueous mixtures of cocoa powder (10 g) or the individual fractions isolated from cocoa powder (10 g) in bottled water (100 mL; pH 6.0) was evaluated on a scale from 0 (not detectable) to 5.0 (strongly detectable). [b] Fractions I–V are the pentane solubles (I), dichloromethane extractables (II), ethyl acetate extractables (III), water solubles (IV), and nonsoluble residue (V) isolated from the corresponding cocoa powder. [c] Yields were determined by weight.

Sequential Solvent Extraction of Cocoa Samples. Nonalkalized roasted cocoa powder (100 g) and alkalized roasted cocoa powder (100 g), respectively, were extracted with *n*-pentane (5 × 300 mL) at room temperature for 30 min. After centrifugation, the organic layers were combined and freed from solvent in vacuum to give the *n*-pentane solubles (fraction I). The residual cocoa material was then extracted five times with a mixture (7:3, v/v; 300 mL each) of acetone and water for 45 min at room temperature with stirring. The acetone/water extract was freed from organic solvent under reduced pressure at 30 °C, the aqueous solution obtained was extracted with dichloromethane (4 × 200 mL), and the combined organic layers were freed from solvent in a vacuum to give the dichloromethane extractables (fraction II). The remaining aqueous layer was adjusted to pH 5.0 with aqueous hydrochloric acid (1 mol/L) and extracted with ethyl acetate (5 × 200 mL), the organic phase was freed from solvent in a vacuum to give the ethyl acetate extractables (fraction III), and the aqueous phase was freeze-dried to give the water solubles (fraction IV). In addition, the insoluble residue of the powdered cocoa was freeze-dried to give fraction V. The individual fractions were freeze-dried two times to remove trace amounts of solvents, their yields were determined by weight, and their taste profiles were evaluated in aqueous solutions as given in **Table 1**.

Gel Permeation Chromatography (GPC). Acetone/water extracts (fractions III/IV; 1 g each) obtained from alkalized (AC) and nonalkalized cocoa (NC) powder, respectively, were dissolved in a mixture (50:50, v/v; pH 3.5; 10 mL) of methanol and water, membrane filtered (45 *μ*m), and then applied onto the top of a water-cooled 100 × 5 cm glass column XK50 (Amersham Pharmacia Biotech, Uppsala, Sweden) filled with a slurry of Sephadex LH 20 material (Amersham Pharmacia Biotech) in the same solvent mixture. Using a flow rate of 2.3 mL/

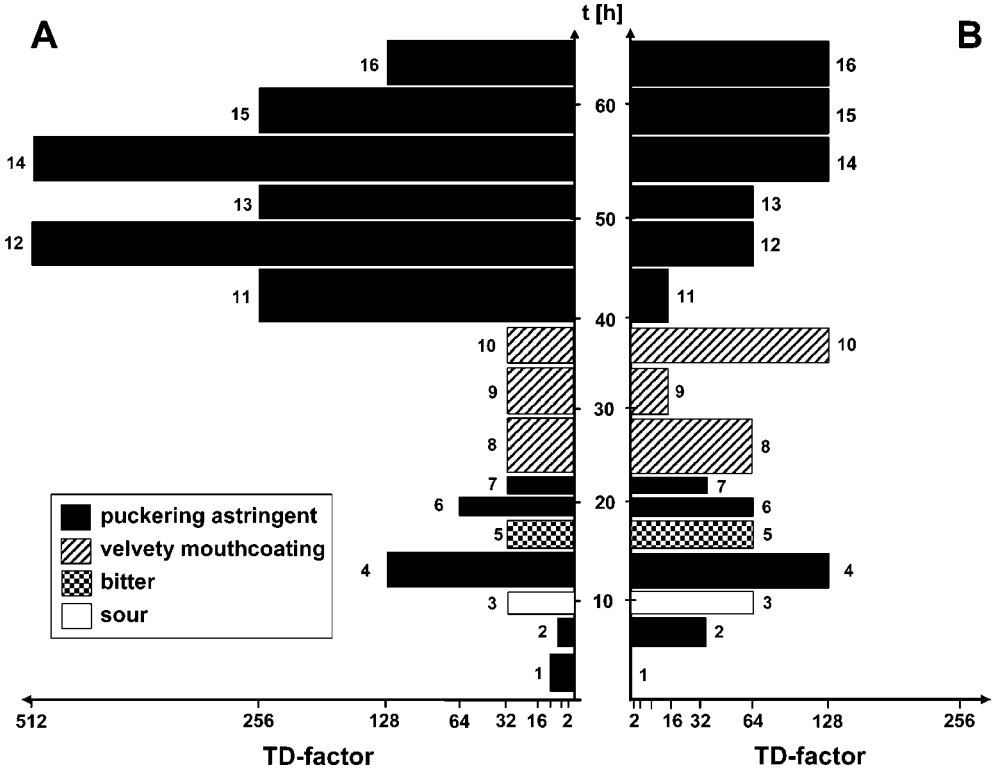

Figure 1. GPC/TDA chromatograms of the solvent fraction III/IV isolated from (**A**) nonalkalized and (**B**) alkalized cocoa powder.

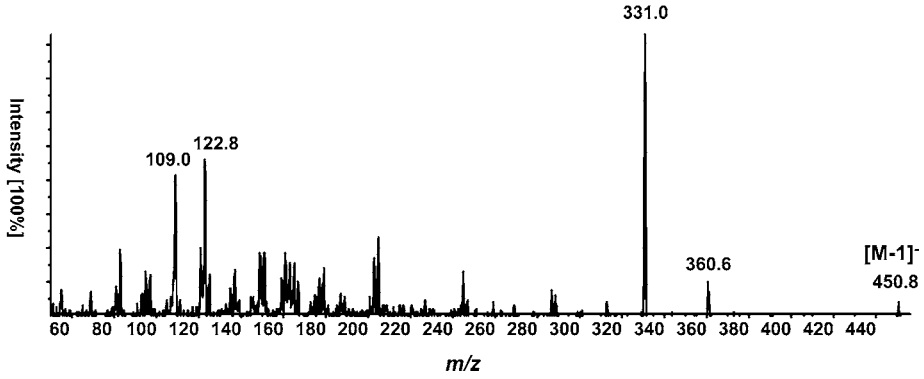

Figure 2. LC-MS/MS (ESI$^-$) spectrum of a velvety astringent compound isolated from GPC fraction 10 of alkalized cocoa.

min, chromatography was performed with methanol/water (50:50, v/v; pH 3.5; 0.8 L), followed by methanol/water (70:30, v/v; pH 3.5; 1.5 L), methanol/water (90:10, v/v; pH 3.5; 1 L), and, finally, methanol (2 L). Monitoring the effluent by means of a UV-1575 type UV−vis detector (Jasco, Gro*β*-Umstadt, Germany) operating at 270 nm, 16 fractions were collected from the AC and NC powders, respectively, by a fraction collector, and the individual fractions were freed from solvent in a vacuum at 30 °C and were then freeze-dried twice. The residue of each GPC fraction was used for the TDA as well as for chemical analysis.

TDA. Aliquots of the GPC fractions and the HPLC fractions, respectively, were dissolved in "natural" ratios in exactly 5.0 mL of bottled water (pH 6.0) and, then, sequentially diluted 1+1 with bottled water. The serial dilutions of each of these fractions were then presented to the sensory panel in order of ascending concentrations, and each dilution was evaluated for astringency by means of the half-mouth test. At the start of the session and before each trial, the subject rinsed with water and expectorated. 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 beside the blank as a proof for the correctness of the data. The geometric mean of the last and the second to last concentrations was calculated and taken as the dilution at which a sensory difference between the diluted extract and the blank sample could just be detected. This dilution was defined as the taste dilution (TD) factor (*8*). The TD factors evaluated by four different assessors in three different sessions were averaged. The TD factors between individuals and separate sessions did not differ by more than one dilution step. On the basis of the TD factors obtained, the individual GPC fractions were rated in their taste impact (**Figure 1**).

HPLC Analysis of GPC Fraction 10. GPC fraction 10 isolated from AC powder was solubilized in a mixture (30:70, v/v, 1 mL) of methanol and formic acid (0.1%) and then analyzed by means of HPLC-DAD as well as LC-MS. A series of compounds were detectable exhibiting UV−vis absorption maxima at 218, 231, and 280 nm and showing the typical mass spectrum exemplified in **Figure 2**.

Preparation of Flavan-3-ol-*C*-glycosides (1−11, Figure 3) Using a Model Alkalization Process. *Analytical Scale*. (−)-Epicatechin (0.04 mmol) was mixed with D-glucose (0.04, 0.08, 0.2, 0.4, 0.8, or 4 mmol, respectively), K_2CO_3 (0.21 mmol), and water (1 g). After homogenization, the alkalization process was performed by heating at 80 °C with stirring. After 10, 20, 40, and 60 min, the alkalization process 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 D-glucose or D-galactose (20 mmol, each), K_2CO_3 (10.42 mmol), and water (25 g). After homogenization, the alkalization process was

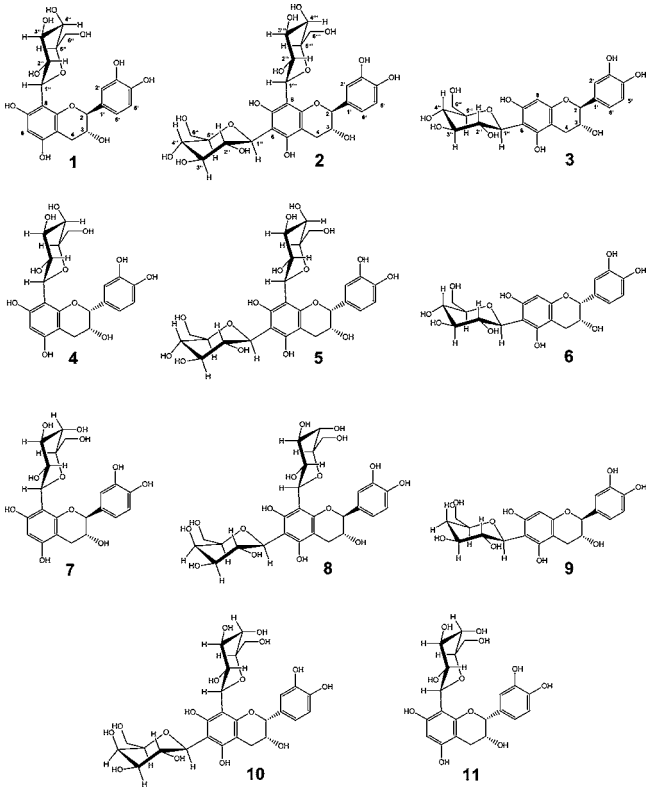

Figure 3. Chemical structures of flavan-3-ol-*C*-glycosides **1**–**11**.

performed for 10 min at 80 °C with stirring. After cooling, the alkalization process 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 (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) 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 a vacuum to give 10 subfractions (f1−f10), freeze-dried, and finally purified by means of HPLC (Jasco) consisting of a HPLC pump system PU 2087, a high-pressure gradient unit, and a PU-2075 UV detector using a preparative RP-18 column, HyperClone micro ODS (C18), 21.2 × 250 mm, 5 μm (Phenomenex, Aschaffenburg, Germany), as the stationary phase. Subfractions f4−f10 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 removal of solvents in a vacuum, the title compounds were suspended in water (10 mL) and freeze-dried two times to afford the corresponding flavan-3-ol-*C*-glycosides **1**−**11** (**Figure 3**) as white, amorphous powders in high purities of >99%.

(−)-Catechin-8-C-β-D-glucopyranoside, ***1*** **(*Figure 3*)**: CD (MeOH) $\lambda_{max}(\Delta\epsilon) = 241$ (+3.09), 284 (+1.32), 297 (−0.45); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) $\lambda_{max} = 218$, 231, 280 nm; MS (ESI^-), *m*/*z* 451 (100%, $[M - 1]^-$), 331 (41%, $[M - 121]^-$); ^1H NMR (400 MHz, MeOD, COSY) δ 2.57 [dd, 1H, $J = 7.0$, 16.0 Hz, H−C(4a)], 2.78 [dd, 1H, $J = 5.2$, 16.0 Hz, H−C(4b)], 3.36−3.42 [m, 3H, H−C(5″, 3″, 4″)], 3.71 [dd, 1H, $J = 4.8$, 12.0 Hz, H−C(6a″)], 3.87 [dd, 1H, $J = 1.6$, 12.0 Hz, H−C(6b″)], 4.01 [ddd, 1H, $J = 5.2$, 6.8, 7.0 Hz, H−C(3)], 4.03 [dd, 1H, $J = 9.2$ Hz, H−C(2″)], 4.77 [d, 1H, $J = 6.8$ Hz, H−C(2)], 4.85 [d, 1H, $J = 9.2$ Hz, H−C(1′)], 6.02 [s, 1H, H−C(6)], 6.71 [dd, 1H, $J = 1.6$, 8.0 Hz, H−C(6′)], 6.77 [d, 1H, $J = 8.0$ Hz, H−C(5′)], 6.85 [d, 1H, $J = 2.0$ Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 27.8 [C-4], 62.8 [C-6″], 68.5 [C-3], 71.7 [C-4″], 73.5 [C-2″], 76.5 [C-1″], 79.4 [C-3″], 82.3 [C-5″], 82.4 [C-2], 97.1 [C-6], 101.0 [C-4a], 104.2 [C-8], 114.7 [C-2′], 116.2 [C-5′], 119.5 [C-6′], 132.4 [C-1′], 146.1 [C-3′], 146.3 [C-4′], 155.1 [C-8a], 157.1 [C-7], 157.5 [C-5].

(−)-Catechin-6-C-β-D,8-C-β-D-diglucopyranoside, ***2*** **(*Figure 3*)**: CD (MeOH) $\lambda_{max}(\Delta\epsilon) = 239$ (−1.79), 286 (+0.46), 301 (−0.39); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v): $\lambda_{max} = 218$, 231, 280 nm; MS (ESI^-), *m*/*z* 613 (100%, $[M - 1]^-$), 493 (13%, $[M - 121]^-$); ^1H NMR (400 MHz, MeOD, COSY) δ 2.45 [dd, 1H, $J = 7.4$, 16.4 Hz, H−C(4a)], 2.73 [dd, 1H, $J = 5.2$, 16.4 Hz, H−C(4b)], 3.24−3.38 [m, 4H, H−C(4, 5″, 5‴, 3‴)], 3.43 [m, 2H, H−C(4, 3″)], 3.54 [dd, 1H, $J = 9.2$, 9.6 Hz, H−C(2″)], 3.62−3.77 [m, 5H, H−C(6‴a,b, 2‴, 6″a,b)], 3.87 [ddd, 1H, $J = 5.2$, 7.2 Hz H−C(3)], 4.61 [d, 1H, $J = 6.8$ Hz, H−C(2)], 4.77 [d, 1H, $J = 9.6$ Hz, H−C(1‴)], 4.80 [d, 1H, $J = 9.6$ Hz, H−C(1″)], 6.59 [dd, 1H, $J = 2.0$, 8.0 Hz, H−C(6′)], 6.65 [d, 1H, $J = 8.0$ Hz, H−C(5′)], 6.71 [d, 1H, $J = 2.0$ Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 26.7 [C-4], 60.6 [C-6‴], 61.0 [C-6″], 67.1 [C-3], 69.8 [C-4‴/4″], 70.1 [C-4″/C-4‴], 72.0 [C-2‴], 73.4 [C-2″], 75.5 [C-1‴/C-1″], 75.9 [C-1″/C-1‴], 77.8 [C-3″/C-3‴], 78.0 [C-3‴/C-3″], 81.0 [C-5″/C-5‴/C-2], 81.1 [C-5″/C-5‴/C-2], 81.2 [C-2/C-5‴/C-5″], 101.0 [C-4a], 103.2 [C-8], 104.6 [C-6], 113.4 [C-2′], 114.7 [C-5′], 118.2 [C-6′], 130.7 [C-1′], 144.8 [C-3′/C-4′], 144.9 [C-4′/C-3′], 153.4 [C-8a], 154.4 [C-7, C-5].

(−)-Catechin-6-C-β-D-glucopyranoside, ***3*** **(*Figure 3*)**: CD (MeOH) $\lambda_{max}(\Delta\epsilon) = 240$ (−5.01), 283 (+1.00), 301 (−0.20); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) $\lambda_{max} = 218$, 231, 280 nm; MS (ESI^-), *m*/*z* 451 (100%, $[M - 1]^-$), 331 (20%, $[M - 121]^-$); ^1H NMR (400 MHz, MeOD, COSY) δ 2.53 [dd, 1H, $J = 8.0$, 16.0 Hz, H−C(4a)], 2.90 [dd, 1H, $J = 5.4$, 16.0 Hz, H−C(4b)], 3.42 [m, 1H, $J = 2.4$, 4.4 Hz, H−C(5″)], 3.50 [m, 1H, $J = 8.8$ Hz, H−C(3″)], 3.54 [m, 1H, H−C(4″)], 3.69 [dd, 1H, $J = 8.8$, 9.6 Hz, H−C(2″)], 3.81 [dd, 1H, $J = 4.4$, 12.0 Hz, H−C(6a″)], 3.88 [dd, 1H, $J = 2.4$, 12.0 Hz, H−C(6b″)], 3.98 [ddd, 1H, $J = 5.4$, 7.6, 8.0 Hz, H−C(3)], 4.59 [d, 1H, $J = 7.6$ Hz, H−C(2)], 4.88 [d, 1H, $J = 9.6$ Hz, H−C(1″)], 6.02 [s, 1H, H−C(8)], 6.72 [dd, 1H, $J = 2.0$, 8.4 Hz, H−C(6′)], 6.77 [d, 1H, $J = 8.4$ Hz, H−C(5′)], 6.83 [d, 1H, $J = 2.0$ Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 28.5 [C-4], 62.2 [C-6″], 68.7 [C-3], 71.2 [C-4″], 74.6 [C-2″], 77.5 [C-1″], 79.6 [C-3″], 82.5 [C-5″], 82.9 [C-2], 96.3 [C-8], 102.2 [C-4a], 105.5 [C-6], 115.2 [C-2′], 116.1 [C-5′], 120.0 [C-6′], 132.1 [C-1′], 146.32 [C-3′/C-4′], 146.35 [C-4′/C-3′], 156.1 [C-8a], 156.4 [C-7/C-5], 156.5 [C-5/C-7].

(−)-Epicatechin-8-C-β-D-glucopyranoside, ***4*** **(*Figure 3*)**: CD (MeOH) $\lambda_{max}(\Delta\epsilon) = 239$ (−3.33), 282 (−1.16), 301 (−0.12); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) $\lambda_{max} = 218$, 231, 280 nm; MS (ESI^-), *m*/*z* 451 (100%, $[M - 1]^-$), 331 (20%, $[M - 121]^-$); ^1H NMR (400 MHz, MeOD, COSY) δ 2.87 [ddd, 2H, $J = 2.0$, 4.0, 16.0 Hz, H−C(4a,b)], 3.37 [m, 1H, H−C(5″)], 3.48 [dd, 1H, $J = 8.4$, 8.8 Hz, H−C(3″)], 3.50 [dd, 1H, $J = 8.8$, 9.6 Hz, H−C(4″)], 3.81 [dd, 1H, $J = 4.4$, 12.0 Hz, H−C(6a″)], 3.88 [dd, 1H, $J = 2.4$, 12.0 Hz, H−C(6b″)], 4.07 [m, 1H, H−C(2″)], 4.16 [s, 1H, H−C(3)], 4.91 [s, 1H, H−C(2)], 4.91 [d, 1H, $J = 9.6$ Hz, H−C(1″)], 6.04 [s, 1H, H−C(6)], 6.78 [d, 1H, 8.4 Hz, H−C(5′)], 6.86 [dd, 1H, $J = 1.6$, 8.4 Hz, H−C(6′)], 7.06 [d, 1H, $J = 1.6$ Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 28.4 [C-4], 61.2 [C-6″], 66.0 [C-3], 70.2 [C-4″], 72.0 [C-2″], 75.2 [C-1″], 78.5 [C-2], 78.6 [C-3″], 80.7 [C-5″], 95.7 [C-6], 98.6 [C-4a], 102.9 [C-8], 113.7 [C-2′], 114.6 [C-5′], 117.5 [C-6′], 131.0 [C-1′], 144.2 [C-3′/C-4′], 144.6 [C-4′/C-3′], 154.1 [C-8a], 155.7 [C-7], 156.7 [C-5].

(−)-Epicatechin-6-C-β-D,8-C-β-D-diglucopyranoside, ***5*** **(*Figure 3*)**: UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) $\lambda_{max} = 218$, 231, 280 nm; MS (ESI^-), *m*/*z* 613 (100%, $[M - 1]^-$), 493 (16%, $[M - 121]^-$); ^1H NMR (400 MHz, MeOD, COSY), 2.86 [ddd, 2H, $J = 2.2$, 4.0, 17.2 Hz, H−C(4a,b)], 3.36−3.42 [m, 2H, H−C(5″, 5‴)], 3.46−3.56 [m, 4H, H−C(3″, 3‴, 4″, 4‴)], 3.64 [dd, 1H, $J = 8.8$, 9.2 Hz, H−C(2″)], 3.76−3.87 [m, 4H, H−C(6‴a,b, 6″a,b)], 3.92 [dd, 1H, $J = 9.6$ Hz H−C(2‴)], 4.17 [m, 1H, H−C(3)], 4.89 [s, 1H, H−C(2)], 4.91 [d, 1H, $J = 9.6$ Hz, H−C(1″)], 4.93 [d, 1H, $J = 9.6$ Hz, H−C(1‴)], 6.77 [d, 1H, $J = 8.0$ Hz, H−C(5′)], 6.83 [dd, 1H, $J = 1.6$, 8.0 Hz, H−C(6′)], 7.03 [d, 1H, $J = 1.6$ Hz, H−C(2′)]; ^{13}C NMR (100

MHz, MeOD, HMQC, HMBC) δ 28.2 [C-4], 60.6 [C-6‴/C-6″], 60.8 [C-6″/C-6‴], 66.0 [C-3], 69.7 [C-4‴/4″], 70.0 [C-4″/C-4‴], 72.1 [C-2‴], 73.3 [C-2″], 75.4 [C-1″], 76.0 [C-1‴], 78.08 [C-3″/C-3‴], 78.12 [C-3‴/C-3″], 78.6 [C-2], 80.8 [C-5″/C-5‴], 81.1 [C-5‴/C-5″], 99.9 [C-4a], 103.5 [C-8], 104.8 [C-6], 113.7 [C-2′], 114.6 [C-5′], 117.6 [C-6′], 130.8 [C-1′], 144.3 [C-3′/C-4′], 144.6 [C-4′/C-3′], 153.1 [C-8a], 154.1 [C-7], 154.6 [C-5].

(−)-Epicatechin-6-C-β-D-glucopyranoside, ***6*** ***(Figure 3):*** UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) λ_{max} = 218, 231, 280 nm; MS (ESI$^-$), *m/z* 451 (100%, [M − 1]$^-$), 331 (20%, [M − 121]$^-$); ^{1}H NMR (400 MHz, MeOD, COSY) δ 2.76 [dd, 1H, *J* = 3.2, 16.4 Hz, H−C(4a)], 2.89 [dd, 1H, *J* = 4.4, 16.4 Hz, H−C(4b)], 3.43 [m, 1H, H−C(5″)], 3.50 [dd, 1H, *J* = 8.4, 8.8 Hz, H−C(3″)], 3.54 [dd, 1H, *J* = 8.0, 8.8 Hz, H−C(4″)], 3.69 [dd, 1H, *J* = 8.8, 9.6 Hz, H−C(2″)], 3.81 [dd, 1H, *J* = 4.6, 12.2 Hz, H−C(6a″)], 3.88 [dd, 1H, *J* = 2.2, 12.2 Hz, H−C(6b″)], 4.21 [m, 1H, *J* = 3.2, 4.4 Hz, H−C(3)], 4.86 [s, 1H, H−C(2)], 4.88 [d, 1H, *J* = 9.6 Hz, H−C(1″)], 6.01 [s, 1H, H−C(8)], 6.77 [d, 1H, *J* = 8.4 Hz, H−C(5′)], 6.81 [dd, 1H, *J* = 1.6, 8.4 Hz, H−C(6′)], 6.98 [d, 1H, *J* = 1.6 Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 27.8 [C-4], 60.7 [C-6″], 66.1 [C-3], 69.8 [C-4″], 73.2 [C-2″], 76.1 [C-1″], 78.2 [C-3″], 78.5 [C-2], 81.1 [C-5″], 95.2 [C-8], 100.0 [C-4a], 103.8 [C-6], 114.0 [C-2′], 114.5 [C-5′], 118.0 [C-6′], 130.7 [C-1′], 144.5 [C-3′/C-4′], 144.6 [C-4′/C-3′], 154.5 [C-8a], 155.1 [C-7/C-5], 155.1 [C-5/C-7].

(−)-Catechin-8-C-β-D-galactopyranoside, ***7*** ***(Figure 3):*** CD (MeOH) $\lambda_{max}(\Delta\epsilon)$ = 239 (+1.94), 283 (+1.15), 301 (−0.30); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) λ_{max} = 218, 231, 280 nm; MS (ESI$^-$), *m/z* 451 (100%, [M − 1]$^-$), 331 (35%, [M-121]$^-$); ^{1}H NMR (400 MHz, MeOD, COSY) δ 2.56 [dd, 1H, *J* = 6.6, 16.4 Hz, H−C(4a)], 2.70 [dd, 1H, *J* = 5.2, 16.4 Hz, H−C(4b)], 3.51 [dd, 1H, J_{ae} = 3.0, 9.4 ($_{aa}$) Hz, H−C(3″)], 3.58 [ddd, 1H, *J* = 1.6, 5.2 Hz, H−C(5″)], 3.68 [dd, 1H, *J* = 4.8, 11.2 Hz, H−C(6a″)], 3.75 [dd, 1H, *J* = 7.0, 11.2 Hz, H−C(6b″)], 3.90 [d, 1H, *J* = 2.8 Hz, H−C(4″)], 4.02 [ddd, 1H, *J* = 5.2, 6.4 Hz, H−C(3)], 4.10 [dd, 1H, *J* = 9.2, 9.6, Hz, H−C(2″)], 4.79 [d, 1H, *J* = 6.0 Hz, H−C(2)], 4.82 [d, 1H, *J* = 9.6 Hz, H−C(1″)], 5.98 [s, 1H, H−C(6)], 6.67 [dd, 1H, *J* = 2.0, 8.4 Hz, H−C(6′)], 6.74 [d, 1H, *J* = 8.4 Hz, H−C(5′)], 6.80 [d, 1H, *J* = 2.0 Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 26.0 [C-4], 61.7 [C-6″], 67.0 [C-3], 69.3 [C-4″], 70.6 [C-2″], 74.8 [C-3″], 75.5 [C-1″], 79.1 [C-5″], 80.9 [C-2], 95.7 [C-6], 99.2 [C-4a], 103.4 [C-8], 113.3 [C-2′], 114.8 [C-5′], 118.0 [C-6′], 131.0 [C-1′], 144.7 [C-3′], 144.9 [C-4′], 152.9 [C-8a], 155.7 [C-7], 156.1 [C-5].

(−)-Catechin-6-C-β-D,8-C-β-D-digalactopyranoside, ***8*** ***(Figure 3):*** CD (MeOH) $\lambda_{max}(\Delta\epsilon)$ = 239 (−1.91), 286 (+0.61), 301 (−0.20); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v): λ_{max} = 218, 231, 280 nm; MS (ESI$^-$), *m/z* 613 (100%, [M − 1]$^-$), 451 (35%, [M − 163]$^-$), 493 (31%, [M − 121]$^-$), 373 (15%, [M − 241]$^-$), 331 (11%, [M − 283]$^-$); ^{1}H NMR (400 MHz, MeOD, COSY) δ 2.57 [dd, 1H, *J* = 6.8, 16.4 Hz, H−C(4a)], 2.81 [dd, 1H, *J* = 5.0, 16.4 Hz, H−C(4b)], 3.55 [dd, 1H, J_{ae} = 2.8, 9.6 ($_{aa}$) Hz, H−C(3‴)], 3.60−3,67 [m, 3H, H−C(3″, 5″, 5‴)], 3.70−3,82 [m, 4H, H−C(6″a,b, 6‴a,b)], 3.94 [d, 1H, *J* = 2.8 Hz, H−C(4‴)], 3.98−4.03 [m, 3H, H−C(3, 2″, 4″)], 4.09 [dd, 1H, *J* = 9.6 Hz, H−C(2‴)], 4.74 [d, 1H, *J* = 6.4 Hz, H−C(2)], 4.84 [d, 1H, *J* = 9.6 Hz, H−C(1‴)], 4.87 [d, 1H, *J* = 9.6 Hz, H−C(1″)], 6.70 [dd, 1H, *J* = 2.0, 8.0 Hz, H−C(6′)], 6.76 [d, 1H, *J* = 8.0 Hz, H−C(5′)], 6.82 [d, 1H, *J* = 2.0 Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 26.4 [C-4], 61.6 [C-6″/C-6‴], 61.7 [C-6‴/C-6″], 67.1 [C-3], 69.21 [C-4″/4‴], 69.25 [C-4‴/C-4″], 69.9 [C-2″], 70.8 [C-2‴], 74.4 [C-3‴], 74.7 [C-3″], 75.5 [C-1″/C-1‴], 75.6 [C-1‴/C-1″], 79.3 [C-5″/C-5‴], 79.4 [C-5‴/C-5″], 81.2 [C-2], 100.2 [C-4a], 103.1 [C-8], 104.9 [C-6], 113.4 [C-2′], 114.7 [C-5′], 118.2 [C-6′], 130.8 [C-1′], 144.8 [C-3′/C-4′], 144.9 [C-4′/C-3′], 152.4 [C-8a], 153.4 [C-7/C-5], 154.6 [C-5/C-7].

(−)-Catechin-6-C-β-D-galactopyranoside, ***9*** ***(Figure 3):*** CD (MeOH) $\lambda_{max}(\Delta\epsilon)$ = 240 (−1.40), 283 (+0.33), 301 (−0.18); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) λ_{max} = 218, 231, 280 nm; MS (ESI$^-$), *m/z* 451 (100%, [M − 1]$^-$), 331 (30%, [M − 121]$^-$); ^{1}H NMR (400 MHz, MeOD, COSY) δ 2.50 [dd, 1H, *J* = 7.8, 16.2 Hz, H−C(4a)], 2.86 [dd, 1H, *J* = 5.4, 16.2 Hz, H−C(4b)], 3.58 [dd, 1H, J_{ae} = 2.8, 9.6 ($_{aa}$) Hz, H−C(3″)], 3.64 [ddd, 1H, *J* = 5.6, 6.4 Hz, H−C(5″)], 3.72 [dd, 1H, *J* = 4.8, 11.6 Hz, H−C(6a″)], 3.79 [dd, 1H, *J* = 7.0, 11.6 Hz, H−C(6b″)], 3.95−4.00 [m, 2H, H−C(3, 4″)], 4.01 [dd, 1H, *J* = 9.6 Hz, H−C(2″)], 4.57 [d, 1H, *J* = 7.2 Hz, H−C(2)], 4.81 [d, 1H, *J* = 10.0 Hz, H−C(1″)], 5.94 [s, 1H, H−C(8)], 6.70 [dd, 1H, *J* = 2.0, 8.4 Hz, H−C(6″)], 6.76 [d, 1H, *J* = 8.4 Hz, H−C(5″)], 6.82 [d, 1H, *J* = 2.0 Hz, H−C(2″)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 27.0 [C-4], 61.6 [C-6″], 67.3 [C-3], 69.2 [C-4″], 70.7 [C-2″], 74.6 [C-3″], 75.8 [C-1″], 79.3 [C-5″], 81.4 [C-2], 94.5 [C-8], 100.4 [C-4a], 104.6 [C-6], 113.8 [C-2′], 114.7 [C-5′], 118.6 [C-6′], 130.7 [C-1′], 144.85 [C-3′/C-4′], 144.86 [C-4′/C-3′], 154.5 [C-8a], 154.8 [C-7/C-5], 154.9 [C-5/C-7].

(−)-Epicatechin-8-C-β-D-galactopyranoside, ***10*** ***(Figure 3):*** UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) λ_{max} = 218, 231, 280 nm; MS (ESI$^-$), *m/z* 451 (100%, [M − 1]$^-$), 331 (33%, [M − 121]$^-$); ^{1}H NMR (400 MHz, MeOD, COSY) δ 2.88 [ddd, 2H, *J* = 2.0, 4.0, 16.4 Hz, H−C(4a,b)], 3.60 [ddd, 1H, *J* = 1.6, 6.0 Hz, H−C(5″)], 3.63 [ddd, 1H, J_{ae} = 3.0, 9.4 ($_{aa}$) Hz, H−C(3″)], 3.70 [dd, 1H, *J* = 5.0, 11.4 Hz, H−C(6a″)], 3.76 [dd, 1H, *J* = 7.0, 11.4 Hz, H−C(6b″)], 3.95 [d, 1H, *J* = 2.8 Hz, H−C(4″)], 4.15 [m, 1H, H−C(3)], 4.16 [dd, 1H, *J* = 8.8, Hz, H−C(2″)], 4.89 [s, 1H, H−C(2)], 4.90 [d, 1H, *J* = 8.8 Hz, H−C(1″)], 6.01 [s, 1H, H−C(6)], 6.78 [d, 1H, *J* = 8.0 Hz, H−C(5′)], 6.80 [dd, 1H, *J* = 2.0, 8.0 Hz, H−C(6′)], 7.03 [d, 1H, *J* = 2.0 Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 28.3 [C-4], 61.6 [C-6″], 66.0 [C-3], 69.1 [C-4″], 70.9 [C-2‴], 74.8 [C-3″], 75.5 [C-1″], 78.6 [C-2], 78.8 [C-5″], 95.9 [C-6], 98.2 [C-4a], 103.5 [C-8], 113.7 [C-2′], 114.6 [C-5′], 117.5 [C-6′], 131.0 [C-1′], 144.3 [C-3′], 144.6 [C-4′], 153.2 [C-8a], 155.7 [C-7], 156.7 [C-5].

(−)-Epicatechin-6-C-β-D,8-C-β-D-digalactopyranoside, ***11*** ***(Figure 3):*** UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) λ_{max} = 218, 231, 280 nm; MS (ESI$^-$), *m/z* 613 (100%, [M − 1]$^-$), 493 (29%, [M − 121]$^-$), 331 (11%, [M − 283]$^-$); ^{1}H NMR (400 MHz, MeOD, COSY) δ 2.77 [ddd, 2H, *J* = 3.2, 4.4, 17.2 Hz, H−C(4a,b)], 3.51 [m, 4H, H−C(3″, 3‴, 5″, 5‴)], 3.63 [m, 4H, H−C(6″a,b, 6‴a,b)], 3.85 [d, 1H, *J* = 2.8 Hz, H−C(4‴)], 3.88 [dd, 1H, *J* = 9.2, 9.6 Hz, H−C(2″)], 3.88 [d, 1H, *J* = 2.8 Hz, H−C(4″)], 4.06 [m, 1H, H−C(3)], 4.07 [dd, 1H, *J* = 9.2 Hz, H−C(2‴)], 4.76 [d, 1H, *J* = 9.6 Hz, H−C(1‴)], 4.78 [s, 1H, H−C(2)], 4.78 [d, 1H, *J* = 9.2 Hz, H−C(1‴)], 6.67 [d, 1H, *J* = 8.4 Hz, H−C(5′)], 6.70 [dd, 1H, *J* = 1.6, 8.0 Hz, H−C(6′)], 6.92 [d, 1H, *J* = 1.6 Hz, H−C(2′)]; ^{13}C NMR (100 MHz, MeOD, HMQC, HMBC) δ 28.0 [C-4], 61.4 [C-6″, C-6‴], 65.9 [C-3], 69.0 [C-4″/4‴], 69.2 [C-4‴/C-4″], 70.6 [C-2‴], 71.4 [C-2″], 74.7 [C-3″, C-3‴], 75.5 [C-1″, C-1‴], 78.5 [C-2], 79.2 [C-5‴, C-5″], 99.2 [C-4a], 103.1 [C-8], 105.0 [C-6], 113.6 [C-2′], 114.6 [C-5′], 117.4 [C-6′], 130.8 [C-1′], 144.2 [C-3′/C-4′], 144.4 [C-4′/C-3′], 153.1 [C-8a], 153.8 [C-7, C-5].

(−)-Catechin: CD (MeOH) $\lambda_{max}(\Delta\epsilon)$ = 232 (+1.91), 280 (+0.51); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) λ_{max} = 218, 231, 280 nm.

(+)-Catechin: CD (MeOH) $\lambda_{max}(\Delta\epsilon)$ = 230 (−0.85), 280 (−0.26); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) λ_{max} = 218, 231, 280 nm.

(−)-Epicatechin: CD (MeOH) $\lambda_{max}(\Delta\epsilon)$ = 239 (−1.41), 279 (−0.63); UV−vis (MeOH/0.1% HCOOH, pH 2.5; 2:8, v/v) λ_{max} = 218, 231, 280 nm.

Identification of Flavan-3-ol-*C*-glycosides 1−11 in Cocoa Powder. Nonalkalized or alkalized roasted cocoa powder (5.0 g) was extracted with *n*-pentane (5 × 30 mL) at room temperature for 30 min. The residual cocoa material was then extracted five times with acetone/water (70:30, v/v; 30 mL each) for 45 min at room temperature with stirring. After centrifugation, the liquid layer was freed from acetone under reduced pressure at 30 °C and then freeze-dried to give the acetone/water extract. Aliquots (∼50 mg) of the acetone/water extract were taken up in a methanol/water mixture (1:1, v/v; 10 mL), which was acidified to pH 2.5 with formic acid (0.1% in water). After membrane filtration, 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) operated with a flow rate of 0.2 mL/min. Chromatography was performed starting with aqueous formic acid (0.1%, pH 2.5), held for 10 min, and the methanol content was increased to 40% in 40 min, increased to 100% within 10 min, and, finally, held at 100% for 10 min. By means of the multiple-reaction monitoring (MRM) mode, the individual flavan-3-ol-*C*-glycosides **1**,

3, **4**, **6**, **7**, **9**, and **11** (*m*/*z* 451.2 → 331.0) and **2**, **5**, **8**, and **10** (*m*/*z* 613.1 → 373.0) were analyzed using the transition reactions monitored for a duration of 150 ms given in brackets.

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 autosampler, 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), 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 mode.

LC−Time-of-Flight Mass Spectrometry (LC-TOF-MS). High-resolution mass spectra of compound **1** measured on a Bruker MicroTOF (Bruker Daltonics, Bremen, Germany) mass spectrometer with flow injection and using sodium formate and PEG 600, respectively, as the reference revealed the molecular formula $C_{21}H_{24}O_{11}$ for the taste compound.

Circular Dichroism (CD) Spectroscopy. For CD spectroscopy, methanolic solutions of the samples were analyzed by means of a Jasco J600 Spectro polarimeter (Hachioji, Japan).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, COSY, HMQC, HMBC, ^{13}C, and DEPT-135 NMR measurements were performed on an DMX 400 spectrometer (Bruker, Rheinstetten, Germany). Evaluation of the experiments was carried out using 1D- and 2D-WIN NMR (version 6.1) as well as MestReC software (version 4.4.6.0, Mestrelab Research, Santiago de Compostela, Spain). Tetramethylsilane was used as the internal standard.

RESULTS AND DISCUSSION

A freshly prepared aqueous suspension (pH 6.0) of nonalkalized, powdered cocoa (NC) imparted the typical complex and attractive cocoa taste and was used as the reference for taste profile analysis. In comparison, an aqueous suspension (pH 6.0) of alkalized cocoa (AC) powder was sensorially evaluated. To achieve this, a trained sensory panel was asked to rate the intensity of the taste qualities bitter, sour, and astringent mouth-coating on a scale from 0 (not detectable) to 5 (intensely detectable). As expected, high scores were found for the intensity of the bitter taste (4.7) and the astringent, mouth-coating taste sensation (3.0) as well as the sourness (2.2) of the nonalkalized cocoa sample (**Table 1**). In contrast, bitterness (3.1), astringency (2.1), and sourness (1.1) were strongly decreased in the AC powder. To gain a first insight into the influence of the alkalization process on the cocoa taste compounds, both cocoa powders were extracted sequentially with solvents of different polarities.

Solvent Fractionation of AC and NC. The cocoa powders were extracted with *n*-pentane to give the pentane solubles (fraction I) after evaporation of the solvent in a vacuum. The residual cocoa material was then extracted with aqueous acetone, and, after removal of the acetone from the aqueous phase in vacuum, the solution obtained was extracted with dichloromethane. The combined organic layers were freed from solvent in vacuum to give the dichloromethane extractables (fraction II). The remaining aqueous layer was extracted with ethyl acetate, the organic phase was freed from solvent to give the ethyl acetate extractables (fraction III), and the aqueous phase was freeze-dried to give the water solubles (fraction IV). In addition, the insoluble residue of the cocoa samples was freeze-dried to give fraction V. Besides the cocoa butter present in fraction I and the nonsoluble residue, the solubles in fraction IV were found with the highest yields of 16.7 and 14.2% in the NC and AC samples, respectively (**Table 1**).

Sensory evaluation of aqueous mixtures of the individual solvent fractions isolated from NC and AC, respectively, by means of taste profile analysis demonstrated the highest scores for bitterness, astringency, and sourness in fraction IV, followed by the ethyl acetate extractables (fraction III) evaluated with somewhat lower taste intensities for bitter taste. Comparison of the samples NC and AC clearly indicated that the alkalization process induced a significant reduction of bitterness (4.0 → 2.5), astringency (2.5 → 2.0), and sourness (1.5 → 1.0) of the cocoa water solubles (fraction IV) (**Table 1**). Similarly to fraction IV, a significant decrease of the intensities of the individual taste modalities was observable for fraction III. Therefore, fractions III and IV were recombined to fraction III/IV prior to further analysis.

Sensory-Guided GPC Separation of Fraction III/IV. To sort out the strongly taste-active compounds from the bulk of less taste-active or tasteless substances and to visualize the differences in the tastant signature of both the cocoa samples, fraction III/IV isolated from NC and AC, respectively, was separated by means of GPC. Using Sephadex LH-20 as the stationary phase and methanol/water mixtures as the mobile phase, the effluent was separated into 16 fractions, respectively, which were collected separately and, then, investigated for the taste impact. To achieve this, the 16 fractions obtained from fraction III/IV of NC (**Figure 1A**) and AC (**Figure 1B**) were freeze-dried, taken up in water, and then analyzed by means of the TDA (*8*) using the recently developed half-mouth test (*1*, *7*).

In the NC sample, the highest TD factors of 512 and 256 were found for the puckering astringent taste of GP fractions 12−15, followed by fractions 4 and 16, which still showed astringent taste after a dilution of 1+127 (**Table 2**). In contrast, GPC fractions 8−11 exhibited a velvety, silky type of astringent mouth-coating sensation evaluated with TD factors between 32 and 256, respectively. In the AC sample, the puckering astringent taste of GPC fractions 4 and 14−16 were judged with the highest TD factor of 128. In addition, the velvety astringent fraction 10 was evaluated with a TD factor of 128 (**Table 2**).

By comparison of the taste dilution chromatograms in **Figure 1** obtained for the solvent fraction III/IV of the two cocoa samples, the sensory data obtained for GPC fractions 11−16 of the AC sample showed clearly lower TD factors for their puckering and rough astringent sensation. As these fractions have been recently demonstrated to contain catechin, epicatechin, and the family of (4β → 8)-linked epicatechin oligomers up to the decamer (*1*), these data show first evidence for the degradation of these so-called procyanidins during the alkalization process. In contrast, the puckering astringent fractions 4, 6, and 8 were evaluated with identical TD factors in both cocoa samples (**Table 2**). As the tastes of these fractions have been recently shown to be due to a family of *N*-phenylpropenoyl-L-amino acids (*1*, *2*), the present study demonstrates first evidence that these cinnamoyl derivatives are relatively stable throughout the alkalization process. In fractions 8−10, exhibiting a velvety astringent taste, LC-MS/MS analysis confirmed our previous findings (*1*) that quercetin, naringenin, luteolin, and apigenin glycopyranosides are present in these fractions (**Table 2**).

The only fraction that showed a higher TD factor for the velvety astringent taste after alkalization was GPC fraction 10. An LC-MS full scan run revealed several compounds exhibiting a molecular mass of 452 Da. Isolation of these peaks by means of semipreparative RP-HPLC, followed by sensory analysis, revealed that these compounds exhibited a smooth and velvety

Table 2. Yield, Taste Quality, and Taste Dilution (TD) Factor of GPC Fractions Obtained from Solvent Fraction III/IV Isolated from Nonalkalized (NC) and Alkalized Cocoa (AC) Powder

fraction[a]	taste quality[b]	TD factor in NC	TD factor in AC	bitter and astringent compounds identified[c]
1	astringent	8	<1	
2	astringent	4	32	
3	sour	32	64	organic acids, sugars
	sweet	4		
4	puckering, astringent, sour	128	128	*N*-phenylpropenoyl amino acids, organic acids
5	bitter	32	64	caffeine, theobromine
6	puckering, astringent	64	64	*N*-phenylpropenoyl amino acids
7	puckering, astringent	32	32	*N*-phenylpropenoyl amino acids
8	velvety, astringent	32	64	apigenin, luteolin, kaempferol, naringenin, and quercetin glycosides
9	velvety, astringent	32	16	
10	velvety, astringent	64	128	
11	puckering, astringent	256	16	epicatechin, catechin
12	puckering, astringent	512	64	epicatechin, catechin
13	puckering, astringent	256	64	procyanidin B2
14	puckering, astringent	512	128	procyanidin C1
15	puckering, astringent	256	128	[epicatechin-(4β→8)]3-epicatechin
16	puckering, astringent	128	128	[epicatechin-(4β→8)]4-epicatechin, polymers

[a] Number of GPC fraction referring to **Figure 1**. [b] Taste quality and TD factor were determined by using a half-mouth test. [c] Compounds were identified by means of HPLC-DAD and LC-MS/MS and were confirmed with reference compounds as reported recently (*1*).

astringent sensation. Electrospray ionization (ESI^-) MS/MS analysis of one representative compound revealed an $[M-1]^-$ ion with *m*/*z* 451 and a cleavage of 120 amu to give the fragment ion *m*/*z* 331 as expected for a catechin *C*-glycoside (**Figure 2**). Because the same compounds were found in significantly lower concentrations in the NC sample, the question arose whether these velvety mouth-coating compounds are formed nonenzymatically during the alkalization process.

Isolation and Structure Elucidation of Velvety Taste Compounds in GPC Fraction 10. To gain more detailed insight into the compounds imparting the velvety astringent sensation in GPC fractions 10 of the AC sample, solutions of (−)-epicatechin and glucose, known as the quantitatively predominant polyphenol and reducing carbohydrate, respectively, were treated in a newly developed "model alkalization process". To achieve this, binary mixtures of (−)-epicatechin and D-glucose in ratios of 1:1 to 1:10 were heated in an aqueous K_2CO_3 solution at 80 °C with stirring. After 10, 20, 40, and 60 min, the alkalization process was stopped by acidification with hydrochloric acid and, after membrane filtration, aliquots of the mixture were analyzed by means of RP-HPLC coupled to a diode array detector and a mass spectrometer, respectively. First, the model alkalization experiments revealed a significant epimerization of (−)-epicatechin into (−)-catechin, thus confirming earlier data reported in the literature (*9*). Second, a series of compounds exhibiting a molecular mass of 452 Da was detectable. The UV−vis and MS spectra as well as the retention times of these compounds fitted well with those isolated from GPC fraction 10 of the alkalized cocoa. Among the model alkalization experiments, a molar ratio of 1:10 of (−)-epicatechin to D-glucose and a heating time of 10 min at 80 °C were found as the conditions showing the most efficient conversion of (−)-epicatechin into these reaction products.

To speed the isolation and full spectroscopic structure determination of the key taste compounds in GPC fraction 10, the alkali-catalyzed (−)-epicatechin/D-glucose reaction was repeated on a preparative scale. The processed mixture was concentrated under reduced pressure and then fractionated by preparative column chromatography on RP-18 material. Monitoring the effluent at 270 nm, the effluents of the individual peaks detected were collected and freed from solvent in a vacuum to give 10 subfractions (f1−f10). Final purification of the individual subfractions was performed by preparative RP-HPLC, thus giving rise to highly pure compounds that were analyzed by means of UV−vis, LC-MS, and NMR spectroscopy.

The velvety astringent compound **1** isolated from fraction f4 was obtained as a white amorphous powder, showed the typical absorption maxima expected for catechin and its epimeric epicatechin, respectively, and showed an $[M-1]^-$ ion with *m*/*z* 451 as well as the fragment ion with *m*/*z* 331 by ESI^- MS/MS as shown in **Figure 2**. High-resolution LC-MS analysis confirmed the target compound to have the molecular formula $C_{21}H_{24}O_{11}$. The ^1H NMR spectrum of compound **1** showed an aromatic singlet for H−C(6) at 6.02 ppm and three aromatic protons resonating at 6.71, 6.77, and 6.85 ppm showing an ABX coupling system. In addition, the four aliphatic protons H−C(4a), H−C(4b), H−C(3), and H−C(2) were observable at 2.57, 2.78, 4.01, and 4.77 ppm, coupling with each other and suggesting a flavan-3-ol aglycon. Besides the signals of the flavan-3-ol aglycone, the ^1H NMR spectrum also exhibited seven aliphatic protons resonating at 3.36−3.42 ppm [H−C(5″, 3″, 4″)], 3.71 ppm [H−C(6a″)], 3.87 ppm [H−C(6b″)], 4.03 ppm [H−C(2″)], and 4.85 ppm [H−C(1″)] as expected for a hexose unit.

Considering all of the coupling constants of the sugar moiety in the molecule and, in particular, the coupling constant of $J = 9.2$ Hz observed for the protons H−C(1″) and H−C(2″) and comparing these values with the H−C(1)/H−C(2) coupling constants reported for β-D-glucopyranosides and α-D-glucopyranosides (*10*, *11*), the sugar moiety was with certainty identified as the β-D-glucopyranose.

A comparison of the ^{13}C NMR spectrum, in which 21 signals appeared, with the results of the DEPT-135 experiment showing 13 signals, revealed 8 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) optimized for $^1J_{C,H}$ coupling constants, respectively. Additionally, the HMBC experiment revealed a correlation between the sugar proton H−C(1″) resonating at 4.85 ppm and neighboring carbon atoms C(7), C(8), and, in particular, C(8a), thus

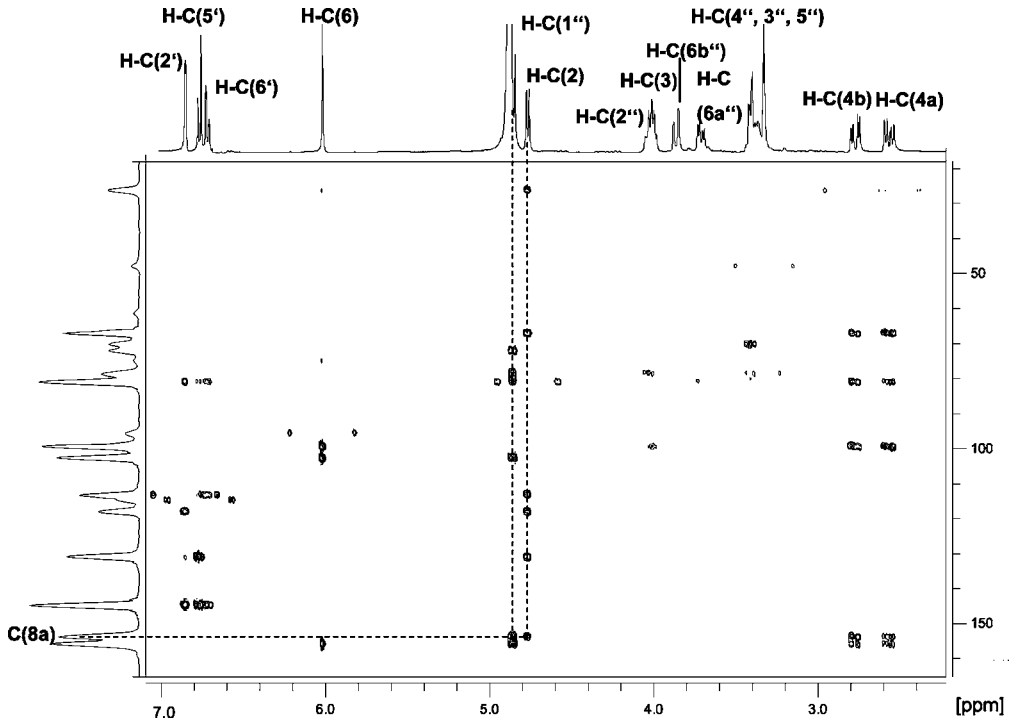

Figure 4. HMBC spectrum (400 MHz, d_3-MeOD) of (–)-catechin-8-*C*-β-D-glucopyranoside (**1**).

demonstrating clearly the intramolecular 8-C-linkage of the β-D-glucopyranose to its aglycone (**Figure 4**). The typical chemical shift of the carbon atom C(1′) at 75.5 ppm confirmed the C-linkage of the sugar part.

To clarify the configuration of the carbon atoms C(2) and C(3) present in the aglycone of compound **1**, in the following (−)-epicatechin and (+)-catechin were investigated by NMR spectroscopy and the unequivocal proton and carbon assignments were successfully achieved by means of ^{1}H, g-COSY, ^{13}C, DEPT-135, HMQC, and HMBC. On comparison of the chemical shifts and coupling constants of the protons H−C(4a,b), H−C(3), and H−C(2), the diastereomers (−)-epicatechin and (+)-catechin were clearly distinguishable; for example, the chemical shift (2.75 ppm) and the vicinal coupling constant (2.8 Hz) of the proton H−C(4a) of (−)-epicatechin are significantly different from those of the corresponding proton H−C(4a) of (+)-catechin (2.52 ppm, 8.2 Hz). The chemical shifts of the protons H−C(3) and H−C(2) of (−)-epicatechin at 4.20 and 4.82 were significantly different from those of the corresponding protons H−C(3) and H−C(2) of (+)-catechin resonating at 4.00 and 4.58 ppm. It is interesting to note that the sequence of increasing chemical shifts of the protons H−C(5′), H−C(6′), and H−C(2′) of (−)-epicatechin is substantially different from that of the (+)-catechin structure showing increasing chemical shifts from H−C(6′) and H−C(5′) to H−C(2′). To complete these investigations, (−)-catechin and (+)-epicatechin, isolated from alkalization model experiments performed with (−)-epicatechin and (+)-catechin, respectively, were analyzed by means of NMR spectroscopy. The same chemical shifts and coupling constants found for the diastereomers (−)-epicatechin and (+)-catechin were found for the corresponding enantiomeric (−)-catechin and (+)-epicatechin. By comparison of the chemical shifts and the coupling constants of the protons H−C(4a), H−C(3), and 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 and (+)-catechin with the isolated *C*-glycoside **1**, its configuration at carbon atom C(2) and at C(3) could be deduced. The coupling constants and the chemical shifts of the protons H−C(2), H−C(3), H−C(4a,b), 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 **1** isolated from fraction f4.

Taking all of these spectroscopic data into consideration, the structure of the velvety astringent compound **1** isolated from fraction f4 could be unequivocally identified as the previously not reported (−)-catechin-8-*C*-β-D-glucopyranoside (**Figure 3**). By means of HPLC degustation, HPLC-MS/MS, and HPLC-DAD, compound **1** was confirmed as an important taste compound in GPC fraction 10, which was evaluated with a high TD factor for its astringent and mouth-coating oral sensation (**Figure 1**).

Using the same analytical strategy, LC-MS/MS studies and 1D- and 2D-NMR experiments led to the identification of compound **2** detected in fraction f4 as (−)-catechin-6-*C*,8-*C*-β-D-diglucopyranoside, compound **3** detected in fraction f5 as (−)-catechin-6-*C*-β-D-glucopyranoside, and compounds **4**−**6** detected in fraction f9 as (−)-epicatechin-8-*C*-β-D-glucopyranoside, (−)-epicatechin-6-*C*,8-*C*-β-D-diglucopyranoside, and (−)-epicatechin-6-*C*-β-D-glucopyranoside (**Figure 3**), respectively.

Because LC-MS/MS experiments with cocoa sample AC revealed, besides compounds **1**−**6**, additional compounds with typical mass transitions for epi- or catechin-*C*-hexopyranosides in cocoa, additional alkalization model experiments were performed with D-galactose as described above for D-glucose. From this model experiment, the corresponding flavan-3-ol galactopyranosides could be isolated and identified as (−)-catechin-8-*C*-β-D-galactopyranoside (**7**), (−)-catechin-6-*C*,8-*C*-β-D-digalactopyranoside (**8**), (−)-catechin-6-*C*-β-D-galactopyranoside (**9**), (−)-epicatechin-6-*C*,8-*C*-β-D-digalactopyranoside (**10**), and (−)-epicatechin-8-*C*-β-D-glucopyranoside (**11**) (**Figure 3**).

As a final proof of the stereochemistry of all these taste compounds, CD spectroscopic measurements were performed with the commercially available reference isomers (−)-epicatechin, (−)-catechin, and (+)-catechin as well as the isolated

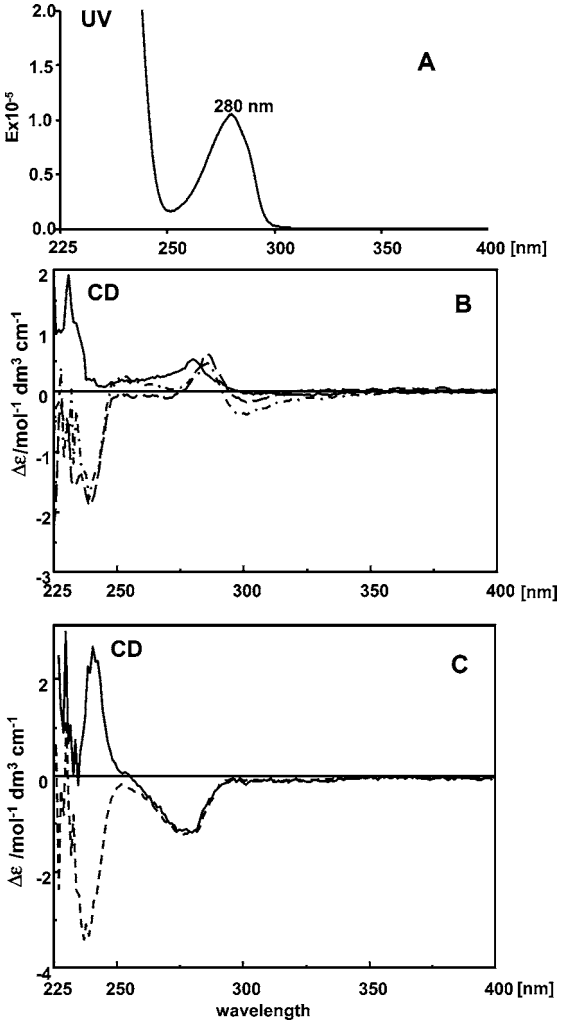

Figure 5. (**A**) UV–vis spectrum; (**B**) CD spectra of (–)-catechin (—), (–)-catechin-6-*C*-*β*-D-galactopyranoside (**9**; - - -) and (–)-catechin-6-*C*,8-*C*-*β*-D-diglucopyranoside (**2**; - – -); and (**C**) CD spectra of (–)-epicatechin (—) and (–)-epicatechin-8-*C*-*β*-D-glucopyranoside (**4**;- - -).

(−)-catechin and the isolated *C*-glycosides **2**, **4**, and **9** (**Figure 5**). The CD spectra of (−)-epicatechin and (+)-catechin were well in line with literature data (*12*). The data obtained clearly demonstrated that the *C*-glycosides **2** and **9** isolated from fractions f4 and f5 showed a positive CD band at about 280 nm, similar to that of (−)-catechin and *C*-glycoside **4** isolated from fractions f9, which showed a negative CD band at about 280 nm similar to that of (−)-epicatechin.

Taking all of these data into consideration, the reaction pathways leading to the formation of the flavan-3-ol-*C*-glycosides by a previously not reported nonenzymatic *C*-glycosylation of flavan-3-ols are proposed in **Figure 6**. Under alkaline conditions, (−)-epicatechin (1) is epimerized to (−)-catechin (2), thus confirming earlier reports (*9*). As exemplified for the formation of the *C*-glycoside **6** in **Figure 6**, the carbonyl atom of the open-chain form of the hexose (3) favorably formed under alkaline conditions (*13*) is attacked by the A-ring of the (−)-epicatechin at the 6-position to give the intermediate adduct 4. Upon water elimination, the unsaturated carbonyl 5 is formed, which is cyclized to give the target glycoside **6** by means of a Michael-type addition. Similarly, the reaction of the (−)-epicatechin at the 8-position leads to the formation of glycoside **4**, whereas the involvement of (−)-catechin instead of (−)-epicatechin results in the corresponding diastereomeric glycosides **1** and **3** (**Figure 6**). On the basis of this type of nonenzymatic glycosylation chemistry, the corresponding bis-glycosylated flavan-3-ols **2**, **5**, **8**, and **10** are formed by an additional glycosylation of a mono-*C*-hexoside involving glucose or galactose.

To the best of our knowledge, compounds **1**−**3**, **5**, and **7**−**10** have not previously been reported in the literature. Although compound **4** has been reported earlier in Pu-er tea (*14*) and, together with compound **6**, was isolated from cassia bark (*Cinnamomum cassia* Blume) (*15*) and compound **11** has been earlier reported in cocoa liquor (*16*), the velvety astringent taste activity of (−)-epicatechin-*C*-glycopyranosides as well as their potential formation by a nonenzymatic *C*-glycosylation of polyphenols has not been previously described.

Identification of Flavan-3-ol-*C*-glycosides 1−11 in Nonalkalized and Alkalized Cocoa Powder. To verify the occurrence of the flavan-3-ol-*C*-glycosides **1**−**11** in cocoa powder, an aqueous acetone extract isolated from cocoa powder was analyzed by RP-HPLC coupled to an LC-MS/MS instrument running in the negative electrospray ionization mode as shown in **Figure 7**. By means of the MRM mode, the mass transition *m*/*z* 451.2 → 331.0 was recorded for the flavan-3-ol-*C*-glycosides **1**, **3**, **4**, **6**, **7**, **9**, and **11** and the mass transition *m*/*z* 613.1 → 373.0 was monitored for the flavan-3-ol-*C*-glycosides **2**, **5**, **8**, and **10**.

LC-MS/MS analysis of the cocoa samples demonstrated for the first time that besides the previously reported (−)-epicatechin-8-*C*-galactopyranoside (**11**) (*16*), the taste compounds **1**−**10** are present in cocoa. These data were further confirmed by comparing the sensory quality, the mass spectra, and retention times (RP-HPLC) with those obtained for the corresponding reference compounds and, finally, by cochromatography. In addition, recording the mass transition *m*/*z* 451.2 → 331.0 and comparing the peak areas obtained for (−)-catechin- and (−)-epicatechin-*C*-hexopyranosides in an aqueous acetone extract isolated from nonalkalized (**Figure 7A**) and alkalized (**Figure 7B**) cocoa powder clearly demonstrated an increase of (−)-catechin-*C*-glycopyranosides (**1**, **3**, **7**, and **9**) upon alkalization. In addition, alkalization induced a decrease of (−)-epicatechin-*C*-glycopyranosides (**4**, **6**, and **11**), which can be explained by an alkali-catalyzed epimerization of (−)-epicatechin-*C*-glycosides into the corresponding (−)-catechin-*C*-glycosides during the alkalization process as already observed for the alkali-treated aglycones as described above. To investigate this observation more precisely, the development of a stable isotope dilution analysis for the accurate quantification of these taste compounds is currently under progress.

Sensory Activity of Flavan-3-ol-*C*-glycosides. Prior to sensory analysis, the purity of all compounds was checked by HPLC-MS as well as ^{1}H NMR spectroscopy. To study the sensory activity of the flavan-3-ol-*C*-glycosides **1**−**11**, the human sensory recognition thresholds were determined in bottled water (pH 6.0) using the half-mouth test (*1*, *7*) (**Table 3**).

Compared to the flavan-3-ols (−)-epicatechin and (−)-catechin exhibiting a puckering astringent as well as bitter taste at threshold concentration levels between 600 and 1000 *μ*mol/L, the flavan-3-ol-*C*-glycosides were found to induce a smooth astringent and velvety mouth-coating sensation at very low threshold concentrations ranging from 1.1 to 99.5 *μ*mol/L without exhibiting any bitter taste (**Table 3**). In particular, the threshold concentrations of 1.1 and 1.3 *μ*mol/L determined for (−)-catechin-6-*C*,8-*C*-*β*-D-digalactopyranoside and (−)-catechin-6-*C*,8-*C*-*β*-D-diglucopyranoside were found to be extraordinarily low, thus confirming our recent findings that the sugar moiety

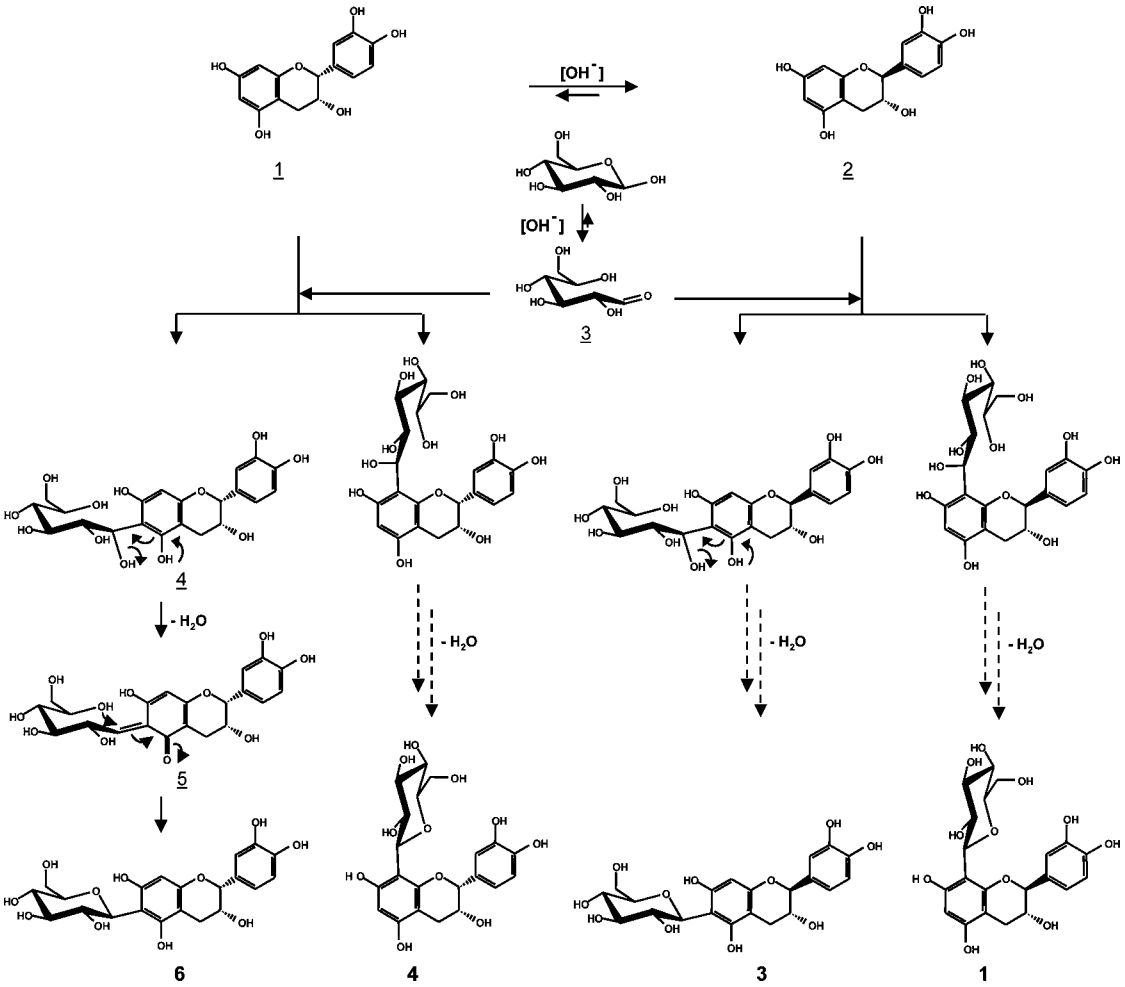

Figure 6. Proposed reaction pathway for the nonenzymatic glycosylation of (–)-epicatechin and (–)-catechin leading to the formation of (–)-epicatechin-6-*C*-β-D-glucopyranoside (**6**), (–)-epicatechin-8-*C*-β-D-glucopyranoside (**4**), (–)-catechin-6-*C*-β-D-glucopyranoside (**3**), and (–)-catechin-6-*C*-β-D-glucopyranoside (**1**).

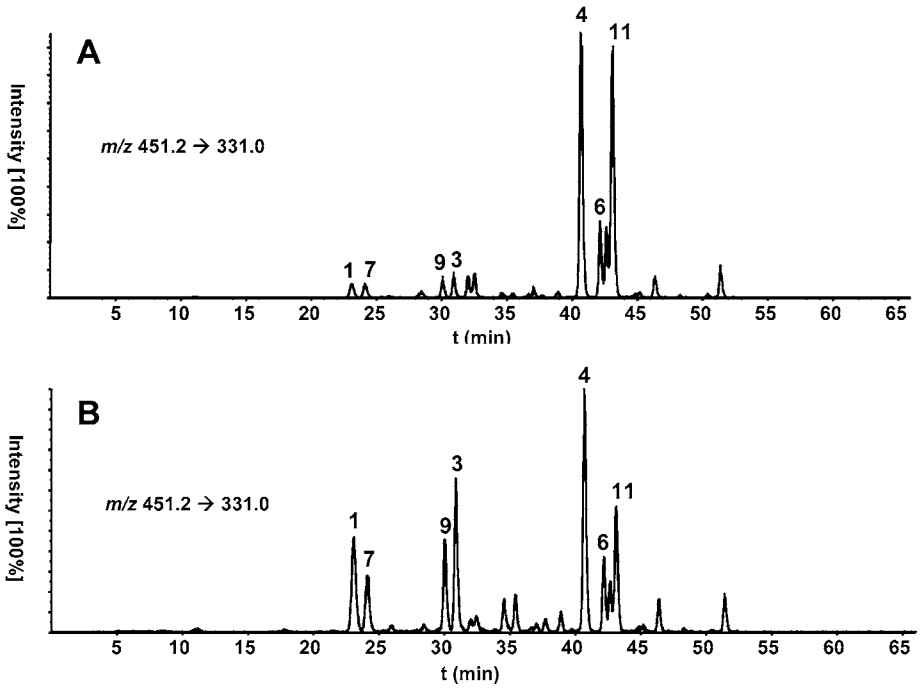

Figure 7. LC-MS/MS analysis of (**A**) nonalkalized and (**B**) alkalized cocoa powder using the multiple reaction monitoring (MRM) mode.

of glycosylated phenols is a key driver for their sensory activity (*1*, *7*). In comparison, the monosubstituted flavan-3-ol-*C*-glycosides showed somewhat higher threshold concentrations ranging from 3.1 to 99.5 μmol/L.

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

compound	threshold concn[a] (μmol/L) for astringency	bitterness
(–)-epicatechin	800.0[b]	800.0
(–)-catechin	600.0[b]	1000.0
(–)-catechin-6-*C*-β-D-galactopyranoside (**9**)	99.5[c]	nd[d]
(–)-catechin-6-*C*-β-D-glucopyranoside (**3**)	63.6[c]	nd
(–)-catechin-8-*C*-β-D-galactopyranoside (**7**)	49.8[c]	nd
(–)-catechin-8-*C*-β-D-glucopyranoside (**1**)	16.2[c]	nd
(–)-epicatechin-8-*C*-β-D-galactopyranoside (**11**)	15.2[c]	nd
(–)-epicatechin-6-*C*-β-D-glucopyranoside (**6**)	10.0[c]	nd
(–)-epicatechin-6-*C*,8-*C*-β-D-digalactopyranoside (**10**)	5.1[c]	nd
(–)-epicatechin-8-*C*-β-D-glucopyranoside (**4**)	3.1[c]	nd
(–)-epicatechin-6-*C*,8-*C*-β-D-diglucopyranoside (**5**)	2.5[c]	nd
(–)-catechin-6-*C*,8-*C*-β-D-digalactopyranoside (**8**)	1.3[c]	nd
(–)-catechin-6-*C*,8-*C*-β-D-diglucopyranoside (**2**)	1.1[c]	nd

[a] Taste threshold concentrations were determined by means of the half-tongue test (*7*). [b] The astringent sensation was described as rough and extremely puckering. [c] The astringent sensation was described as a very smooth and velvety mouthcoating. [d] Not detectable.

Besides the structure of the sugar, the aglycone moiety has a significant influence on the perception of astringency. Comparison of (−)-epicatechin-8-*C*-β-D-glucopyranoside (**4**) and (−)-epicatechin-6-*C*-β-D-glucopyranoside (**6**) with the corresponding 8-*C*-β-D-glucopyranoside (**1**) and 6-*C*-β-D-glucopyranoside (**3**) of (−)-catechin showed that the aglycone strongly influences the taste intensity of these compounds; for example, (−)-catechin-8-*C*-β-D-glucopyranoside (**1**) and (−)-catechin-6-*C*-β-D-glucopyranoside (**3**) possess threshold concentrations of 16.2 and 63.6 μmol/L, respectively, which are 5.3 and 6.4 times above the threshold concentration determined for the corresponding (−)-epicatechin derivative.

In addition to the sugar species and the aglycone, the position of the individual monosaccharides at the aglycone also seems to have an influence on the sensory activity of the flavan-3-ol-*C*-glycosides; for example, all compounds bearing the glucose or galactose linked at position 6 to the aglycone were evaluated with relatively high thresholds of 99.5, 63.6, and 10.0 μmol/L, respectively, whereas (−)-catechin-8-*C*-β-D-galactopyranoside (**7**), (−)-catechin-8-*C*-β-D-glucopyranoside (**1**), and (−)-epicatechin-8-*C*-β-D-glucopyranoside (**4**), respectively, all bearing the sugar moiety at position 8 next to the aglycone, showed significantly lower thresholds of 49.8, 16.2, and 3.1 μmol/L, respectively (**Table 3**).

The data obtained for the flavan-3-ol-*C*-glycosides clearly show that the sensory activity changes with variations in the stereochemistry of the flavan-3-ol moiety and the position of the linkage between aglycone and carbohydrate as well as in the glycosylation pattern, thereby illustrating that oral thresholds

Table 4. Influence of Flavan-3-ol-*C*-glycosides on the Taste Profile of a Cocoa Beverage[a]

additive[b]	PDD[c]	taste profile[d]
no additive (control)		typical cocoa-like bitterness
(–)-catechin–8-*C*-β-D-glcp (**1**)	6	softer, more pleasant, less bitter (++) than control
(–)-catechin–6-*C*-β-D-glcp (**3**)	6	softer, more pleasant, less bitter (++) than control
(–)-catechin–8-*C*-β-D-galp (**7**)	6	softer, more pleasant, less bitter (++) than control
(–)-catechin–6-*C*-β-D-galp (**9**)	6	softer, more pleasant, less bitter (++) than control
(–)-catechin–6,8-*C*-β-D-digalp (**8**)	8	very mild and pleasant, less bitter (+++) than control
(–)-catechin–6,8-*C*-β-D-diglcp (**2**)	8	very mild and pleasant, less bitter (+++) than control

[a] The cocoa beverage was freshly prepared from cocoa powder (25 g/L) and bottled water stirred for 15 min at 50 °C prior to use. [b] The individual *C*-glycoside (111 μmol/L) was used as additive. [c] Number of panelists detecting the difference from the control. Eight panelists were asked to compare the cocoa beverage containing no additive (control) with the cocoa beverage spiked with an individual flavan-3-ol-*C*-glycoside by means of a triangle test. [d] If the sample was detected correctly (cf. footnote *c*), the changes in taste and taste intensities were evaluated as being weak (+), medium strong (++), and strong (+++).

Table 5. Influence of the Polyphenol Structure on the Bitterness of an Aqueous Theobromine Solution

additive	PDD[a]	taste (intensity)[b]
no additive (control)		bitter (5.0)
(–)-catechin-8-*C*-β-D-glcp (**1**)		
2.2 μmol/L	1	softer, more pleasant, and less intense bitterness (4.5)
22.2 μmol/L	5	softer, more pleasant, and less intense bitterness (3.5)
222.2 μmol/L	8	very mild, pleasant, and weakly bitter (1.0)
(+)-catechin		
2.2 μmol/L	0	bitter (5.0)
22.2 μmol/L	4	more cocoa-like, but intensely bitter (5.0)
222.2 μmol/L	4	more cocoa-like, but rather bitter (4.0)
(–)-epicatechin		
2.2 μmol/L	0	bitter (5.0)
22.2 μmol/L	4	more cocoa-like, but intensely bitter (5.0)
222.2 μmol/L	4	more cocoa-like, but intensely bitter (4.5)
apigenin-8-*C*-β-D-glucopyranoside		
2.2 μmol/L	0	bitter (5.0)
22.2 μmol/L	0	bitter (5.0)
222.2 μmol/L	4	bitter (4.5)
apigenin–6-*C*-β-D-glucopyranoside		
2.2 μmol/L	0	bitter (5.0)
22.2 μmol/L	0	bitter (5.0)
222.2 μmol/L	4	bitter (4.5)

[a] Number of panelists detecting the difference from the control. Eight panelists were asked to compare a solution of theobromine (3 mmol/L) with the a solution of theobromine (3 mmol/L) spiked with different amounts of individual additives. [b] If the sample was detected correctly (cf. footnote *a*), the changes in taste and taste intensities were evaluated in comparison to the control.

of astringent compounds cannot be predicted from chemical structures but have to be investigated on the basis of systematic sensory studies with purified reference compounds.

Taste-Modifying Activity of Flavan-3-ol-*C*-glycosides. To directly evaluate the influence of purified flavan-3-ol-*C*-glycosides on the taste of cocoa powder, in the following, flavan-3-ol-*C*-glycosides were added to a cocoa beverage and aqueous theobromine solutions, respectively, and the taste profiles of these solutions were compared to those of the corresponding nonspiked samples as the control (**Tables 4** and **5**).

As summarized in **Table 4**, the addition of 111 μmol/L of one of the mono-*C*-glycosides **1**, **3**, **7**, or **9**, respectively, to the cocoa beverage was detected by six of eight panelists. Substitution of the mono-*C*-glycosides by the di-*C*-glycosides **2** and **8**, respectively, could be detected by all of the panelists. The individual panelists reported that the bitterness of the cocoa beverage was perceived as being significantly milder, softer, and more pleasant in the presence of the *C*-glycosides.

To answer the question of whether the taste-modifying activity of flavan-3-ol-*C*-glycoside is due to an interaction with the theobromine perception, an aqueous solution of theobromine (3 mmol/L) was spiked with increasing concentrations of (−)-catechin-8-*C*-β-D-glucopyranoside (**1**) (**Table 5**). Spiking a solution of theobromine (3 mmol/L) with an amount of 222 μmol of **1** was detected by all of the panelists and induced a significant decrease of the bitterness (5.0 → 1.0) of theobromine, thus indicating that the bitterness-mellowing effect of the glycosides is due to a partial bitterness suppression of theobromine. The addition of only 22 or 2.2 μmol of the glycoside could be perceived by just five or one of eight panelists.

To investigate the influence of the polyphenol structure on their modifying effect on theobromine bitter taste, the (−)-catechin-8-*C*-β-D-glucopyranoside (**1**) was substituted by (+)-catechin, (−)-epicatechin, apigenin-8-*C*-β-D-glucopyranoside, and apigenin-6-*C*-β-D-glucopyranoside, respectively. As given in **Table 5**, the addition of either (+)-catechin or (−)-epicatechin to the theobromine at a concentration of 222.2 μmol/L was detectable by just four of eight panelists, who described the taste difference as a more "cocoa-like bitterness" with somewhat lower bitter taste intensity (5.0 → 4.0). In comparison, apigenin-8-*C*-β-D-glucopyranoside and apigenin-6-*C*-β-D-glucopyranoside did not show any significant influence on the bitterness of the theobromine.

In summary, this is the first report demonstrating that alkalization of cocoa is inducing the nonenzymatic C-glycosylation of flavan-3-ols to give the newly identified flavan-3-ol-*C*-glycosides. Human sensory experiments revealed that these glycosides modify the bitter taste profile and decrease the bitter taste intensity of cocoa beverages as well as theobromine solutions, whereas the aglycones or other *C*-glycosides, such as apigenin-8-*C*-β-D-glucopyranoside and apigenin-6-*C*-β-D-glucopyranoside, did not show any significant activity. Aimed at gaining a more comprehensive understanding of the sensory contribution of the flavan-3-ol-*C*-glycosides to the typical velvety astringent and mild bitter taste of alkalized cocoa powder, the development of a stable isotope dilution analysis is currently under progress.

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