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# Degradation of Neohesperidin Dihydrochalcone by Human Intestinal Bacteria

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The degradation of neohesperidin dihydrochalcone by human intestinal microbiota was studied in vitro. Human fecal slurries converted neohesperidin dihydrochalcone anoxically to 3-(3-hydroxy-4-methoxyphenyl)propionic acid or 3-(3,4-dihydroxyphenyl)propionic acid. Two transient intermediates were identified as hesperetin dihydrochalcone 4′-β-D-glucoside and hesperetin dihydrochalcone. These metabolites suggest that neohesperidin dihydrochalcone is first deglycosylated to hesperetin dihydrochalcone 4′-β-D-glucoside and subsequently to the aglycon hesperetin dihydrochalcone. The latter is hydrolyzed to the corresponding 3-(3-hydroxy-4-methoxyphenyl)propionic acid and probably phloroglucinol. *Eubacterium ramulus* and *Clostridium orbiscindens* were not capable of converting neohesperidin dihydrochalcone. However, hesperetin dihydrochalcone 4′-β-D-glucoside was converted by *E. ramulus* to hesperetin dihydrochalcone and further to 3-(3-hydroxy-4-methoxyphenyl)propionic acid, but not by *C. orbiscindens*. In contrast, hesperetin dihydrochalcone was cleaved to 3-(3-hydroxy-4-methoxyphenyl)propionic acid by both species. The latter reaction was shown to be catalyzed by the phloretin hydrolase from *E. ramulus*.

**KEYWORDS: Neohesperidin dihydrochalcone; hesperetin dihydrochalcone 4′-β-D-glucoside; hesperetin dihydrochalcone; 3-(3-hydroxy-4-methoxyphenyl)propionic acid; 3-(3,4-dihydroxyphenyl)propionic acid; *Eubacterium ramulus*; *Clostridium orbiscindens*; phloretin hydrolase**

## INTRODUCTION

Neohesperidin dihydrochalcone (3,5-dihydroxy-4-(3-hydroxy-4-methoxyhydrocinnamoyl)phenyl 2-*O*-β-L-rhamnopyranosyl-β-D-glucopyranoside) is a sweetener which is up to 1800 times sweeter than sucrose at threshold concentrations and has synergistic effects with other table sweeteners such as aspartame and saccharin (*1*). Neohesperidin dihydrochalcone is allowed as a food ingredient by the European Union and used as a sugar substitute in a wide range of foodstuffs (sweets, snacks, beverages) at concentrations of 10−400 mg/kg or mg/L (*2*, *3*). Due to its bitterness-suppressing and flavor-modifying properties at concentrations below the sweetness threshold, this compound is also used for taste improvement in pharmaceutical products and feedstuffs. An acceptable daily intake (ADI) of 5 mg of neohesperidin dihydrochalcone/kg of body mass has been defined (*4*). Neohesperidin dihydrochalcone has not been found in nature so far but is obtained by alkaline hydrogenation of neohesperidin, the main flavonoid present in bitter oranges (*Citrus aurantium*) (*5*).

So far, current data on the absorption and the metabolism of neohesperidin dihydrochalcone in humans are not available. Early work suggested that neohesperidin dihydrochalcone is not converted to phenolic acids by the gut microbiota since the major metabolite found in rats was the aglycon (*6*). Whereas the transformation of other flavonoids, including glycosides of flavonols, flavones, and isoflavones, in humans has been studied in detail (*7*−*10*), information on the conversion of dihydrochalcone glycosides is limited (*11*). So far, dihydrochalcones have been identified in a large variety of plants (*12*, *13*). For example, phloretin glycosides are characteristic of apples and derived products (*14*), which are frequently consumed by humans. Dihydrochalcones are postulated to have beneficial effects on human health based mainly on their antioxidant activity as described for a number of other flavonoids (*15*). To judge these effects, information on the bioavailability of flavonoids including dihydrochalcones is needed. The fate of these polyphenols in the gastrointestinal tract is influenced by intestinal bacteria to a great extent. For example, phloretin was shown to be degraded by human intestinal anaerobes, and it is also an intermediate in the degradation of both the flavone apigenin and the flavanone naringenin (*16*, *17*).

The aim of this work was to study the capability of human gut bacteria to transform neohesperidin dihydrochalcone. Since this compound represents one of the few dihydrochalcone glycosides commercially available, it has been used as a model substance representing the corresponding flavonoid subclass to elucidate the basic mechanisms of anaerobic flavonoid metabolism by bacteria.

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## MATERIALS AND METHODS

**Chemicals.** Neohesperidin dihydrochalcone was purchased from Sigma (Deisenhofen, Germany). Phloretin was purchased from Roth (Karlsruhe, Germany). 3-(3,4-Dihydroxyphenyl)propionic acid and 3-(4-hydroxyphenyl)propionic acid were purchased from Fluka (Deisenhofen, Germany). High-performance liquid chromatographic (HPLC)-grade methanol (Roth) was used throughout the experiments.

**Preparation of Standard Substances.** Hesperetin dihydrochalcone 4′-*β*-D-glucoside and hesperetin dihydrochalcone are not commercially available and were prepared from neohesperidin dihydrochalcone by acid hydrolysis (*18*) according to Bloor et al. (*19*). Neohesperidin dihydrochalcone was dissolved in 2 M HCl/methanol (50:50, v/v) to final concentrations of 0.05−2.4 mM. The solution was heated for up to 150 min at 80 or 95 °C or in a steam bath. Aliquots were taken at intervals to follow the hydrolysis of neohesperidin dihydrochalcone by HPLC and LC/MS analyses. For transformation experiments, hesperetin dihydrochalcone 4′-*β*-D-glucoside and hesperetin dihydrochalcone were purified by solid-phase extraction using a reversed-phase Bakerbond SPE C18 column (J. T. Baker, Phillipsburg, NJ).

For synthesis of 3-(3-hydroxy-4-methoxyphenyl)propionic acid, malonic acid (100 mmol) was dissolved in a mixture of pyridine (15 mL) and piperidine (0.6 mL). 3-Hydroxy-4-methoxybenzaldehyde (69 mmol) was added, and the mixture was stirred at 100 °C for 3 h and refluxed for an additional 30 min. After cooling, the mixture was poured into ice−water (150 mL) and acidified to pH 1. The precipitate was collected with a filter, washed with diluted HCl and $H_2O$, and recrystallized from methanol to obtain *trans*-3-hydroxy-4-methoxycinnamic acid (isoferulic acid) as pale yellow needles (yield 52%): mp 231−232 °C (lit. (*20*) mp 226 °C). Isoferulic acid (2.5 mmol) and palladium−charcoal (10%, 0.25 mmol) were placed in a Schlenk tube. After evacuation and washing with argon, the headspace gas of the tube was replaced with $H_2$. Degassed methanol (100 mL) was injected via the septum. The mixture was stirred at room temperature for 2 h and collected with a filter. The filtrate was evaporated to dryness and dissolved in $H_2O$ (50 mL), and the pH was adjusted to 8. The solution was extracted with ethyl acetate (2 × 25 mL), and the aqueous layer was acidified to pH 3 with HCl. The precipitate was filtered off to give 3-(3-hydroxy-4-methoxyphenyl)propionic acid as a colorless solid substance (yield 33%): mp 146−147 °C (lit. (*21*) mp 146 °C). $^1H$ NMR (500 MHz) and $^{13}C$ NMR (125 MHz) spectra were recorded on a Bruker Avance DRX 500 in DMSO-$d_6$. $^1H$ NMR: $\delta$ 2.43 (t, $J$ = 7.6 Hz, 2H, $CH_2COOH$), 2.66 (t, $J$ = 7.6 Hz, 2H, $CH_2CH_2COOH$), 3.71 (s, 3H, $CH_3$), 6.56 (dd, $J$ = 8.2 Hz, 2.2 Hz, 1H, 6′-H), 6.62 (d, $J$ = 2.2 Hz, 1H, 2′-H), 6.78 (d, $J$ = 8.2 Hz, 1H, 5′-H), 8.74 (s, 1H, 3′-OH), 12.01 (s, 1H, COOH). $^{13}C$ NMR: $\delta$ 29.88 ($CH_2CH_2COOH$), 35.68 ($CH_2$-COOH), 55.88 ($CH_3$), 112.55 (C-5′), 115.80 (C-2′), 118.74 (C-6′), 133.67 (C-1′), 146.10, 146.48 (C-3′, C-4′), 173.92 (COOH).

**Growth of Bacterial Cultures.** The bacterial cultures (10 mL) were grown under strictly anoxic conditions at 37 °C in 16 mL tubes using a complex medium (ST medium) (*22*). In addition, a defined bicarbonate-buffered medium (medium B) (*23*) supplemented with 10 or 20 mM glucose or without glucose addition was used for fermentation experiments. Both media were inoculated (5%) with fecal slurry, which was prepared by suspending 1 g of freshly collected feces anoxically in a final volume of 10 mL of ST medium. Pure cultures of *Eubacterium ramulus* strain wK1 (DSM 16296) (*23*, *24*) and *Clostridium orbiscindens* strain I1 (*17*) were grown in ST medium at 37 °C. The anoxic techniques were as described elsewhere (*22*).

**Fermentation Experiments.** Fermentation experiments with neohesperidin dihydrochalcone were performed by adding 100 $\mu$L of a 50 mM stock solution of neohesperidin dihydrochalcone dissolved in dimethyl sulfoxide (DMSO) with a syringe to 10 mL of medium in 16 mL tubes. The media were inoculated with 1−5% of a 10% fecal suspension or pure bacterial culture and incubated at 37 °C in a tube rotator. In some of the experiments, a second dose of neohesperidin dihydrochalcone was added after 43−48 h of incubation. Fermentation experiments with the intermediates of neohesperidin dihydrochalcone degradation were performed on a minor scale by addition of 20 $\mu$L of the respective stock solution dissolved in DMSO with a syringe to 2 mL of ST medium in 5 mL tubes. The medium was inoculated with 5% of an exponentially growing *E. ramulus* culture or 10% of an exponentially growing *C. orbiscindens* culture and incubated at 37 °C. In some of the experiments, the bacteria were grown for 4−22 h before addition of the flavonoid substrate. At the times indicated, aliquots of 200 $\mu$L were taken with a syringe and centrifuged for 5 min at 12000*g*, and the supernatant (30 $\mu$L) was analyzed without further processing by HPLC. To prove the solubility of neohesperidin dihydrochalcone and its bacterial metabolites in aqueous medium, complete aliquots and pellets resulting from centrifugation of selected samples were analyzed after lyophilization and extraction with DMSO.

**Conversion Experiments with Phloretin Hydrolase.** Neohesperidin dihydrochalcone, hesperetin dihydrochalcone 4′-*β*-D-glucoside, hesperetin dihydrochalcone, and phloretin, respectively, were incubated with a partially purified preparation of the phloretin hydrolase from *E. ramulus*. The recombinant enzyme had been enriched from a cell-free extract of *Escherichia coli* DH5α (pPH3) expressing the phloretin hydrolase gene (*phy*) of *E. ramulus* by ammonium sulfate fractionation and column chromatography on DEAE-Sephacel according to Schoefer et al. (*25*). The assay contained a 0.12 mM concentration of the respective flavonoid added from a stock solution in DMSO (final concentration 1% DMSO) in 50 mM potassium phosphate buffer (pH 6.8) and was performed at 23 °C. The reaction was started by the addition of the phloretin hydrolase preparation (final concentration 2.31 $\mu$g of protein/mL). Samples were taken at the times indicated and mixed with 1 volume of methanol−$H_2O$−acetic acid (50:45:5, v/v/v) to stop the reaction. Subsequently, 60 $\mu$L of the mixture was analyzed by HPLC.

**HPLC Analysis.** Neohesperidin dihydrochalcone and aromatic metabolites were analyzed by reversed-phase HPLC. The HPLC system (Gynkotek, Munich, Germany) was equipped with a high-precision pump (M480G), a degasser (GT-103), an autosampler (GINA 160), a column oven (STH 585), a diode array detector (UVD 320), and a 250 × 4 mm i.d., 5 $\mu$m, LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany). The column temperature was maintained at 37 °C. Aqueous 0.1% trifluoroacetic acid (TFA; solvent system A) and methanol (solvent system B) served as the mobile phase. The HPLC was run in gradient mode (solvent B from 5% to 30% in 20 min, from 30% to 50% in 5 min, from 50% to 80% in 10 min, and from 80% to 100% in 4 min) at a flow rate of 1 mL/min and detection at 280 nm. In addition, UV spectra were recorded in the range of 200−355 nm. External standard substances were used for calibration.

**MS Analysis.** Selected incubation supernatants from degradation experiments were used for compound identification by electrospray ionization mass spectrometry (ESI-MS). For analysis, a triple-quadrupole mass spectrometer fitted with a Z-spray API electrospray source (Quattro II, Micromass, U.K.) was used. The model 2960 HPLC system (Waters, Milford, MA) was equipped with a 250 × 4 mm i.d., 5 $\mu$m, LiChrospher 100 RP-18 column (Merck) and a diode array detector (PDA 996). The mobile phase was a mixture of aqueous 1.6% formic acid (FA; solvent system A) and methanol (solvent system B). The gradient mode described above was used at a flow rate of 0.7 mL/min. The column temperature was maintained at 35 °C. The flow was split 6:1 prior to introduction into the mass spectrometer. MS analyses were carried out in positive ionization mode. The temperature of the ion source was maintained at 100 °C. The cone and capillary voltages used were 20 V and 3.0 kV, respectively. The desolvation temperature was 350 °C, and the desolvation gas ($N_2$) was held at 400 L/h.

In parallel with NMR analyses, the 3-(3-hydroxy-4-methoxyphenyl)-propionic acid preparation was subjected to MS with electron impact ionization (EI-MS; 70 eV) using an MS-50 spectrometer (AEI, Manchester, U.K.). EI-MS of 3-(3-hydroxy-4-methoxyphenyl)propionic acid: (*m/z*, rel intens) 196 ($M^+$, 32), 150 (11), 137 (62), 91 (38), 78 (100), 63 (82).

**NMR Analysis.** The structure of 3-(3-hydroxy-4-methoxyphenyl)-propionic acid, formed during neohesperidin dihydrochalcone degradation by fecal samples, was elucidated by $^{13}C$ and $^1H$ NMR analysis. A sample of a representative fermentation experiment (∼7.5 mL) was lyophilized and resuspended in 1 mL of DMSO. Aliquots of 50 $\mu$L each were run on the HPLC system using the TFA/methanol gradient. Fractions containing the corresponding metabolite were manually collected, pooled, and dried by vacuum centrifugation. $^1H$ NMR (500

MHz) and $^{13}C$ NMR (125 MHz) spectra were recorded on a Bruker Avance DRX 500 in DMSO-$d_6$. $^1H$ NMR: $\delta$ 2.43 (t, $J$ = 7.6 Hz, 2H, C$H_2$COOH), 2.66 (t, $J$ = 7.6 Hz, 2H, C$H_2$CH$_2$COOH), 3.71 (s, 3H, CH$_3$), 6.56 (dd, $J$ = 8.2 Hz, 2.1 Hz, 1H, 6′-H), 6.62 (d, $J$ = 2.1 Hz, 1H, 2′-H), 6.78 (d, $J$ = 8.2 Hz, 1H, 5′-H), 8.75 (s, 1H, 3′-OH), 12.01 (s, 1H, COOH). $^{13}C$ NMR: $\delta$ 29.85 ($C$H$_2$CH$_2$COOH), 35.66 ($C$H$_2$-COOH), 55.87 (CH$_3$), 112.53 (C-5′), 115.78 (C-2′), 118.72 (C-6′), 133.64 (C-1′), 146.08, 146.46 (C-3′, C-4′), 173.90 (COOH).

## RESULTS

**Fermentation of Neohesperidin Dihydrochalcone by Human Fecal Slurries.** To test the ability of human intestinal bacteria to transform neohesperidin dihydrochalcone, the conversion of this flavonoid diglycoside by fecal slurries was investigated. Under the conditions used, it turned out that neohesperidin dihydrochalcone could not be metabolized as the sole source of carbon and energy. Using a defined medium without an organic carbon source (medium B), neohesperidin dihydrochalcone (0.5 mM) was not converted within 93 h by any of the fecal slurries prepared from feces of four different human subjects. In contrast, the supplementation of medium B with 20 mM glucose resulted in a rapid conversion of 0.5 mM neohesperidin dihydrochalcone within 22 h by all four fecal slurries. Under identical conditions, the kinetics of neohesperidin dihydrochalcone degradation by the different fecal suspensions diverged largely. For example, in ST medium the time required for complete degradation of neohesperidin dihydrochalcone ranged from 44 to more than 142 h. Most probably, these differences reflect the individual variation in bacterial composition of the fecal samples. Interestingly, the type of medium used also influenced the rate of degradation. Whereas neohesperidin dihydrochalcone degradation in medium B supplemented with 20 mM glucose was completed within 22 h, nearly no degradation of this substrate by the same fecal sample was observed within 142 h using ST medium. These differences between media may be explained by different substrate requirements for optimal growth and activity of fecal bacteria in different samples.

The bacterial neohesperidin dihydrochalcone conversion was followed by HPLC/UV analysis of samples taken in the course of fermentation. The samples were centrifuged, and the supernatants were examined. Compared with the complete samples or the pellets, the recovery of neohesperidin dihydrochalcone and its metabolites was found to be similar or even better with the untreated supernatants.

Fecal slurries from four different human donors converted 0.4−0.5 mM neohesperidin dihydrochalcone ($R_t$ = 30.8 min, UV $\lambda_{max}$ 228 and 288 nm) to equimolar amounts of 3-(3-hydroxy-4-methoxyphenyl)propionic acid ($R_t$ = 26.8 min, UV $\lambda_{max}$ 225 and 285 nm). The degradation of neohesperidin dihydrochalcone was accompanied by the formation of a transient intermediate, which was observed in the course of all fermentation experiments. This metabolite ($R_t$ = 33.0 min, UV $\lambda_{max}$ 229 and 293 nm) was identified as the aglycon hesperetin dihydrochalcone. The maximal hesperetin dihydrochalcone concentration observed during fermentation was 0.24 mM.

Another transient metabolite was observed under certain conditions but only in some of the fermentation experiments, suggesting that this intermediate was further converted very quickly. Following the complete conversion of neohesperidin dihydrochalcone in medium B supplemented with 20 mM glucose, a second dose of neohesperidin dihydrochalcone was added to the fermentation tubes. Depending on the donor of the fecal sample, the added neohesperidin dihydrochalcone was degraded within 3.5−23 h. A compound that eluted near neohesperidin dihydrochalcone ($R_t$ = 31.0 min) was detected

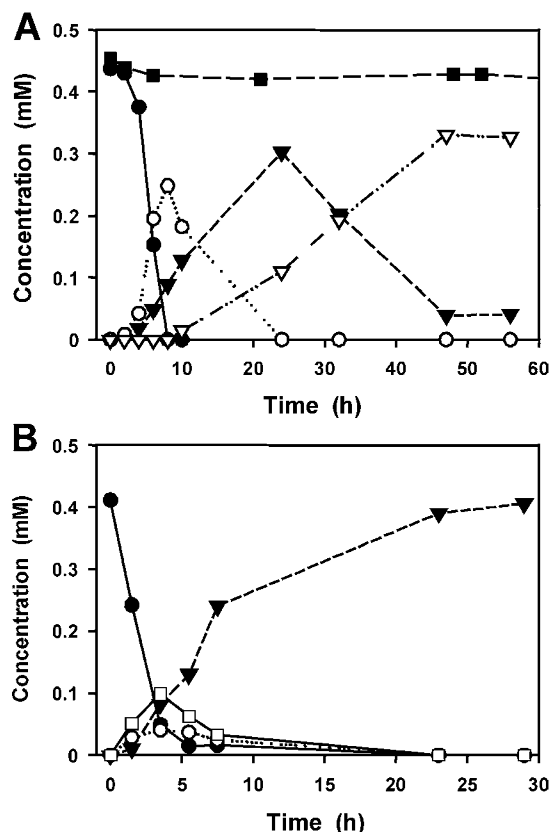


**Figure 1.** (**A**) Time course of neohesperidin dihydrochalcone fermentation by a human fecal slurry in a defined medium (medium B supplemented with 10 mM glucose). Neohesperidin dihydrochalcone (0.47 mM) was added after 22 h of bacterial cell growth. From neohesperidin dihydrochalcone (●), 3-(3,4-dihydroxyphenyl)propionic acid (▽) was formed via hesperetin dihydrochalcone (○) and 3-(3-hydroxy-4-methoxyphenyl)-propionic acid (▼). Neohesperidin dihydrochalcone without fecal inoculum (■) served as a control. (**B**) Time course of neohesperidin dihydrochalcone fermentation by a human fecal slurry in a defined medium (medium B supplemented with 20 mM glucose). Neohesperidin dihydrochalcone was added at the beginning of incubation (not shown) and after 43 h of bacterial cell growth. The figure depicts the time course of the degradation following the addition of neohesperidin dihydrochalcone at 43 h. In the figure, the latter time point was set at 0 h. From neohesperidin dihydrochalcone (●), 3-(3-hydroxy-4-methoxyphenyl)propionic acid (▼) was formed via hesperetin dihydrochalcone 4′-$\beta$-D-glucoside (□) and hesperetin dihydrochalcone (○).

in addition to the metabolites already identified. This metabolite was identified as hesperetin dihydrochalcone 4′-$\beta$-D-glucoside and exhibited a UV spectrum ($\lambda_{max}$ 228 and 288 nm) similar to those of neohesperidin dihydrochalcone and hesperetin dihydrochalcone. The maximal concentration of this intermediate during fermentation was 0.10 mM.

Whereas 3-(3-hydroxy-4-methoxyphenyl)propionic acid was not further degraded within a period of up to 4 d in most of the fermentation experiments, the demethylation of 3-(3-hydroxy-4-methoxyphenyl)propionic acid was observed in some of the fermentation experiments, giving rise to 3-(3,4-dihydroxyphenyl)propionic acid ($R_t$ = 17.8 min, UV $\lambda_{max}$ 222 and 286 nm).

The time course of neohesperidin dihydrochalcone degradation for two fermentation experiments is shown in **Figure 1**. While **Figure 1A** demonstrates the subsequent formation of 3-(3,4-dihydroxyphenyl)propionic acid from neohesperidin dihydrochalcone via hesperetin dihydrochalcone and 3-(3-hydroxy-4-methoxyphenyl)propionic acid, **Figure 1B** shows the

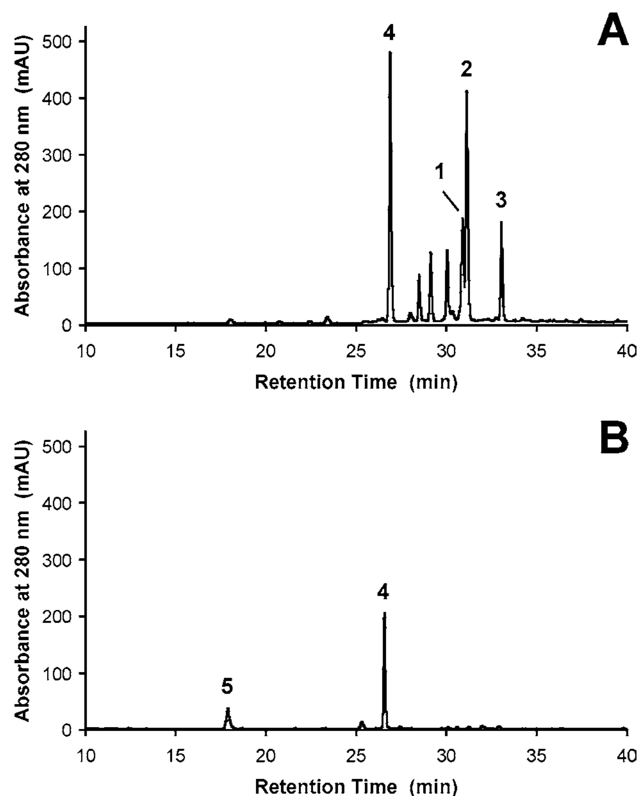


**Figure 2.** HPLC elution profiles (excerpt) of supernatants of the fermentation experiments shown in **Figure 1**: (**A**) sample taken at 3.5 h of incubation in the experiment depicted in **Figure 1B**, (**B**) sample taken at 24 h of incubation in the experiment depicted in **Figure 1A**, (1) neohesperidin dihydrochalcone, (2) hesperetin dihydrochalcone 4′-$\beta$-D-glucoside, (3) hesperetin dihydrochalcone, (4) 3-(3-hydroxy-4-methoxyphenyl)propionic acid, (5) 3-(3,4-dihydroxyphenyl)propionic acid.

degradation of neohesperidin dihydrochalcone to 3-(3-hydroxy-4-methoxyphenyl)propionic acid as the final product. Besides the aglycon hesperetin dihydrochalcone, hesperetin dihydrochalcone 4′-$\beta$-D-glucoside was formed transiently. HPLC elution profiles of fermentation supernatants that show the peaks of neohesperidin dihydrochalcone and its metabolites are depicted in **Figure 2**.

**Identification of Metabolites of Neohesperidin Dihydrochalcone Fermentation.** The products of bacterial deglycosylation hesperetin dihydrochalcone 4′-$\beta$-D-glucoside and hesperetin dihydrochalcone were identified and quantified by comparison with standard compounds prepared from neohesperidin dihydrochalcone by acid hydrolysis. Under conditions combining high acidity with high temperature, neohesperidin dihydrochalcone yielded hesperetin dihydrochalcone via the formation of hesperetin dihydrochalcone 4′-$\beta$-D-glucoside. The retention times and UV spectra in HPLC/DAD analysis and the LC/MS data confirmed the identity of both structures. Under the conditions used, a characteristic in-source fragmentation of neohesperidin dihydrochalcone (**Figure 3A**) and its deglycosylated derivatives (**Figure 3B,C**) was obtained. Besides the protonated molecules $[M + H]^+$ and the corresponding sodium adduct ion $[M + Na]^+$, specific fragments were formed by cleavage of the molecules, the most characteristic of which are indicated in **Figure 3A−C**. The MS spectra of both metabolites were identical to those obtained for the reference substances hesperetin dihydrochalcone 4′-$\beta$-D-glucoside and hesperetin dihydrochalcone (data not shown).

The structure of 3-(3-hydroxy-4-methoxyphenyl)propionic acid was unambiguously elucidated by $^1$H and $^{13}$C NMR analysis. For this purpose, the compound was purified from the fermentation supernatant of fecal slurries by fractionation following HPLC. In addition, 3-(3-hydroxy-4-methoxyphenyl)-propionic acid was characterized by LC/ESI-MS and EI-MS. The LC/MS analysis gave the expected $[M + Na]^+$ and $[M + H]^+$ of $m/z$ 219 and 197, respectively. In-source fragmentation led to the formation of additional characteristic ions (**Figure 3D**). The resulting mass spectrum (**Figure 3D**) was identical to that of the standard substance synthesized subsequently (data not shown). EI-MS analysis of 3-(3-hydroxy-4-methoxyphenyl)-propionic acid showed the respective molecular ion peak of $m/z$ 196.

An HPLC retention time and UV and ESI-MS spectra identical with those of the authentic substance confirmed the identity of 3-(3,4-dihydroxyphenyl)propionic acid. LC/MS analysis revealed the respective peaks of the protonated molecule, $m/z$ 183 $[M + H]^+$, and the sodium adduct ion, $m/z$ 205 $[M + Na]^+$. In addition, characteristic fragments of $m/z$ 165 and 123 were formed from the compound by ESI-MS analysis.

**Fermentation of Neohesperidin Dihydrochalcone, Hesperetin Dihydrochalcone 4′-$\beta$-D-Glucoside, and Hesperetin Dihydrochalcone by Pure Cultures of Human Intestinal Bacteria.** The flavonoid-degrading human intestinal bacterial species *E. ramulus* and *C. orbiscindens* were tested for their ability to degrade neohesperidin dihydrochalcone. Since hesperetin dihydrochalcone 4′-$\beta$-D-glucoside and hesperetin dihydrochalcone had been identified as intermediates of neohesperidin dihydrochalcone degradation by human fecal suspensions, they were included in fermentation studies with pure cultures of *E. ramulus* and *C. orbiscindens*. Neohesperidin dihydrochalcone was incubated at a concentration of 0.52 mM in ST medium with *E. ramulus* and *C. orbiscindens*, respectively. Neither of the strains converted neohesperidin dihydrochalcone within 43 h of incubation. Since hesperetin dihydrochalcone 4′-$\beta$-D-glucoside and hesperetin dihydrochalcone were not commercially available, they were prepared by acid hydrolysis and subsequently purified. The maximum concentrations of the compounds used in ST medium were 0.15 and 0.26 mM, respectively. Bacterial transformation was monitored by both disappearance of the substrate and formation of specific fermentation products. *E. ramulus* completely converted 0.15 mM hesperetin dihydrochalcone 4′-$\beta$-D-glucoside within 18 h of incubation to an equimolar amount of 3-(3-hydroxy-4-methoxyphenyl)propionic acid (0.14 mM). The aglycon hesperetin dihydrochalcone was transiently formed, reaching a maximum concentration of 24 $\mu$M after 4 h of fermentation. When hesperetin dihydrochalcone was used in fermentation experiments, *E. ramulus* cleaved the compound (0.26 mM) within 1 h to yield 0.23 mM 3-(3-hydroxy-4-methoxyphenyl)-propionic acid. The formation of 3-(3-hydroxy-4-methoxyphenyl)propionic acid from the aglycon occurred at least at a 10-fold higher rate than that from the corresponding glucoside ($\geq$229 $\mu$M/h versus 23 $\mu$M/h). In contrast to *E. ramulus*, *C. orbiscindens* was not able to convert hesperetin dihydrochalcone 4′-$\beta$-D-glucoside within a period of 43 h, whereas the aglycon was cleaved by this species. *C. orbiscindens* formed 0.18 mM 3-(3-hydroxy-4-methoxyphenyl)propionic acid from 0.26 mM hesperetin dihydrochalcone within 23 h of incubation. However, the product was only detected after 2−4 h of fermentation. From this point on, its formation proceeded at a rate of ~30 $\mu$M/h, which was 7.6-fold lower than that observed with *E. ramulus*.

**Figure 3.** Mass spectra of neohesperidin dihydrochalcone and bacterial metabolites formed during neohesperidin dihydrochalcone degradation: (**A**) neohesperidin dihydrochalcone (*m*/*z* 451, $Z_1$ fragment), (**B**) hesperetin dihydrochalcone 4′-*β*-D-glucoside (*m*/*z* 305, $Y_0$ fragment), (**C**) hesperetin dihydrochalcone, (**D**) 3-(3-hydroxy-4-methoxyphenyl)propionic acid.

Neither *E. ramulus* nor *C. orbiscindens* was able to demethylate this phenolic acid to 3-(3,4-dihydroxyphenyl)propionic acid as observed with fecal slurries.

**Transformation of Hesperetin Dihydrochalcone by Phloretin Hydrolase from *E. ramulus*.** The recombinant phloretin hydrolase from *E. ramulus* that catalyzes the hydrolytic cleavage of phloretin was tested for its ability to convert hesperetin dihydrochalcone. This compound differs from phloretin by the presence of a methoxy group instead of a hydroxy group at C-4 and an additional hydroxy group at C-3. HPLC analysis demonstrated the phloretin hydrolase-dependent hydrolysis of hesperetin dihydrochalcone. Its conversion led to the formation of an equimolar amount of 3-(3-hydroxy-4-methoxyphenyl)-propionic acid (**Figure 4**). Compared with the initial rate of phloretin hydrolysis of 4.06 (*μ*mol/min)/mg of protein, the recombinant enzyme catalyzed the hydrolysis of hesperetin dihydrochalcone at an 8-fold lower rate of 0.50 (*μ*mol/min)/mg of protein. In contrast to the aglycon, the glycosylated derivatives neohesperidin dihydrochalcone and hesperetin dihydrochalcone 4′-*β*-D-glucoside did not serve as substrates for the phloretin hydrolase from *E. ramulus*.

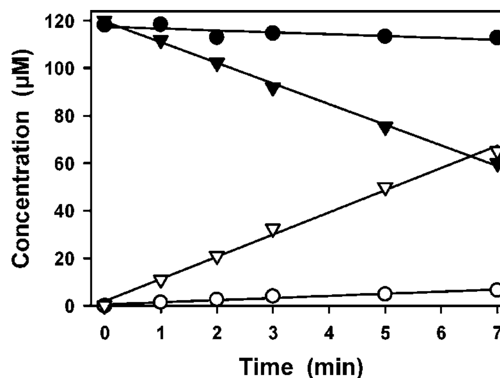


**Figure 4.** Transformation of hesperetin dihydrochalcone (●) to 3-(3-hydroxy-4-methoxyphenyl)propionic acid (○) by the recombinant phloretin hydrolase from *E. ramulus*. For comparison, the hydrolysis of phloretin (▼) to 3-(4-hydroxyphenyl)propionic acid (▽) by this enzyme is shown.

## DISCUSSION

Although neohesperidin dihydrochalcone has been used as a sweetener and flavor modifier in food and pharmaceutical products for more than a decade, there is a lack of data on the absorption and metabolism of this dihydrochalcone glycoside following ingestion by humans. Neohesperidin dihydrochalcone belongs to the large group of flavonoids. The attached sugar moiety neohesperidose represents a disaccharide composed of an L-rhamnose residue which is α-1,2-linked to a *β*-glucosidic residue. Human tissue is devoid of α-rhamnosidase activity (*26*, *27*), and so far, there is no evidence for mammalian enzymes

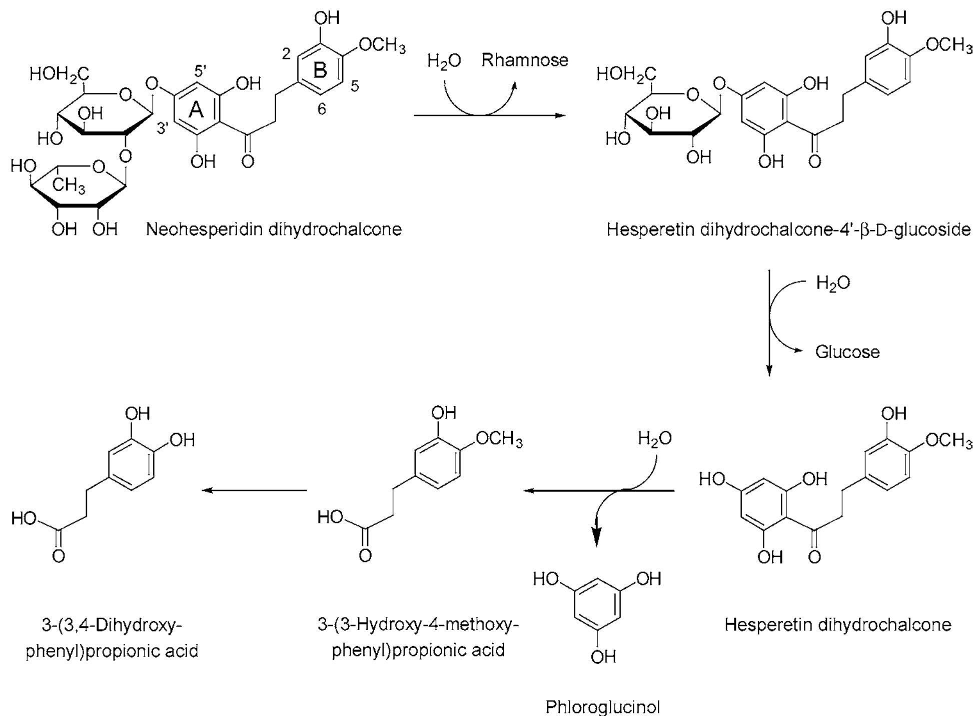


**Figure 5.** Proposed degradation pathway of neohesperidin dihydrochalcone by the human intestinal microbiota.

that cleave the flavonoid backbone itself. Therefore, the metabolism of neohesperidin dihydrochalcone and very likely its absorption depend on the action of gut bacteria. Since the major metabolite produced from neohesperidin dihydrochalcone in rats was the corresponding aglycon, it was suggested that neohesperidin dihydrochalcone does not undergo further conversions to phenolic acids by the gut microbiota (*6*). This is the first study about the conversion of neohesperidin dihydrochalcone by human gut bacteria.

To investigate the potential of human intestinal bacteria to convert neohesperidin dihydrochalcone and to elucidate the underlying pathway, the fermentation of this flavonoid glycoside by human fecal samples was studied. All of the fecal samples tested were able to completely degrade neohesperidin dihydrochalcone, indicating that the bacteria responsible for the degradation are common inhabitants of the human gut. Although the source of the fecal sample and the growth medium influenced the kinetics of neohesperidin dihydrochalcone conversion, the transformation products formed from neohesperidin dihydrochalcone were identical.

Identification of the metabolites formed was accomplished with HPLC/DAD analysis and reference to standard substances. MS and NMR analyses were used to confirm the results. From the data collected in this study, we were able to deduce the neohesperidin dihydrochalcone degradation pathway (**Figure 5**). It starts with the stepwise deglycosylation of neohesperidin dihydrochalcone, resulting in the formation of the aglycon hesperetin dihydrochalcone and the transient formation of hesperetin dihydrochalcone 4′-*β*-D-glucoside. The ability of human intestinal bacteria to cleave off a *β*-neohesperidosyl moiety has already been shown in vitro and in vivo for other flavonoids, such as neohesperidin and naringin (*28*−*30*). Flavonoid neohesperidosides and rutinosides, the latter of which represent α-1,6-linked rhamnoglucosides, are known to elude hydrolysis by human enzymes: In contrast to the respective glucoside derivatives, neither are rutin and naringin hydrolyzed by cell-free extracts of the small intestine or liver tissue nor are they substrates for cytosolic *β*-glucosidase (*26*) or lactase phlorizin hydrolase (*27*). Consequently, these flavonoid glycosides are generally not absorbed in the small intestine, and thus, they migrate further down the gastrointestinal tract and reach the colon, where they are exposed to the colonic microbiota. The kinetics of the appearance of hesperidin and narirutin in plasma indicate their absorption from the distal part of the intestine. Thus, these rutinosides are probably only absorbed in the colon after their hydrolysis by the microbiota (*31*). Accordingly, it has been shown in rats that neohesperidosides and rutinosides are not deglycosylated or absorbed in the small intestine, but converted to the corresponding aglycons by intestinal bacteria, and then absorbed in the caecum (*32*−*34*).

The aglycon hesperetin dihydrochalcone formed from neohesperidin dihydrochalcone by human fecal bacteria was further cleaved to 3-(3-hydroxy-4-methoxyphenyl)propionic acid and most likely the A-ring metabolite phloroglucinol. Whereas 3-(3-hydroxy-4-methoxyphenyl)propionic acid was further demethylated to 3-(3,4-dihydroxyphenyl)propionic acid (**Figure 5**), phloroglucinol is known to undergo rapid degradation to butyrate and/or acetate by *E. ramulus* (data not shown) and probably other bacterial species as well (*35*). These results do not support the notion of DeEds (*6*) that hesperetin dihydrochalcone represents the final product of bacterial metabolism. This notion was based on an in vivo study in rats harboring a rat microbiota. However, when phloretin, a dihydrochalcone, which in comparison with hesperetin dihydrochalcone carries a hydroxy group at C-4 and lacks the hydroxy group at C-3, was administered to rats, the cecal microbiota converted it to 3-(4-hydroxyphenyl)-propionic acid and phloroglucinol, both of which were detected in the urine of the animals (*36*). However, small changes in the structure of phloretin may result in a greatly reduced bacterial degradation in the rat intestine (*37*), which could be an explanation for the diverging results obtained for hesperetin dihydrochalcone and phloretin in rats.

Dihydrochalcones have been shown to be intermediates of flavone transformation by human gut bacteria (*17*). Therefore, the formation of identical phenolic acids from hesperetin dihydrochalcone and the corresponding flavone and flavone

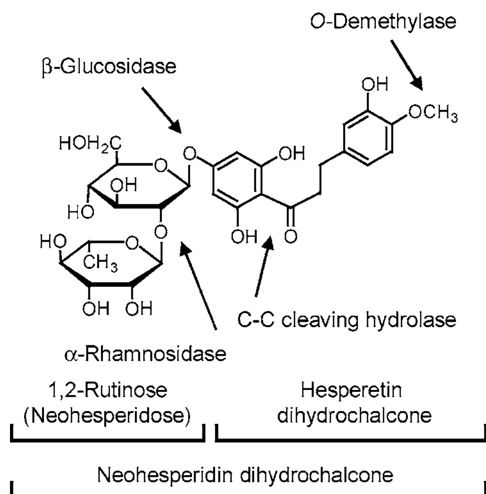


**Figure 6.** Enzymes involved in the degradation of neohesperidin dihydrochalcone.

glycosides might be expected. However, the main metabolite resulting from the hesperidin conversion in rats was 3-(4-hydroxyphenyl)propionic acid (*38*), indicating that an additional dehydroxylation occurred at C-3 of the final product of neohesperidin dihydrochalcone conversion, 3-(3,4-dihydroxyphenyl)propionic acid. In a human test subject, the main metabolite of both hesperidin and hesperetin observed in urine was 3-hydroxy-4-methoxyphenylhydracrylic acid (*38*). Although the presence of the latter compound suggested that the B-ring of hesperidin remains intact and demethylation does not necessarily need to occur, the structural differences indicate that mammalian enzymes catalyzed additional changes.

So far, bacterial species capable of completely degrading neohesperidin dihydrochalcone have not yet been described. Such bacteria would have to harbor deglycosylating enzymes, such as α-rhamnosidases and *β*-glucosidases, as well as dihydrochalcone-cleaving enzymes (**Figure 6**), and should be prevalent in the human intestinal tract. Two species, *E. ramulus* and *C. orbiscindens*, known to degrade flavonoid compounds and to be common inhabitants of the human intestine (*17*, *39*) were tested for their ability to transform neohesperidin dihydrochalcone. Pure cultures of *E. ramulus* did not degrade neohesperidin dihydrochalcone but converted the intermediates formed by human fecal slurries from neohesperidin dihydrochalcone: hesperetin dihydrochalcone 4′-*β*-D-glucoside and hesperetin dihydrochalcone. Both compounds were transformed to 3-(3-hydroxy-4-methoxyphenyl)propionic acid. In the case of the glucoside, the transient formation of the aglycon hesperetin dihydrochalcone could be observed. These results are in accordance with the presence of *β*-D-glucosidase activity and the absence of an α-L-rhamnosidase in cell extracts from *E. ramulus*. Consequently, naringenin 7-neohesperidoside is not converted by *E. ramulus*, whereas a number of flavonoid glucosides and aglycons are degraded by this gut bacterium (*16*). Although *E. ramulus* lacks an α-L-rhamnosidase, it has been reported to attack rutin (*16*) probably by hydrolysis of the *β*-glucosidic bond of the disaccharide attached to C-3 of the flavonol structure. However, a comparable cleavage was not observed with neohesperidin dihydrochalcone, in which the disaccharide is attached to C-4′ (equivalent to C-7 in the basic flavonoid structure). The cleavage of hesperetin dihydrochalcone which yields 3-(3-hydroxy-4-methoxyphenyl)propionic acid is analogous to the cleavage of the dihydrochalcone phloretin to 3-(4-hydroxyphenyl)propionic acid reported earlier (*16*). The latter reaction is catalyzed by phloretin hydrolase. The encoding gene was heterologously expressed in *E. coli* and subsequently purified and characterized (*25*). This C−C cleaving hydrolase from *E. ramulus* was also able to hydrolyze the hesperetin dihydrochalcone. As already shown for phloretin (*25*), the enzyme formed the corresponding phenylpropionic acid. However, probably owing to the presence of the methoxy group, the conversion rate was reduced by 88% compared to that of phloretin. Sugar moieties attached to the hesperetin dihydrochalcone, as present in neohesperidin dihydrochalcone and hesperetin dihydrochalcone 4′-*β*-D-glucoside, seem to prevent their cleavage by the phloretin hydrolase.

Pure cultures of *C. orbiscindens* were only able to transform the aglycon hesperetin dihydrochalcone but none of the glycosides. These results are not surprising, as the glycosidic bonds of several other flavonoid glycosides tested, such as the neohesperidoside naringin and the rutinoside rutin, cannot be cleaved by *C. orbiscindens* (*17*). In agreement with these findings, *C. orbiscindens* has been described as an asaccharolytic organism (*40*, *41*). However, a number of flavonoid aglycons are degraded by this species, yielding the corresponding phenolic acids (*17*). For example, the dihydrochalcone phloretin is converted to phloroglucinol and 3-(4-hydroxyphenyl)propionic acid (*17*). This reaction is analogous to the hydrolytic cleavage of hesperetin dihydrochalcone to 3-(3-hydroxy-4-methoxyphenyl)propionic acid investigated in this study. An enzyme similar to the phloretin hydrolase from *E. ramulus* (*25*), which was also found to hydrolyze hesperetin dihydrochalcone, might be responsible for the conversion of both dihydrochalcones. The delayed formation of 3-(3-hydroxy-4-methoxyphenyl)propionic acid from hesperetin dihydrochalcone by *C. orbiscindens* might be related to antibacterial effects as reported for phloretin incubated with *C. orbiscindens* (*17*).

Enzymes possibly involved in neohesperidin dihydrochalcone transformation (**Figure 6**) and previously described in bacterial species other than *E. ramulus* and *C. orbiscindens* include glycosidases and demethylases. Whereas *β*-glucosidases have been detected in a number of species, α-rhamnosidases are less common among bacteria. This agrees with the finding that α-rhamnosidase activity in fecal material is lower than that of *β*-glucosidase (*42*). *Bacteroides distasonis* and *Bacteroides* JY-6 were shown to hydrolyze flavonoid glycosides with a rhamnosyl moiety to their aglycons by producing α-rhamnosidase and *β*-glucosidase (*43, 44*). The thermostable enzyme of the thermophilic *Clostridium stercorarium* represents one of the best characterized α-L-rhamnosidases. This glycosidase was shown to hydrolyze both the neohesperidoside naringin and the rutinoside hesperidin (*45*).

Regarding the anaerobic *O*-demethylation of phenyl methyl ethers, a number of human intestinal bacteria with *O*-demethylating activity have been described, including the acetogenic *Eubacterium limosum* (*46*, *47*) and members of the genera *Enterobacter* and *Escherichia* (*48*). Various corrinoid-dependent methyl transferases, which catalyze the transfer of the methyl group to tetrahydrofolate, have been purified and characterized (*49*, *50*). In the course of neohesperidin dihydrochalcone fermentation by human fecal slurries only the *O*-demethylation of 3-(3-hydroxy-4-methoxyphenyl)propionic acid was observed. Demethylated metabolites of neither the glycosides nor the aglycon were detected. Whereas *E. limosum* was shown to demethylate methoxylated isoflavonoid aglycons (*47*), there are no reports on the demethylation of flavonoid glycosides by bacteria.

In summary, so far no single bacterium isolated from the human gut has been identified which is able to degrade flavonoid

rhamnoglucosides to the respective phenolic acids. Therefore, the transformation of these flavonoid glycosides is probably brought about by cooperative action of different bacterial species present in the complex intestinal microbiota. For example, the cleavage of hesperidin by methanogenic consortia enriched from digested municipal sludge led to the formation of phloroglucinol, 3-(3,4-dihydroxyphenyl)propionic acid, and 3-(3-hydroxy-4-methoxyphenyl)propionic acid as a transient intermediate (*51*). Since only bacteria are able to attack flavonoid rhamnoglucosides and the absorption of these glycosides has not been observed in most of the studies performed, it may be concluded that the bioavailability of neohesperidin dihydrochalcone and similar compounds depends on the activity of gut bacteria. However, in one human study a fraction of only 0.02% of the administered dose of naringin was recovered intact from urine (*30*). When released from the bacteria, the glucosides or aglycons formed can be either absorbed by the human host or further transformed by other gut bacteria. However, the cleavage of flavonoid structures as shown for hesperetin dihydrochalcone by *E. ramulus* and *C. orbiscindens* herein is exclusively catalyzed by bacterial enzymes. By releasing phenolic acids, such as 3-(3-hydroxy-4-methoxyphenyl)propionic and 3-(3,4-hydroxyphenyl)propionic acid, bacteria influence not only the bioavailability but also the effects of the ingested flavonoids considered to be bioactive.

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