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Complex Flavonoids in Cocoa: Synthesis and Degradation by Intestinal Microbiota

Gordon van't Slot, Wera Mattern, Sebastian Rzeppa, Dagmar Grewe, and Hans-Ulrich Humpf*

Institute of Food Chemistry, University of Münster, Corrensstrasse 45, 48149 Münster, Germany

Rarely occurring flavan-3-ol derivatives such as *C*-glycosides can be generated during food processing, for example, by cocoa production. These astringent taste compounds may also exert interesting behavior toward microbial metabolism, as other *C*-glycosides have been shown to be quite stable. Oligomeric flavan-3-ols, the procyanidins, bear also a C−C bond between the main moieties and are suspected to resist microbial metabolism for a prolonged time compared to other flavonoids. This paper describes a semisynthetic approach for the generation of flavan-3-ol *C*-glycosides. Results of incubation experiments studying five flavan-3-ol *C*-glycosides bearing different sugars, linkage positions, and stereochemistries are presented as well as the behavior of di- and trimeric B-type procyanidins toward intestinal microbiota. Low molecular weight degradation products are considered as well as concentration−time courses of degraded and liberated compounds. All metabolic studies were performed with the well-proven pig cecum model.

KEYWORDS: Flavonoids; flavan-3-ol; *C*-glycoside; procyanidin; degradation; gastrointestinal tract; cocoa; microbiota; intestinal metabolism; pig cecum; bioavailability

INTRODUCTION

Flavonoids are important secondary plant metabolites with polyphenol structure. Most of them occur naturally as glycosides. There are two types of glycoside bonds: *O*- and *C*-glycosides. The *C*-glycosides bear a C−C bond, usually in position C6 or C8 (see **Figure 1**), between the aglycon and the sugar moiety (*1*). Whereas flavonoid *O*-glycosides are widely found in nature, the *C*-glycosides occur less frequently. *C*-Glycosylflavones have been identified, for example, in sugar cane, wheat bran, and dates (*2*−*4*). In rooibos tea the *C*-glucosidically linked dihydrochalcone aspalathin can be found and is extracted into the beverage with amounts around 1.2 mg/L (*5*). The flavan-3-ol derivatives (−)-epicatechin-8-*C*-glucopyranoside and (−)-epicatechin-6-*C*-*β*-D-glucopyranoside have been isolated from cinnamon or rhubarb (*6*, *7*). They also are produced during Dutch processing of cocoa and can be found in cocoa products (*8*).

Procyanidins consist of two or more units of flavan-3-ols. B-Type procyanidins are linked via a C4−C8 bond, but C4−C6 linkages also occur. A-Type procyanidins bear an additional ether bond between C2 and C7. Procyanidins are, besides other substances, responsible for the oral sensation of astringency and can be found in ripening fruits (apples, peaches, grapes, berries), beverages (tea, cocoa, wine), and chocolate (*9*).

Intestinal metabolism and absorption of flavonoids and their glycosides have been examined intensively. These compounds resist the acidic conditions in the stomach, but some hydrolysis may occur in the small intestine (*10*, *11*). The stability of procyanidins during gastrointestinal passage is high; there is no degradation in the stomach (*11*). In the small intestine also slight degradation seems to take place (*12*). To overcome the intestinal barrier the necessity of previous cleavage of the glycosides (*13*, *14*) is discussed as vividly as the possibility of an active transport of intact glycosides by sodium-dependent glucose transporter 1 (SGLT1) (*15*−*17*). The resorption of flavonoid *C*-glycosides is not well characterized until now, but most of the compounds reach the proximal colon (*16*, *18*). The absorption of procyanidins is also unclear and quantitatively even lower than the absorption of flavonoid monomers (*19*, *20*). Flavonoids that are not absorbed can be extensively metabolized into various low molecular weight aromatic compounds by the colonic microbiota (*21*−*23*). Bacterial metabolites absorbed in the colon can reach high plasma concentration and may also have physiological effects (*24*). However, knowledge of the microbial biotransformation of flavonoid *C*-glycosides in the colon is rather scarce (*25*, *26*). Thus, determining the intestinal metabolism of flavan-3-ol *C*-glycosides constitutes an important field of research toward a better understanding of the bioavailability and potential physiological effects of these substances.

In this paper, we report the nonenzymatic synthesis of *C*-glycosides from (+)-catechin and *β*-D-glucose. Furthermore, we present the results of incubation experiments of five different flavan-3-ol *C*-glycosides and three procyanidins of the B-type in the pig cecum model. These substances are (−)-catechin-6-*C*-glucoside, -8-*C*-glucoside, and -8-*C*-galactoside as well as (+)-epicatechin-6-*C*-glucoside and -6,8-*C*-diglucoside. B-Type procyanidins tested include procyanidin B2, its isomer B5, and the trimer C1 (see **Figure 1**).

The pig cecum model, developed and constantly improved in our group, showed its applicability several times and has been described in detail in the literature (*22*, *27*).

*Corresponding author (phone +49 251 83 33 391; fax +49 251 83 33 396; e-mail humpf@uni-muenster.de).

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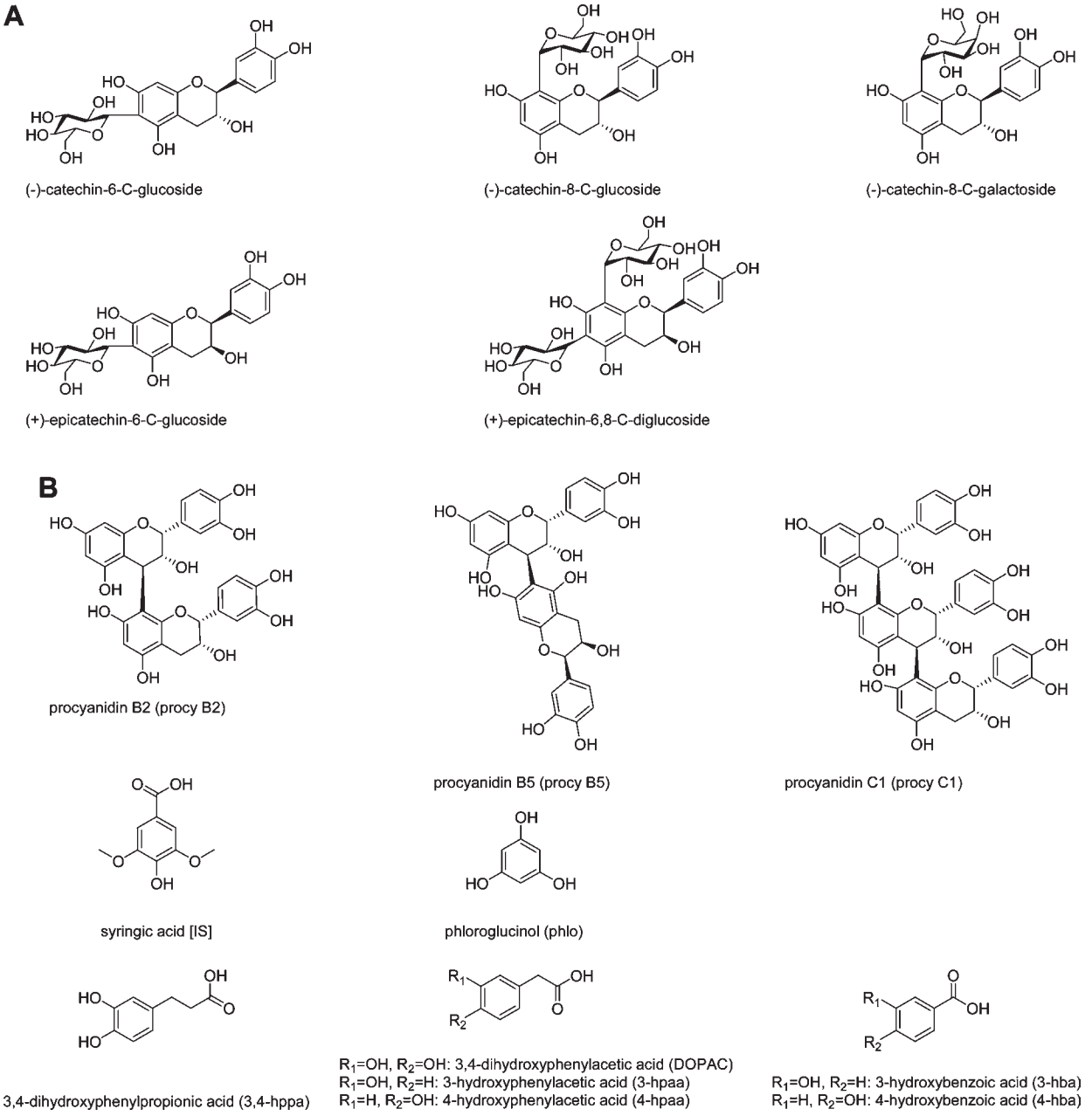

Figure 1. Chemical structures of (**A**) the used flavan-3-ol *C*-glycosides and (**B**) the used procyandins and occurring degradation products.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased in p.a. quality. 3,4-Dihydroxyphenylpropionic acid (3,4-hppa) as well as 3- and 4-hydroxyphenylacetic acid (3-/4-hpaa) were purchased from Sigma-Aldrich (Steinheim, Germany). (+)-Catechin, (−)-epicatechin, and glucose were provided from Roth (Karlsruhe, Germany). *N*,*O*-Bis(trimethylsilyl)acetamide (BSA) was obtained from Fluka (Buchs, Switzerland). 3,4-Dihydroxyphenylacetic acid (DOPAC) was provided from Roth. Phloroglucinol (phlo) and 3- and 4-hydroxybenzoic acid (3-/4-hba) were ordered from Merck (Darmstadt, Germany). (−)-Catechin-6-*C*-glucoside, (−)-catechin-8-*C*-glucoside, and (−)-catechin-8-*C*-galactoside were kindly provided by Dr. Timo Stark (Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich). Procyanidin B2 (procy B2) was a donation of Prof. Dr. A. Hensel (Institute for Pharmaceutical Biology and Phytochemistry, University of Muenster).

All other chemicals were obtained, in p.a. quality, from Merck and Sigma-Aldrich. The water was demineralized by a Milli-Q Gradient A10-system (Millipore, Schwalbach, Germany).

Methods. *Synthesis of Flavan-3-ol C-Glucosides.* The flavan-3-ol *C*-glucosides were synthesized as described in the literature (*8*). Briefly (+)-catechin (2 mmol) was mixed with D-glucose (20 mmol), K_2CO_3 (10.42 mmol), and water (25 mL). This reaction mixture was heated, and a temperature of 80 °C was held for 8 min. After an alkalization process, the mixture was adjusted to pH 5.0 under cooling to stop the reaction. The concentrated batch was fractionated via RP-18 column chromatography (LiChroprep 25−40 μm (Merck)) and preparative HPLC. The system consisted of two HPLC pumps, Jasco PU 2087 (Jasco, Gross-Umstadt, Germany), and a Jasco PU 2075 UV detector using a preparative RP-18 column, Varian Microsorb 100-5 C18, 150 × 21.2 mm i.d., 5 μm (Varian, Darmstadt, Germany). After removal of the solvents under vacuum and freeze-drying, three compounds, (−)-catechin-6-*C*-glucoside, -8-*C*-glucoside, and -6,8-*C*-diglucoside, were yielded as white, amorphous powders in high purities of >99%. The mass spectrometric and ^{1}H and ^{13}C NMR data, as well as circular dichroism spectra, are in accordance with the literature (*9*).

Isolation of Procyanidins. Procyanidins B5 and C1 (see **Figure 1**) were isolated from cocoa. The detailed method will be published elsewhere. In brief, the ground and defatted cocoa beans were extracted with acetone/water (7:3, v/v). After removal of acetone, the aqueous residue was extracted by ethyl acetate. The ethyl acetate extract was macerated with methanol to remove theobromine. This extract was further separated by

column chromatography with Sephadex LH-20 (GE Healthcare, Freiburg, Germany) and ethanol as eluent. The fraction containing procyanidins B5 and C1 was further purified by column chromatography with MCI CHP20P (Mitsubishi Chemical Corp., Tokyo, Japan) and a water/methanol gradient. The mass spectrometric and ^{1}H and ^{13}C NMR data, as well as circular dichroism spectra, are in accordance with the literature (*28*−*30*). The purities were 99.0% for procyanidin B5 and 97.2% for procyanidin C1. This was determined with an analytical Eclipse XDB-C18 column 150 × 4.6 mm i.d., 5 μm; Agilent, Waldbronn, Germany) using a binary gradient generated by two Shimadzu LC-20AT HPLC pumps (Shimadzu, Duisburg, Germany) with methanol as solvent A and 1% (v/v) formic acid as solvent B after degassing with a Shimadzu DGU-20A3. For separation, the following gradient at room temperature was used: 0 min, 85% (v/v) solvent B; 35 min, 60% (v/v) solvent B; 45 min, 0% (v/v) solvent B; 55 min, 0% (v/v) solvent B, 55.5 min, 85% (v/v) solvent B, equilibrating the column for a further 7 min. The flow rate was 1 mL/min. For injection (10 μL), a Shimadzu SIL-20A autosampler was used. A Shimadzu UV−vis SPD-20AV detector was applied as detector monitoring the wavelength 280 nm, followed by an Shimadzu ELSD-LT low-temperature evaporative light scattering detector at 40 °C with a nitrogen stream of 2.5 bar. Acquisition of the data was carried out with Shimadzu LC-Solution software 1.21 SP1.

Stock Solutions. Stock solutions (1 mM) of the analytes were prepared in methanol.

Preparation of Inoculum. All conditions have already been described in ref *22*.

Sample Preparation and Analysis

(*a*) *HPLC-DAD and -FLD Analysis*. The frozen samples were thawed quickly in a water bath. The samples were diluted immediately with an equal amount of methanol/hydrochloric acid (99:1 (v/v) with or without 2% (w/v) sodium dodecyl sulfate (SDS) to prevent any further microbial degradation and to extract the analytes. After 15 min of ultrasonic treatment, the samples were centrifuged at 12000*g* for 15 min. Aliquots of the supernatant were used for HPLC analysis. The compounds were separated on an analytical Halo Fused Core C18 column (50 × 2.1 mm i.d., 2.7 μm; Advanced Materials Technology, Wilmington, DE) using a binary gradient generated by an Agilent/HP 1100 low-pressure gradient HPLC pump (G1311A) (Agilent/HP, Böblingen, Germany) with methanol as solvent A and 0.1% (v/v) formic acid as solvent B. For separation the following gradient at room temperature was used: 0 min, 95% (v/v) solvent B; 10 min, 0% (v/v) solvent B; 12 min, 0% (v/v) solvent B; 12.50 min, 95% (v/v) solvent B, equilibrating the column for a further 3.5 min. The flow rate was 0.4 mL/min. For injection (5 μL), an Agilent/HP 1100 autosampler (G1329A) was used. An Agilent/HP 1100 diode array detector (G1315A) was applied as detector monitoring the wavelength range of 200−800 nm, followed by an Agilent/HP 1100 fluorescence detector (G1321A), if fluorescence occurred. Acquisition of the data was carried out with ChemStation software A.08.03 (Agilent/HP). Identification of the compounds was achieved by comparison of UV−vis spectra and retention times with authentic references. Concentrations were calculated using calibration curves ranging from 0 to 150 μM for all substances. The calibration curves were linear in this range for all compounds with correlation coefficients ranging from 0.9937 to 0.9999. The recovery was determined by spiking blank cecal samples with the compounds under study followed by the extraction method described above. The recovery ranged from 86 to 104% (mol/mol). Each sample was injected at least three times. The following wavelengths were monitored for quantitative analysis: 280 and 360 nm for all flavan-3-ol *C*-glycosides. For possibly occurring deglycosylated flavan-3-ols the emission was measured at 330 nm after excitation at 290 nm, and for procyanidins the emission was measured at 310 nm after excitation at 270 nm.

(*b*) *GC-MS Analysis*. See preparation of the samples in ref *22*.

(*c*) *High-Resolution Fourier Transformation Mass Spectrometry*. A flow of 10 μL/min was directly injected to a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) in negative electrospray ionization mode (ESI^-). Experiments in collision-induced dissociation mode (CID) with fragmentation energy values ranging from 0 to 35% were performed for structural elucidation via fragmentation into pseudomolecular ions. Further adjustments were as follows: capillary voltage, −25.00 V; tube lens voltage, 138.71 V; AGC target setting full MS, 2.0×10^5; multipole 00 offset, 3 V; lens 0 voltage, 8.5 V; multipole 0 offset, 5.5 V; lens 1 voltage, 19.0 V; gate lens voltage, 30.0 V; multipole 1 offset, 8.5 V; front lens, 5.7 V; resolution, unit. Installed software was Xcalibur 2.0.7 SP1 (Thermo Fisher Scientific).

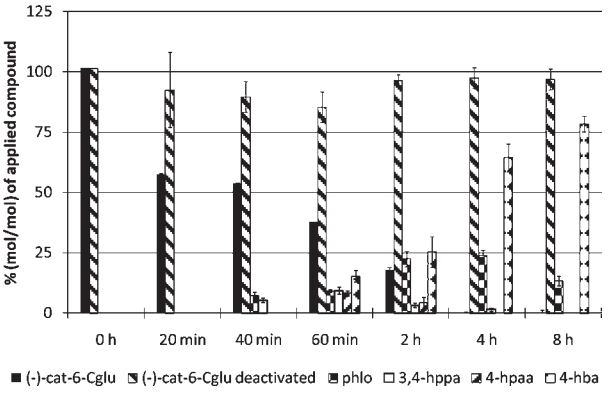

Figure 2. Relative proportions (% (mol/mol)) of (−)-catechin-6-*C*-glucoside ((−)-cat-6-Cglu) and its microbial metabolites phloroglucinol (phlo), 3,4-dihydroxyphenylpropionic acid (3,4-hppa), 4-hydroxyphenylacetic acid (4-hpaa), and 4-hydroxybenzoic acid (4-hba) in active cecal suspension in reference to that in sterilized control samples ((−)-cat-6-Cglu deactivated) (n = 6, mean ± SD; measured by HPLC-UVD and GC-MS).

(*d*) *Statistics*. Student's *t* test was used to test if the amount of applied compounds was significantly changed in time, if production of hydroxylated phenylcarboxylic acids took part, and if there were significant differences in recovery after extraction. A probability below 0.05 ($p < 0.05$) was considered to be significant. Error bars are depicted as mean ± SD. Statistical analysis was carried out with Excel 2003 (Microsoft Corp., Redmond, WA) and OriginPro 7G (Microcal Software, Northampton, MA).

RESULTS

Synthesis of (+)-Epicatechin *C*-Glycosides. The synthesis of (+)-epicatechin *C*-glycosides was performed on the basis of an alkalization process known as Dutch processing (*31*). For this (+)-catechin and D-glucose were heated with aqueous K_2CO_3 according to ref *8*. During Dutch processing an epimerization occurs and (+)-epicatechin is generated from (+)-catechin. This phenomenon is caused by the epimerization of the stereocenter at position C3. Whereas the linkage between the C- and B-rings (see **Figure 1**) is not influenced, the hydroxy group in position C3 is epimerized (*47*, *48*). For this reason (+)-catechin-6-*C*-glucoside and -8-*C*-glucoside were found only in very low amounts and have not been collected (data not shown). Besides this, three glucosides of epimerized flavan-3-ol (+)-epicatechin were formed as main products. (+)-Epicatechin-8-*C*-glucoside, -6-*C*-glucoside, and -6,8-*C*-diglucoside were isolated by preparative HPLC.

Incubation of (−)-Catechin *C*-Glycosides. (−)-*Catechin-8-C-glycosides*. During the incubation time of 8 h the detected amount of (−)-catechin-8-*C*-glucoside was 96 ± 2% (mol/mol) of the applied compound. For the deactivated control samples recovery was slightly higher (data not shown). Therefore, it can be concluded that (−)-catechin-8-*C*-glucoside is stable under these conditions and not degraded by the cecal microbiota.

For (−)-catechin-8-*C*-galactoside the results were the same. The concentration did not lower significantly ($p < 0.05$) during the incubation time of 8 h. Measured concentrations remained relatively constant at 97 ± 3 μM for the cecal samples and 105 ± 4 μM for the deactivated control samples (data not shown). These results also show that (−)-catechin-8-*C*-galactoside resists microbial decomposition.

(−)-*Catechin-6-C-glucoside*. The concentration−time course for (−)-catechin-6-*C*-glucoside ((−)-cat-6-Cglu) differs from the two others described above, as can be seen from **Figure 2**. Within an incubation time of 20 min 43 ± 1% (mol/mol) of the applied

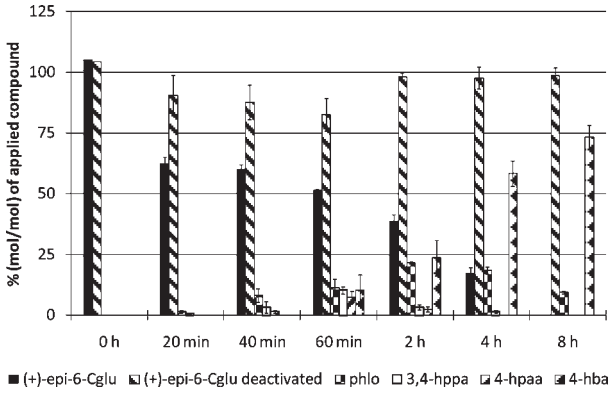

Figure 3. Relative proportions (% (mol/mol)) of (+)-epicatechin-6-*C*-glucoside ((+)-epi-6-Cglu) and its microbial metabolites phloroglucinol (phlo), 3,4-dihydroxyphenylpropionic acid (3,4-hppa), 4-hydroxyphenylacetic acid (4-hpaa), and 4-hydroxybenzoic acid (4-hba) in active cecal suspension in reference to that in sterilized control samples ((+)-epi-6-Cglu deactivated) (n = 6, mean ± SD; measured by HPLC-UVD and GC-MS).

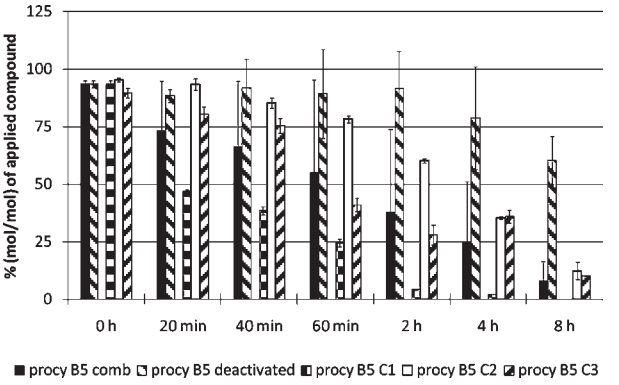

Figure 4. Relative proportions (% (mol/mol)) of procyanidin B5 in active cecal suspension (procy B5 active combined (n = 6)) in reference to that in sterilized inoculum (procy B5 sterilized (n = 6)) and the single incubation experiments in three different ceca (C1−C3) (procy B2 C1−3 (mean ± SD; measured by HPLC-UVD/-FLD and GC-MS).

compound was degraded. Within the next 40 min an additional 6% (mol/mol) was metabolized. At a time point between 2 and 4 h the compound is totally degraded by intestinal microorganisms. Occurring metabolites were, on the one hand, phloroglucinol (phlo) as residue of the A-ring (see **Figure 1**). Its amount alternated from 7 ± 1% (mol/mol) at 40 min to 24 ± 2% (mol/mol) after 4 h in correlation to the degradation of the applied (−)-catechin-6-*C*-glucoside after 4 h. After this time point, degradation prevailed and the amount lowered. On the other hand, liberation of 3,4-dihydroxyphenylpropionic acid (3,4-hppa) took part. This was also an intermediate metabolite with the highest concentration of 9 ± 1 μM after an incubation time of 60 min. The compound was degraded via shortening of the aliphatic chain and dehydroxylation leading to mainly 4-hydroxyphenylacetic acid (4-hpaa) and 4-hydroxybenzoic acid (4-hba) with highest concentrations after 1 and 8 h, respectively. The 4-hydroxybenzoic acid could be found in amounts up to 78 ± 3% (mol/mol) of the applied 6-*C*-glucoside. The corresponding isomer 3-hydroxybenzoic acid (3-hba) was detectable only in traces as well as 3,4-dihydroxyphenylacetic acid (DOPAC).

Incubation of (+)-Epicatechin *C*-Glucosides. *(+)-Epicatechin-6-C-glucoside*. Incubation of (+)-epicatechin-6-*C*-glucoside (see **Figure 3**, (+)-epi-6-Cglu) yielded results slightly different from the flavan-3-ol isomer (−)-catechin-6-*C*-glucoside (see **Figure 2**, (−)-cat-6-Cglu). Within the incubation time of 60 min the applied compound was degraded to an amount of 51 ± 1% (mol/mol). After the physiologically relevant time of maximum 4 h, only 17 ± 3% (mol/mol) remained. Degradation took part via C-ring cleavage and liberation of phloroglucinol (phlo) and 3,4-dihydroxyphenylpropionic acid (3,4-hppa). Very small amounts could be detected after 20 min, for both. Phloroglucinol accumulated until a time point of 2 h with 22 ± 1% (mol/mol), when degradation overbalanced liberation. 3,4-Dihydroxyphenylpropionic acid was degraded further. This led to the microbial production of 4-hydroxyphenylacetic acid (4-hpaa) with a maximum amount of 7 ± 2% (mol/mol) after 60 min, whereas ongoing degradation generated high amounts of 4-hydroxybenzoic acid (4-hba), which was not degraded further. An amount of 73 ± 5% (mol/mol) accumulated within a total incubation time of 8 h. Again, amounts below 1% (mol/mol) of 3-hydroxybenzoic acid (3-hba) and 3,4-dihydroxyphenylacetic acid (DOPAC) were found.

(+)-Epicatechin-6,8-C-diglucoside. For (+)-epicatechin-6,8-*C*-diglucoside the results differ compared to the 6-*C*-glucoside. Similar to the results for the (−)-catechin-8-*C*-glycosides mentioned above, the compound could be detected in high amounts at all sampled time points. For the cecal suspension containing viable microbiota 94 ± 3% (mol/mol) was recovered after 20 min, whereas 91 ± 3% (mol/mol) was detected after an incubation time of 8 h. For the inactivated control samples the concentration−time course did not differ significantly ($p < 0.05$), with recoveries between 96 ± 3 and 92 ± 2% (mol/mol) over the whole examined time (data not shown). These results indicate that (+)-epicatechin-6,8-*C*-diglucoside is also not metabolized by the intestinal microbiota.

Incubation of Procyanidins. *Procyanidin B5*. Procyanidin B5 was degraded more slowly than the 6-*C*-glucosides. The combination of values measured during incubation of procyanidin B5 (see **Figure 4**, procy B5 comb) in three different cecal suspensions led to high standard deviations. Within 2 h 62 ± 36% (mol/mol) of procyanidin B5 was degraded. Afterward, the degradation rate lowered. Because of the large differences among the results of the three ceca the obtained results for each cecum were not combined. This is caused by huge interindividual differences in the microbial composition in each animal. The individual concentration−time courses of each cecum (C1−C3) are also shown in **Figure 4** (procy B5 C1, procy B5 C2, and procy B2 C3). One pig (see **Figure 4**, procy B5 C1) donated a microbiota that was able to degrade procyanidin B5 directly and nearly totally within 4 h. The two others were more similar to each other. The largest differences existed at time points of 1 and 2 h (see **Figure 4**, procy B5 C2, procy B5 C3) with ∼35 percentage points each. At time points of 4 and 8 h both values were statistically indifferent ($p < 0.05$) for both ceca at 35 ± 2% (mol/mol) and 12 ± 4% (mol/mol), respectively. Degradation of procyanidin B5 did not proceed via cleavage into flavan-3-ol monomers, which could not be detected at any time point. Even if monomers had been formed inside the microorganisms, they should have been detected by using SDS in the extraction solvent as this detergent also leads to the disintegration of microbial cell membranes. In sterilized inoculum suspension a loss of procyanidin B5 was observed. After 8 h, nearly 40% (mol/mol) of the applied concentration (100 μM) disappeared (see **Figure 4**, procy B5 sterilized) without formation of any metabolites.

Formation of hydroxylated phenylcarboxylic acids and phloroglucinol was in the same magnitude compared to monomers, taking into account that per mole of procyanidin B5 2 mol of metabolites were formed. The main metabolite was 4-hydroxybenzoic acid with an amount of 81 ± 16% (mol/mol) after 8 h of incubation (data not shown).

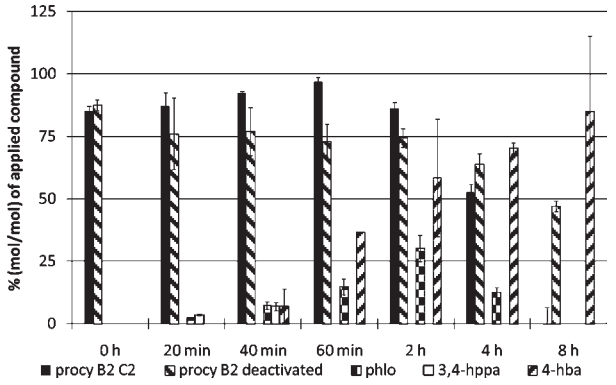

Figure 5. Relative proportions (% (mol/mol)) of procyanidin B2 (procy B2 C2 (n = 6)) and its microbial metabolites phloroglucinol (phlo), 4-hydroxyphenylpropionic acid (4-hppa), and 4-hydroxyphenylacetic acid (4-hpaa) in active cecal suspension (cecum C2) in reference to that in sterilized inoculum (procy B2 deactivated (n = 6)) (mean ± SD; measured by HPLC-FLD and GC-MS) (*22*).

Procyanidin B2. Procyanidin B2 was also degraded with large interindividual differences, as published before (*22*). As can be seen exemplarily in **Figure 5**, for one of the tested ceca, procyanidin B2 (procy B2 C2) was degraded nearly totally within 4 h. Liberated compounds after C-ring cleavage were phloroglucinol and 3,4-dihdroxyphenylpropionic acid. The main metabolite was 4-hydroxybenzoic acid with an amount of 85 ± 30% (mol/mol) after 8 h of incubation (see **Figure 5**, 4-hba). As previously described (*22*), the degradation proceeded via formation of an unknown metabolite that was generated in low amounts. The structure of this unknown metabolite (*22*) could now be elucidated by advanced fragmentation experiments with high-resolution Fourier transformation mass spectrometry. Due to its also unknown optical properties it was not possible to quantitate the amount. As liberated hydroxylated phenylcarboxylic acids and phloroglucinol were in the same magnitude compared to monomers, the amount of the unknown metabolite was assumed to be little. Liberated compounds after C-ring cleavage were phloroglucinol and 3,4-dihdroxyphenylpropionic acid. The main metabolite was 4-hydroxybenzoic acid, with an amount of 85 ± 30% (mol/mol) after 8 h of incubation (see **Figure 5**, 4-hba).

Structural Elucidation of an Occurring Metabolite of Procyanidin B2. For structure elucidation the unknown metabolite was collected from several analytical HPLC-FLD runs after injection of enlarged aliquots of 50 μL of the pig cecum samples. The combined fractions were concentrated with a rotary evaporator by a factor of 300. After this, the sample was directly injected with 10 μL/min into the high-resolution Fourier transformation mass spectrometer with an integrated syringe pump. A product ion spectrum and postulated structure are shown in **Figure 6**. The compound gave m/z 291.08668 ($M - H^+$; ESI^-) with an absolute deviation of 1.25 ppm. This leads to the molecular formula of $(C_{15}H_{15}O_6)^-$. Fragmentation in CID mode with a fragmentation energy of 30% yielded pseudomolecular ions of m/z (%) 291.08668 (12) ($M - H^+$), 247.09698 (100), 167.03488 (30), 151.04007 (5), 123.04531 (27), and 109.02971 (7). These have been used for a structural characterization via reactions described in the literature (*32*−*34*). Due to the limited amount of sample structure, elucidation using NMR was not possible.

Procyanidin C1. The concentration−time course of procyanidin C1 is shown in **Figure 7**. The compound is neither degraded in active cecal suspension nor bound to the matrix in higher amount, as can be seen from deactivated control samples. Within the incubation time of 4 h only 12 ± 5% (mol/mol) of the applied compound

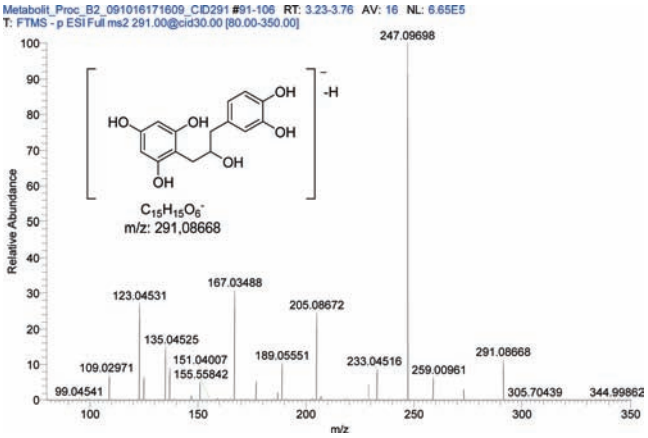

Figure 6. ESI-FTMS-MS/MS product ion spectrum with suggested structure of unknown metabolite formed during incubation of procyanidin B2 with m/z 291.00 (isolation width 1.5; relative fragmentation energy (CID) 30%).

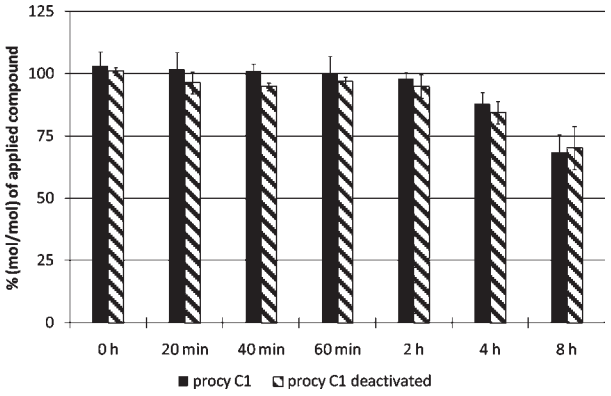

Figure 7. Relative proportions (% (mol/mol)) of procyanidin C1 (procy C1) in active cecal suspension in reference to sterilized control samples (procy C1 deactivated) (n = 6, mean ± SD; measured by HPLC-FLD.

was no longer detectable; however, no degradation products could be found. To sum up, the course for the active cecal suspension was statistically not significantly different ($p < 0.05$) from the deactivated control sample.

DISCUSSION

Metabolism of *C*-Glycosides. In unprocessed cocoa beans a lot more (−)-epicatechin can be found than (+)-catechin. However, as already described above, during Dutch processing an epimerization occurs, and for this reason (−)-catechin prevails over (+)-epicatechin (*35*). Therefore, the incubation of (−)-catechin *C*-glycosides has a higher relevance compared to (+)-epicatechin *C*-glycosides as the latter are found in lower concentrations.

(−)-Catechin-6-*C*-glucoside was degraded more rapidly than (+)-epicatechin-6-*C*-glucoside. More than 60% (mol/mol) of the first was degraded within 60 min, and total degradation could be detected for a time point between 2 and 4 h. After 60 min, only 49 ± 1% (mol/mol) of (+)-epicatechin-6-*C*-glucoside was degraded, and total disappearance took between 4 and 8 h. The reason for this phenomenon is unclear, as former results of our group showed a degradation independent from stereoisomeric effects for flavan-3-ols (*22*). However, in the former experiments no (+)-epicatechin was studied as it is unstable and uncommon in food.

The fact that the flavan-3-ol *C*-glycosides with a *C*-glycosidic bond at C8, in detail, (−)-catechin-8-*C*-glucoside, -galactoside, and (+)-epicatechin-6,8-*C*-diglucoside, were not degraded is

interesting with regard to results of the degradation of vitexin (apigenin-8-*C*-glucoside). Vitexin was degraded like the 6-*C*-glucosides under study within a comparable time period, and similar phenolic compounds of lower molecular weight were liberated (*22*). As a hypothesis an influence of the structural properties of the flavon apigenin as aglycone of vitexin could be an explanation. In general, results presented in this publication are in accordance with the literature. Mangiferin (norathyriol-6-*C*-glucoside) was degraded in a study using human stool samples as source of intestinal microorganisms (*36*), whereas puerarin (daidzein-8-*C*-glucoside) was also not degraded by microorganisms from human stool samples or in rats (*37*, *38*). Liberated hydroxylated phenolic compounds were qualitatively comparable to each other. Further research activities toward the detailed background of the position- and linkage-dependent microbial degradation pathways could be done, for example, with luteolin derivatives, where it is possible to have a total set of both *O*- and *C*-glycosides, or in studies additional to those presented here with flavan-3-ol *O*-glycosides (*39*).

Metabolism of Procyanidins. Degradation of procyanidins in the experiments shown here only took part up to dimers. The procyanidin C1 trimer was not degraded by the intestinal microbiota. To our best knowledge no data according the intestinal microbial degradation of a single trimer have been published until now. Studies according the stability of procyanidins in the gastrointestinal tract of ileostomists showed a pH-dependent reduction of the median degree of polymerization of ~2 (*40*). The pH in the distal ileum is higher than in the proximal colon (*41*), which is reflected in the pig cecum model and illustrates the stability. This observation of a pH-dependent chemical degradation is reinforced by results of Zhu et al., who studied this in detail (*42*).

The data for the degradation of procyanidins B5 and B2 show a dependence of the origin of the specifically used microbiota. These interindividual differences are well-known (*43*−*45*) and normally were overcome by pooling of samples (*46*). The metabolic profile of the microbiota is unique for each human and is influenced strongly by nutrition, host−microorganism cross talk, and interaction between different microorganisms (*47*, *48*). For this reason normally a single incubation experiment with later combination of the data reflects the in vivo situation quite well. However, the combination of the data is limited in the cases where large differences between different ceca occur as was shown, for example, for the dimeric procyanidins. This leads to the difficulty of stating a trend for everyone, but reflects the in vivo situation excellently, as the interindividual differences observed in our studies are expected to be the same in humans.

The degradation of procyanidins B5 and B2 did not proceed via cleavage into flavan-3-ol monomers, which could not be detected at any time point. This is in accordance with Donovan et al., who found no monomers studying the metabolism of procyanidin B3 and a grapeseed extract containing several procyanidins (*20*). Even if monomers are formed inside the microorganism, they should be detected by using SDS in the extraction solvent as this detergent also leads to the disintegration of microbial cell membranes.

Loss of dimeric procyanidins in sterilized inoculum suspension is much stronger than for monomers (*22*). After 8 h, between 30 and 50% (mol/mol) of the applied concentration (100 μM) disappeared (see **Figures 4**, **5**, **7**, procy deactivated) without formation of any metabolites. This can be explained by binding of procyanidins to proteins, which is supported by the observation that more complex flavonoids interfere more strongly with proteins (*49*).

Formation of hydroxylated phenylcarboxylic acids and phloroglucinol is in the same magnitude compared to monomers, taking into account that per mole of procyanidin dimer 2 mol of metabolites is formed. The main metabolite is 4-hydroxybenzoic acid with amounts near 80% (mol/mol) after 8 h of incubation (data not shown). The accumulation of hydroxybenzoic acids is an interesting aspect. In vivo hydroxybenzoic acids are taken up quickly through the ileal epithelium by monocarboxylic acid transporters (MCT), which cannot be mimicked in our model. This leads to low amounts that cannot profitably be utilized for microbial metabolism, which might be why there are no microorganisms present that are able to degrade hydroxybenzoic acids. However, the increasing amounts of benzoic acid derivatives did not have any influence on the metabolic activity of the microbiota, which could be shown by co-incubation of 3- or 4-hydroxybenzoic acid with well-studied model substances such as quercetin-3-*O*-β-D-glucopyranoside (*22*). 4-Hydroxybenzoic acid was formed in much higher concentrations compared to 3-hydroxybenzoic acid in all incubation experiments. Favored production of one hydroxylated compound is well-known from other studies (*43*). Relatively low detected amounts of 3,4-dihydroxyphenylacetic acid compared to the results of Appeldoorn et al. (*46*) were caused presumably by the lower dilution of microorganisms in the approach described here, leading to much faster ongoing degradation of this intermediate compound. Also in contrast to this (*46*), but also to other studies (*50*, *51*), phenylvalerolactones and their corresponding phenylvaleric acids could not be detected in our incubation experiments. This was already discussed in detail in ref *22*.

The occurrence of the described previously unknown metabolite was unexpected, as no liberation of the intact monomer could be observed. The appearance with rising, but still very low amounts, after 4−8 h of incubation leads to the assumption that this is possibly caused by changes in metabolic abilities of the microbiota or a change in the composition of the microbiota or in modified enzyme expression in responsible microorganisms. The concentration was very low during incubation experiments, as other hydroxylated phenylcarboxylic compounds represented high amounts of the applied compounds. The structural analogy of the generated metabolite leads to the hypothesis that this compound can be absorbed through the intestinal barrier, comparable to chalcones (*18*).

ABBREVIATIONS USED

cat, catechin; BSA, *N*,*O*-bis(trimethylsilyl)acetamide; DOPAC, 3,4-dihydroxyphenylacetic acid; EI, electron impact; epi, epicatechin; FLD, fluorescence detector/detection; glu, glucose; hba, hydroxybenzoic acid; hppa, hydroxyphenylacetic acid; 3,4-hppa, 3,4-dihydroxyphenylpropionic acid; i.d., inner diameter; IS, internal standard; nd, not detectable; p.a., pro analysis; phlo, phloroglucinol; procy B2, procyanidin B2; procy B5, procyanidin B5; procy C1, procyanidin C1; SD, standard deviation; SDS, sodium dodecyl sulfate; UV-vis, ultraviolet-visible; UVD, ultraviolet detector/detection; vit, vitexin.

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