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RESEARCH ARTICLE

Flavan-3-ol *C*-glycosides – Preparation and model experiments mimicking their human intestinal transit

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In order to study the human intestinal transit of flavan-3-ol *C*-glycosides, several *C*-glycosyl derivatives were prepared by non-enzymatic reaction of (+)-catechin with α-D-glucose, α-D-galactose and α-D-rhamnose, respectively. In contrast to literature data, we propose that the reaction mechanism proceeds in analogy to the rearrangement of flavan-3-ols during epimerization under alkaline conditions. Four of the 12 synthesized flavan-3-ol *C*-glycosides were incubated under aerobic conditions at 37°C using saliva (2 min) and simulated gastric juice (3 h). To simulate human intestine, the *C*-glycosides were also incubated under anaerobic conditions at 37°C both in human ileostomy fluid (10 h) and colostomy fluid (24 h), respectively. The flavan-3-ol *C*-glycosides under study, *i.e.* (+)-epicatechin 8-*C*-β-D-glucopyranoside (1a), (+)-epicatechin 6-*C*-β-D-glucopyranoside (1d), (+)-catechin 6-*C*-β-D-galactopyranoside (2b), (+)-catechin 6-*C*-β-D-rhamnopyranoside (3b) were analyzed in the incubation samples by HPLC-DAD and HPLC-DAD-MS/MS. They were found to be stable in the course of incubation in saliva, simulated gastric juice and ileostomy fluid and underwent degradation in colostomy fluid. While the 6-*C*-β-D-glucopyranoside 1d was completely metabolized between 2 and 4 h, decomposition of the 6-*C*-β-D-galactopyranoside 2b reached only 16 ± 2% within 4 h of incubation. Linear degradation rates of 1d and 2b in colostomy fluid differed significantly. As microbial metabolism of flavan-3-ols is known not to be influenced by the stereochemistry of the aglycon, varying degradation rates are ascribed to the effect of the sugar moiety. Based on these results we assume that flavan-3-ol *C*-glycosides pass through the upper gastrointestinal tract (oral cavity, stomach and small intestine) unmodified and are then metabolized by the colonic microflora.

Keywords:
Colostomy fluid / Flavan-3-ol *C*-glycosides / Gastric Juice / Ileostomy fluid

1 Introduction

Flavonoids, important secondary plant metabolites with polyphenol structure, occur in nature predominantly as glycosides. There are two types of glycoside bondings: *O*- and *C*-glycosides. *C*-glycosides, in which the sugar moiety is attached to the aglycon by a carbon-carbon bond, usually in position C-6 or C-8, are more resistant towards acid, alkaline and enzymatic hydrolysis than their corresponding *O*-glycosides. Even prolonged exposure to acid does not cleave the glycosyl bond of *C*-glycosides, to give the aglycon [1]. While the flavonoid *O*-glycosides are widely spread in nature, the *C*-glycosides occur less frequently. Various *C*-β-D-glycopyranosylluteolin- and apigenin derivatives, *e.g.* orientin, vitexin and vicenin-2, have been identified in sugarcane, wheat bran and dates [2–4]. *C*-glucosidically linked dihydrochalcone aspalathin as well as (iso)vitexin and (iso)orientin occur in the processed leaves and stems of rooibos tea. An aspalathin content of 7 mg/L in "ready to drink"

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Abbreviation: CD, circular dichroism

fermented rooibos tea (82 mg/L in unfermented tea) was determined. Vitexin, isovitexin, orientin and isoorientin showed concentrations of 2, 2, 15 and 11 mg/L in fermented tea, respectively [5, 6]. The flavan-3-ol derivatives (−)-epicatechin 8-*C*-glucopyranoside and (−)-epicatechin 6-*C*-β-D-glucopyranoside have been isolated from the bark of *Cinnamomum cassia* and Pu-er tea [7, 8]. In addition, *C*-glycosides can be formed non-enzymatically during food processing. Stark and Hofmann [9] have identified powder (−)-catechin- and (−)-epicatechin *C*-mono- and *C*-diglycopyranosides substituted with glucopyranoside or galactopyranoside moieties in position C-6 or C-8 in cacao. In addition to their natural occurrence, their formation was proposed as a consequence of cacao processing at alkaline conditions.

Worldwide flavonoids are in the focus of intensive research, as they have been shown to possess powerful antioxidative, antiinflammatory and anticancerogenic properties in various *in vitro* test systems and small animal models [10, 11]. But up to date, it has not been fully clarified whether they are able to exhibit the same effects in the human body. The prerequisite for *in vivo* effects of a substance is its bioavailability that is defined as the rate and extent to which the active compound is absorbed and reaches the systemic circulation [12]. Intestinal metabolism and absorption of flavonoid *O*-glycosides have been examined intensively [13–18]. They take place predominantly in the small intestine where aglycons and a few glucosides are able to penetrate the gut membrane by passive diffusion or by the help of proton driven active transport mechanisms [13, 19]. Other glycosides, esters or polymers, predominantly present in foods, must be hydrolyzed to enable their absorption. As the majority of compounds resist the acid conditions in the stomach, hydrolysis occurs enzymatically by intestinal enzymes or microbiota. Substances that are not absorbed are extensively metabolized into various low molecular aromatic compounds by the colonic microflora. Bacterial metabolites absorbed in the colon can reach high plasma concentration and may also have physiological effects [18]. However, knowledge of the biotransformation of flavonoid *C*-glycosides during the passage of the gastrointestinal tract is rather scarce [14, 20], with studies on flavan-3-ol *C*-glycosides entirely missing. Thus, determining intestinal metabolism of flavan-3-ol *C*-glycosides constitutes an important field of research towards a better understanding of the bioavailability and potential physiological effects of these substances.

In this paper, we report the non-enzymatic biomimetic preparation of *C*-glycosides from (+)-catechin and α-D-glucose, α-D-galactose and α-D-rhamnose, respectively. Furthermore, we present the results of the human intestinal transit and metabolism of four (of 12 synthesized) flavan-3-ol *C*-glycosides obtained by *in vitro* and *ex vivo* studies, *i.e.* using human saliva, simulated gastric juice, as well as ileostomy and colostomy fluids, respectively. In previous investigations, the use of ileostomy fluid has been found to reliably determine the availability of physiologically active compounds [21, 22].

2 Materials and methods

2.1 Subjects

Saliva samples from three healthy subjects (25–29 years old) were collected in the morning with the subjects abstaining from tooth brushing since the previous evening.

Ileostomy fluids were provided by three healthy subjects (36–41 years old, all suffering from Crohn's disease without an involvement of the ileum) with a terminal ileostomy undergone colectomy 7–8 years prior to the study. No ileal resection was performed by surgery.

Colostomy effluents were provided by two healthy subjects (40 and 70 years old), both with left-sided colostomies after suffering from colon cancer or prestages undergone colectomy 15–32 years previous to the study. After removal of the affected part of the colon no further symptoms of colon cancer were noticed. All subjects have been without pathological findings. None of the patients was treated with antibiotics within the last 4 wk prior to the study.

2.2 Chemicals

All compounds were obtained in ''analytical grade'' quality from Sigma-Aldrich (Steinheim, Germany), Roth (Karlsruhe, Germany), Fluka (Deisenhofen, Germany), Merck (Darmstadt, Germany) and EurisoTop (Gif sur Yvette, France). Ultra pure water was obtained from a Millipore™ purification system (Millipore, Bedford, MA, USA). Solvents were ''gradient grade'' (Fisher-Scientific, Ulm, Germany and Merck, LiChrosolv®).

2.3 Biomimetic preparation of flavan-3-ol *C*-glycosides

α-D-Glucose (as well as in separate essays α-D-galactose and α-L-rhamnose, respectively, each of 10 mmol) and 5.21 mmol K_2CO_3 were mixed with 12.5 mL of water. After homogenization, the reaction mixture was heated to 40°C and 1 mmol (+)-catechin was added. The mixture was stirred for 120 min at 40°C. After cooling, the reaction in alkaline medium was stopped by the addition of hydrochloric acid (5 mol/L) until a pH of 5.0 was reached. Thereafter, the reaction mixture was concentrated at reduced pressure to about 5 mL. The reaction mixture was applied onto a 200 cm × 2.5 cm Sephadex™ LH-20 column (103 μm, Amersham Pharmacia Biotech, Uppsala, Sweden) preconditioned with methanol/water (50:50 v/v). Chromatography was performed using methanol/water (50:50 v/v) as eluent for 10 h, followed by methanol for 5 h at a flow rate of 3 mL/min. Fractions with a volume of 9 mL were collected and analyzed by means of HPLC-DAD-MS. A Symmetry™ C18 column (150 mm × 3.9 mm, 5 μm; Waters, Milford, USA)

was applied. Chromatography was performed using 0.1% v/v formic acid as solvent A and ACN as solvent B with a gradient of 0 min, 1% solvent B; 35 min, 30% solvent B; 40 min, 99% solvent B; 43 min, 99% solvent B; 46 min, 1% solvent B; 49 min, 1% solvent B. The flow rate was 1 mL/min, reduced by split to 0.2 mL before entering the mass spectrometer, which was run in full scan mode (*m/z* 100–700). The fractions containing the desired substances were collected and solvent was removed under reduced pressure to provide, after final freeze drying, the flavan-3-ol *C*-glycosides 1a-d, 2a-d and 3a-d (Fig. 1) as amorphous orange colored powders.

(+)-*Epicatechin 8-C*-β-D-*glucopyranoside* **(1a)**: Yield: 3.4% (15.6 mg, 0.034 mmol) of an orange powder; purity: 95%; UV/Vis:λ_{max} = 220, 230, 280 nm; MS (ESI$^-$): *m/z* (%) 451.2 (45) [M-H]$^-$); ^{1}H NMR (600 MHz, MeOH-d$_4$, COSY):δ = 2.76 [dd, 1 H, *J* = 1.8, 16.8 Hz, H-C(4)a], 2.89 [dd, 1 H, *J* = 4.2, 16.8 Hz, H-C(4)b], 3.41–3.47 [m, 3 H, H-C(5″, 3″, 4″)], 3.67 [d, 1 H, *J* = 11.4 Hz, H-C(6″)a], 3.84 [d, 1 H, *J* = 12.0 Hz, H-C(6″)b], 4.01 [dd, 1 H, *J* = 7.2, 13.8 Hz, H-C(3)], 4.19 [dd, 1 H, *J* = 7.2 Hz, H-C(2″)], 4.83 [d, 1 H, *J* = 9.6 Hz, H-C(2)], 4.96 [d, 1 H, *J* = 9.6 Hz, H-C(1″)], 6.24 [s, 1 H, H-C(8)], 7.02 [dd, 1 H, *J* = 1.2, 8.0 Hz, H-C(6′)], 7.06 [d, 1 H, *J* = 8.4 Hz, H-C(5′)], 7.14 [s, 1 H, H-C(2′)]; ^{13}C-NMR (150 MHz, MeOH-d$_4$, DEPT-135, HSQC, HMBC):δ = 29.5 [C-4], 62.7 [C-6″], 69.5 [C-3], 71.6 [C-4″], 72.3 [C-2″], 75.8 [C-1″], 79.5 [C-3″], 79.6 [C-5″], 82.0 [C-2], 96.8 [C-6], 100.6 [C-4a], 104.2 [C-6], 115.2 [C-2′], 116.4 [C-5′], 119.5 [C-6′], 132.3 [C-1′], 145.2 [C-3′], 145.5 [C-4′], 156.1 [C-8a], 156.5 [C-7], 157.4 [C-5].

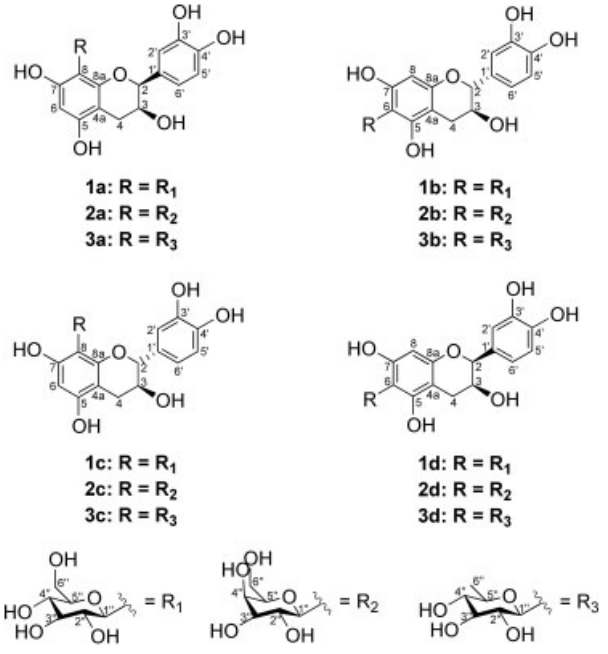

Figure 1. Chemical structures of prepared flavan-3-ol *C*-glycosides.

(+)-*Catechin 6-C*-β-D-*glucopyranoside* **(1b)**: Yield: 5.1% (23.2 mg, 0.051 mmol) of an orange powder; purity: 96%; UV/Vis:λ_{max} = 210, 234, 280 nm; MS (ESI$^-$): *m/z* (%) 451.2 (80) [M-H]$^-$); ^{1}H NMR (600 MHz, MeOH-d$_4$, COSY):δ = 2.54 [dd, 1 H, *J* = 7.2, 16.2 Hz, H-C(4)a], 2.74 [dd, 1 H, *J* = 4.8, 16.2 Hz, H-C(4)b], 3.34–3.45 [m, 3 H, H-C(5″, 3″, 4″)], 3.71 [dd, 1 H, *J* = 4.8, 12.0 Hz, H-C(6″)a], 3.84 [d, 1 H, *J* = 12.0 Hz, H-C(6″)b], 4.07 [dd, 1 H, *J* = 6.6, 12.6 Hz, H-C(3)], 4.15 [dd, 1 H, *J* = 7.2 Hz, H-C(2″)], 4.52 [dd, 1 H, *J* = 7.8, 12.6 Hz, H-C(2)], 4.78 [d, 1 H, *J* = 9.6 Hz, H-C(1″)], 6.05 [s, 1 H, H-C(8)], 6.81 [dd, 1 H, *J* = 7.2, 10.2 Hz, H-C(6′)], 6.82 [d, 1 H, *J* = 1.8 Hz, H-C(5′)], 6.99 [d, 1 H, *J* = 1.8 Hz, H-C(2′)]; ^{13}C-NMR (150 MHz, MeOH-d$_4$, DEPT-135, HSQC, HMBC):δ = 27.6 [C-4], 62.5 [C-6″], 67.9 [C-3], 71.4 [C-4″], 72.5 [C-2″], 75.7 [C-1″], 79.5 [C-3″], 80.4 [C-5″], 81.7 [C-2], 96.8 [C-6], 101.2 [C-4a], 104.3 [C-6], 115.2 [C-2′], 116.4 [C-5′], 119.8 [C-6′], 132.2 [C-1′], 145.4 [C-′], 145.6 [C-4′], 155.2 [C-8a], 155.4 [C-7], 155.8 [C-5].

(+)-*Catechin 8-C*-β-D-*glucopyranoside* **(1c)**: Yield: 1.2% (5.5 mg, 0.012 mmol) of an orange powder; purity: 96%; UV/Vis:λ_{max} = 204, 228, 280 nm; MS (ESI$^-$): *m/z* (%) 451.2 (50) [M-H]$^-$); MS/MS (ESI$^-$, 15eV): *m/z* (%) 331.2 (100) [M-121]$^-$, 451.2 (80) [M-H]$^-$, 361.2 (18) [M-91]$^-$; ^{1}H NMR (600 MHz, MeOH-d$_4$, COSY):δ = 2.57 [dd, 1 H, *J* = 7.2, 16.8 Hz, H-C(4)a], 2.75 [dd, 1 H, *J* = 5.4, 16.2 Hz, H-C(4)b], 3.40–3.43 [m, 3 H, H-C(5″, 3″, 4″)], 3.80 [dd, 1 H, *J* = 2.4, 7.2 Hz, H-C(6″)a], 3.86 [dd, 1 H, *J* = 1.8, 12.0 Hz, H-C(6″)b], 4.03 [dd, 1 H, *J* = 6.6, 13.8 Hz, H-C(3)], 4.17 [dd, 1 H, *J* = 6.6, 12.0 Hz, H-C(2‘‘)], 4.50 [d, 1 H, *J* = 7.2 Hz, H-C(2)], 4.75 [d, 1 H, *J* = 7.2 Hz, H-C(1″)], 6.04 [s, 1 H, H-C(6)], 6.75 [dd, 1 H, *J* = 1.8, 7.8 Hz, H-C(6′)], 6.80 [d, 1 H, *J* = 8.4 Hz, H-C(5′)], 6.83 [d, 1 H, *J* = 2.4 Hz, H-C(2′)]; ^{13}C-NMR (150 MHz, MeOH-d$_4$, DEPT-135, HSQC, HMBC):δ = 26.0 [C-4], 61.7 [C-6″], 66.8 [C-3], 70.7 [C-4″], 73.7 [C-2″], 76.7 [C-1″], 79.0 [C-3″], 79.5 [C-5″], 81.8 [C-2], 96.8 [C-6], 101.6 [C-4a], 105.3 [C-6], 115.3 [C-2′], 116.4 [C-′], 119.7 [C-6′], 132.0 [C-1′], 145.2 [C-3′], 145.3 [C-4′], 155.4 [C-8a], 155.9 [C-7], 156.4 [C-5].

(+)-*Epicatechin 6-C*-β-D-*glucopyranoside* **(1d)**: Yield: 4.2% (19.2 mg, 0.042 mmol) of an orange powder; purity: 93 %; UV/Vis:λ_{max} = 208, 226, 280 nm; MS (ESI$^-$): *m/z* (%) 451.2 (100) [M-H]$^-$; MS/MS (ESI$^-$, 15eV): *m/z* (%) 331.2 (100) [M-121]$^-$, 451.2 (70) [M-H]$^-$, 361.2 (15) [M-91]$^-$; ^{1}H NMR (600 MHz, MeOH-d$_4$, COSY):δ = 2.77 [dd, 1 H, *J* = 2.4, 16.8 Hz, H-C(4)a], 2.89 [dd, 1 H, *J* = 4.8, 16.8 Hz, H-C(4)b], 3.46–3.49 [m, 3 H, H-C(5″, 3″, 4″)], 3.80 [dd, 1 H, *J* = 4.2, 16.8 Hz, H-C(6″)a], 3.86 [dd, 1 H, *J* = 2.4, 12.6 Hz, H-C(6″)b], 4.02 [dd, 1 H, *J* = 6.6, 12.8 Hz, H-C(3)], 4.24 [dd, 1 H, *J* = 4.2 Hz, H-C(2″)], 4.55 [d, 1 H, *J* = 9.0 Hz, H-C(2)], 4.87 [d, 1 H, *J* = 10.2 Hz, H-C(1″)], 6.08 [s, 1 H, H-C(8)], 6.64 [d, 1 H, *J* = 1.2, 7.8 Hz, H-C(6′)], 6.66 [d, 1 H, *J* = 7.8 Hz, H-C(5′)], 6.89 [d, 1 H, *J* = 1.2 Hz, H-C(2′)]; ^{13}C-NMR (150 MHz, MeOH-d$_4$, DEPT-135, HSQC, HMBC):δ = 26.0 [C-4], 61.7 [C-6″], 66.8 [C-3], 70.7 [C-4″], 73.7 [C-2″], 76.7 [C-1″], 79.0 [C-3″], 79.5 [C-5″], 81.8 [C-2], 96.8 [C-6], 101.6 [C-4a], 105.3 [C-6], 115.3 [C-2′], 116.4

[C-5′], 119.7 [C-6′], 132.0 [C-1′], 145.2 [C-3′], 145.3 [C-4′], 155.4 [C-8a], 155.9 [C-7], 156.4 [C-5].

(+)-*Epicatechin 8-C*-β-D-*galactopyranoside* **(2a)**: Yield: 8.0%, (81.4 mg, 0.180 mmol) of a brown powder; purity: 78%; UV/Vis:λ_{max} = 204, 227, 279 nm; MS (ESI$^-$): *m/z* (%) 451.2 (40), [M-H]$^-$; ^{1}H NMR (600 MHz, MeOH-d_4):δ = 2.76 [dd, 1 H, *J* = 1.2, 16.8 Hz, H-C(4)a], 2.93 [dd, 1 H, *J* = 4.2, 16.8 Hz, H-C(4)b], 3.44–3.50 [m, 3 H, H-C(5″, 3″, 4″)], 3.64 [dd, 1 H, *J* = 3.00, 9.6 Hz, H-C(6″)a], 3.73 [dd, 1 H, *J* = 5.4, 9.6 Hz, H-C(6″)b], 3.99 [d, 1 H, *J* = 3.0 Hz, H-C(3)], 4.08 [dd, 1 H, *J* = 1.8, 6.6 Hz, H-C(2″)], [hidden: H-C(2), H-C(1″)], 6.09 [s, 1 H, H-C(8)], 6.85 [bs, 1 H, H-C(6′)], 6.91 [bs, 1 H, H-C(5′)], 7.14 [d, 1 H, *J* = 7.8 Hz, H-C(2′)]; ^{13}C-NMR (150 MHz, MeOH-d_4): undetectable

(+)-*Catechin-6-C*-β-D-*galactopyranoside* **(2b)**: Yield: 6.1% (27.5 mg, 0.061 mmol) of an orange powder; purity: 79%; UV/Vis: λ_{max} = 204, 225, 280 nm; MS (ESI$^-$): *m/z* (%) 451.2 (65) [M-H]$^-$, MS/MS (ESI$^-$, 15 eV): *m/z* (%) 331.2 (100) [M-121]$^-$, 451.2 (75) [M-H]$^-$, 361.2 (25) [M-91]$^-$.

(+)-*Catechin 8-C*-β-D-*galactopyranoside* **(2c)**: Yield: 3.9% (17.5 mg, 0.039 mmol) of an orange powder; purity: 71%; UV/Vis: λ_{max} = 204, 228, 280 nm; MS (ESI$^-$): *m/z* (%) 451.2 (75), [M-H]$^-$.

(+)-*Epicatechin 6-C*-β-D-*galactopyranoside* **(2d)**: Yield: 5.2% (23.3 mg, 0.052 mmol) of a dark orange powder; purity: 55%; UV/Vis: λ_{max} = 204, 226, 279 nm; MS (ESI$^-$): *m/z* (%) 451 (100) [M-H]$^-$.

(+)-*Epicatechin 8-C*-β-D-*rhamnopyranoside* **(3a)**: Yield: 1.0% (4.5 mg, 0.010 mmol) of an orange powder; purity: 86%; UV/Vis:λ_{max} = 202, 230, 280 nm; MS (ESI$^-$): *m/z* (%) 435.3 (50) [M-H]$^-$; ^{1}H NMR (600 MHz, MeOH-d_4):δ = 2.52 [dd, 1 H, *J* = 8.4, 16.2 Hz, H-C(4)a], 2.83 [dd, 1 H, *J* = 5.4, 16.2 Hz, H-C(4)b], 3.41–3.48 [m, 3 H, H-C(5″, 3″, 4″)], 3.87 [d, 3 H, *J* = 7.2 Hz, H-C(6″)], 3.94 [dd, 1 H, *J* = 7.2, 13.8 Hz, H-C(3)], 4.13 [dd, 1 H, *J* = 2.4, 7.8 Hz, H-C(2″)], 4.50 [d, 1 H, *J* = 7.8 Hz, H-C(2)], 4.60 [d, 1 H, *J* = 7.8 Hz, H-C(1″)], 6.05 [s, 1 H, H-C(8)], 6.83 [dd, 1 H, *J* = 1.2, 6.0 Hz, H-C(6′)], 6.85 [d, 1 H, *J* = 5.4 Hz, H-C(5′)], 6.91 [d, ^{1}H, H, *J* = 1.2 Hz, H-C(2′)]; ^{13}C-NMR (150 MHz, MeOH-d_4): = 28.0 [C-4], 62.0 [C-6″], 69.1 [C-3], 71.9 [C-4″], 73.5 [C-2″], 76.3 [C-1″], 79.5 [C-3″], 81.9 [C-5″], 84.6 [C-2], 96.6 [C-6], 101.5 [C-4a], 105.5 [C-6], 115.4 [C-2′], 116.6 [C-5′], 120.4 [C-6′], 131.7 [C-1′], 145.6 [C-3′], 145.6 [C-4′], 155.4 [C-8a], 156.1 [C-7], 156.2 [C-5].

(+)-*Catechin 6-C*-β-D-*rhamnopyranoside* **(3b)**: Yield: 3.2% (14.0 mg, 0.032 mmol) of an orange powder; purity: 94%; UV/Vis:λ_{max} = 204, 228, 280 nm, MS (ESI$^-$): *m/z* (%) 435.3 (35) [M-H]$^-$, MS/MS (ESI$^-$, 25 eV): *m/z* (%) 331.2 (100) [M-121]$^-$, 435.3 (40) [M-H]$^-$, 361.3 (40) [M-91]$^-$.

(+)-*Catechin 8-C*-β-D-*rhamnopyranoside* **(3c)**: Yield: 4.3% (18.6 mg, 0.043 mmol) of an orange powder; purity: 74%; UV/Vis:λ_{max} = 204, 227, 280 nm; MS (ESI$^-$): *m/z* (%) 435.3 (60) [M-H]$^-$.

(+)-*Epicatechin 6-C*-β-D-*rhamnopyranoside* **(3d)**: Yield: 0.96% (4.2 mg, 9.62×10^{-3} mmol) of an orange powder; purity: 90%; UV/Vis:λ_{max} = 204, 226, 280 nm; MS (ESI$^-$): *m/z* (%) 435.3 (100) [M-H]$^-$.

2.4 Preparation of saliva

For the incubations, collected saliva (directly transferred from the oral cavity into tubes) was diluted with the same volume of distilled water and shaken to reduce viscosity [23].

2.5 Preparation of simulated gastric juice

Conditions in the stomach during the incubations were simulated with artificial gastric juice (pH 1.8) according to reference [24].

2.6 Preparation of inoculum (ileostomy/colostomy fluids)

After removal, the ileostomy/colostomy bag was immediately placed in an anaerobic jar containing AnaeroCult™ from Merck to create an anaerobic atmosphere. It was transported straight into the laboratory where the jar was directly transferred into an anaerobic chamber (self-constructed) flushed with a N_2/CO_2 gas mixture (80:20 v/v). The ileostomy/colostomy fluid was diluted with the same volume of anaerobic carbonate-phosphate buffer (pH 6.3 for ileostomy fluid, pH 7.4 for colostomy fluid) according to Labib *et al.* [25]. The ileostomy/colostomy fluid was mixed and coarse particles were removed by filtration using glass wool. The filtrate was used as inoculum.

2.7 Incubation conditions

2.7.1 Saliva

For incubation with the *C*-glycosides **1a**, **1d**, **2b** and **3b**, 1.0 mL diluted saliva was added to 20 μL of each *C*-glycoside stock solution (25 mM). The incubation vessels were sealed and after shaking stored at 37°C for 0, 0.5, 1, 1.5 and 2 min. Parallel determinations were carried out for all incubations. To stop enzymatic reactions the vessels were placed in liquid nitrogen. For blank assays adequate volumes of distilled water instead of *C*-glycoside stock solution were applied. The samples were freeze-dried.

2.7.2 Simulated gastric juice

Simulated gastric juice (1.25 mL) was added to 20 μL *C*-glycoside stock solutions (25 mM) of the *C*-glycosides **1a**, **1d**, **2b** and **3b**. The incubation vessels were sealed tightly and after shaking stored at 37°C for 0, 0.5, 1, 1.5, 2, 2.5 and 3 h. Parallel determinations were carried out for all incubations. To stop enzymatic reactions the vessels were placed in liquid nitrogen and were then lyophilized. For blank assays adequate volumes of distilled water instead of *C*-glycoside stock solution were applied.

2.7.3 Ileostomy/colostomy fluids

Inoculum (1.25 mL) was added to pregassed (N_2) incubation vessels, each containing 20 μL *C*-glycoside stock solution (25 mM) of **1a**, **1d**, **2b** and **3b**. The incubation vessels were sealed tightly and after shaking stored at 37°C for 0, 0.5, 1, 2, 5, 7 and 10 h (ileostomy fluid) and 0, 2, 4, 6, 8, 10 and 24 h (colostomy fluid). Parallel determinations were carried out for all incubations. To stop enzymatic reactions the vessels were placed in liquid nitrogen and were then lyophilized. For blank assays adequate volumes of distilled water instead of *C*-glycoside stock solution were applied.

2.8 Sample preparation

The freeze-dried samples were extracted twice using 1.25 mL methanol (70% v/v in 1% v/v formic acid). The samples were shaken and sonicated. After centrifugation at 2700 *g* for 10 min (Hettrich EBA-12, Tuttlingen, Germany) the supernatants were pooled and filtered (polyvinylidene difluoride, 0.45 μm). Blanks (without substrates) were treated identically. The filtrate was diluted with the same volume of internal standard 3,4,5-*trans*-trimethoxycinnamic acid (0.05 g/L in methanol/water 50:50 v/v).

2.9 Analysis of *C*-glycosides in the incubation samples

RP-HPLC analysis of *C*-glycosides was performed using a Symmetry™ C18 column (150 mm × 3.9 mm, 5 μm; Waters). The mobile phase consisted of 0.1% v/v formic acid as solvent A and ACN as solvent B. The following gradient was applied: 0 min, 1% solvent B; 35 min, 30% solvent B; 38 min, 80% solvent B; 40 min, 80% solvent B; 43 min, 1% solvent B; 45 min, 1% solvent B. Flow rate and injection volume were set to 1 mL/min and 50 μL, respectively. The content of *C*-glycosides in the incubation samples was determined by external calibration using the obtained reference substances and 3,4,5-*trans*-trimethoxycinnamic acid (0.05 g/L in methanol/water (50:50 v/v) as an internal standard. Degradation rates of each substrate were evaluated by drawing the tangent through the linear domain of the degradation curves. The resulting slope of each linear regression corresponds to the degradation rate in %/h.

2.10 HPLC

The HPLC system used was a HP 1100 HPLC gradient pump equipped with a HP 1100 photodiode array detector (Waldbronn, Germany) and a Waters Wisp 712b autosampler (Eschborn Germany). Data acquisition was carried out with HP ChemStation software.

2.11 LC-MS and LC MS/MS

LC-MS and LC-MS/MS analyses were performed with a TSQ 7000 Triple-Stage-Quadrupol tandem mass spectrometer with ESI-interface (Finnigan MAT, Bremen, Germany) equipped with a HP 1100 HPLC gradient pump with a HP 1100 photodiode array detector (Waldbronn, Germany) and a Waters Wisp 712b autosampler (Eschborn Germany). The analyses were performed in the negative ionization mode with a spray capillary voltage of 3.2 kV and a capillary temperature of 250°C. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 units). For MS/MS experiments a collision energy of 15–25 eV was applied with argon (2 mTorr) serving as collision gas. Data acquisition was carried out with HP ChemStation software and ICIS 8.1 Data System (Finnigian MAT, Bremen, Germany) on DECstation 5000/33 (Digital Equipment, Unterföhring, Germany).

2.12 NMR spectroscopy

^{1}H, COSY, HMQC, HMBC, ^{13}C and DEPT-135 NMR measurements were recorded on a DMX 600 spectrometer (Bruker, Rheinstetten, Germany). Evaluation of the experiments was carried out using 1-D and 2-D-WIN NMR (version 6.1) as well as MestReC software (version 4.4.6.0, Mestrelab Research, Santiago de Compostela, Spain). The chemical shift δ is declared in ppm relative to residual MeOH-d_4.

2.13 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed on a J600 Spectro polarimeter (Jasco, Hachioji, Japan). Methanolic solutions of the samples were used.

3 Results and discussion

3.1 Preparation of catechin *C*-glycosides

Catechin *C*-glycosides were prepared from (+)-catechin and α-D-glucose, α-D-galactose and α-D-rhamnose, respectively, in an alkalized aqueous system according to Stark and Hofmann with some modifications [9]. Catechin is known to degrade and epimerize fast at high temperatures in an alkaline milieu [26]. Thus, the reaction temperature was reduced to 40°C and, to increase the yield, the reaction time was extended up to 120 min. The formation of flavan-3-ol *C*-glycosides was confirmed by HPLC-MS (data not shown). In each case four products were isolated and identified: (+)-epicatechin 8-*C*-β-D-glucopyranoside **(1a)**, (+)-catechin 6-*C*-β-D-glucopyranoside **(1b)**, (+)-catechin 8-*C*-β-D-glucopyranoside **(1c)** and (+)-epicatechin 6-*C*-β-D-glucopyranoside

(1d), (+)-epicatechin 8-*C*-β-D-galactopyranoside **(2a)**, (+)-catechin 6-*C*-β-D-galactopyranoside **(2b)**, (+)-catechin 8-*C*-β-D-galactopyranoside **(2c)** and (+)-epicatechin 6-*C*-β-D-galactopyranoside **(2d)**, (+)-epicatechin 8-*C*-β-D-rhamnopyranoside **(3a)**, (+)-catechin 6-*C*-β-D-rhamnopyranoside **(3b)**, (+)-catechin 8-*C*-β-D-rhamnopyranoside **(3c)** and (+)-epicatechin 6-*C*-β-D-rhamnopyranoside **(3d)** (Fig. 1).

The formation of the four isomers each time resulted both from epimerization of catechin under alkaline conditions [26, 27] as well as the sugar moiety's ability to be connected to the aglycon at the C-6 or C-8 position [28]. The mechanism of epimerization is not completely known up to date. The formation of a stereoselective chinonmethide intermediate has been assumed, out of which the corresponding epimer arises [27, 29].

The signals in the ^{1}H-NMR spectra of catechin *C*-glucosides and *C*-galactosides were in accordance with those reported in the literature [9]. To the best of our knowledge there is no literature NMR data for catechin *C*-rhamnosides available so far. In comparison to the ^{1}H-NMR spectra of catechin *C*-glucosides and *C*-galactosides that of catechin *C*-rhamnosides differed only for the three H-C(6″)-atoms where a doublet instead of a doublet of doublet was observable at 3.87 ppm.

To identify the configuration of the substances, CD spectroscopy was applied using the prepared catechin and epicatechin *C*-glucosides, exhibiting positive CD signals at 285 nm for epicatechin *C*-glucosides and a negative signal at 282 nm for catechin *C*-glucosides. In comparison with literature data [30, 31] these results suggest the existence of (+)-(epi)catechin derivatives. This hypothesis is confirmed by the studies of Kiatgrajai *et al.* [31] and Stark and Hofmann [9] showing the epimerization of (+)-catechin to (+)-epicatechin as well as the formation of (−)-(epi)catechin *C*-glycosides from (−)-epicatechin without change of configuration.

In MS/MS experiments catechin *C*-glucosides **(1a,d)**, catechin *C*-galactoside **(2b)** and the catechin *C*-rhamnoside **(3b)** showed the identical main fragment of *m/z* 331 $[M\text{-}121]^-$, corresponding to epi(catechin) substituted with a hydroxyethyl moiety in position C-6 or C-8. This indicates a fragmentation at the carbon atom 2 of the sugar moiety independently from the attached glycoside.

3.1.1 Reaction mechanism

Using α-D-glucose, additional experiments to synthesise *C*-glycosides were carried out with several flavonols (quercitin, rutin, morin, fisetin), the flavanone naringenin, as well as the flavone chrysine. The experimental setup was identical to that used for the synthesis of flavan-3-ol *C*-glycosides. Solely naringenin reacted with glucose to the expected *C*-glucoside (data not shown).

Stark and Hofmann [9] have postulated a Michael-type addition mechanism for the formation of flavan-3-ol *C*-glycosides whereby the carbonyl atom of the open-chain form of the glycoside is attacked by the flavonoid A-ring at position C-6 or C-8. Their proposed mechanism is independent of the molecular structure of the rings B and C. The unique reaction requirement is that C-6 and C-8 are not substituted. Our experiments, however, showed that only flavan-3-ols and flavanones with a single bond between C2 and C3 reacted with the sugar, whereas flavonols and flavons did not. Thus, we propose that a mechanism of *C*-glycoside formation takes place as shown in Fig. 2, *i.e.* analogously to the rearrangement during epimerization.

3.2 *In vitro* and *ex vivo* studies of the gastrointestinal metabolism of flavan-3-ol *C*-glycosides

To investigate their behavior in the human gastrointestinal tract, four of the synthesized substances, *i.e.* (+)-epicatechin 8-*C*-β-D-glucopyranoside **(1a)**, (+)-epicatechin 6-*C*-β-D-glucopyranoside **(1d)**, (+)-catechin 6-*C*-β-D-galactopyranoside **(2b)** and (+)-catechin 6-*C*-β-D-rhamnopyranoside **(3b)** were incubated with saliva and simulated gastric juice under aerobic conditions as well as with ileostomy and colostomy fluids under anaerobic conditions. Compounds **(1a)**, **(1d)**, **(2b)** and **(3b)**, representing all three glycoside moieties, were chosen for incubation experiments due to their high purity and yield. The identification of flavan-3-ol *C*-glycosides in the incubation samples was performed by comparison of retention time, MS data and UV-spectrum with that of the authentic reference substances.

3.2.1 *Ex vivo* incubations in human saliva

The *C*-glycosides **1a**, **1d**, **2b** and **3b** were incubated *ex vivo* for 0, 0.5, 1, 1.5 and 2 min at 37°C in diluted human saliva. In the course of these experiments no significant decline in *C*-glycoside concentration was observed (data not shown), indicating the stability of flavan-3-ol *C*-glycosides in the oral cavity. Fast hydrolysis of different flavonoid *O*-glucosides by microflora and epithelial cells during incubation in human saliva was shown by Walle *et al.*, but flavonoid *O*-rhamnosides and *O*-rutinosides, respectively, were not degraded [23]. Our studies indicate that glucose becomes also resistant to hydrolysis by human saliva when bound *C*-glycosidically to the flavonoid molecule.

3.2.2 *In vitro* incubations in simulated gastric juice

The *C*-glycosides **1a**, **1d**, **2b** and **3b** were also tested for their stability in the acid milieu of the stomach. The samples were incubated in simulated gastric juice (pH 1.8) at 37°C for 0, 0.5, 1, 1.5, 2 and 3 h. The substrates were found to be stable during the whole duration of the experiment (data not

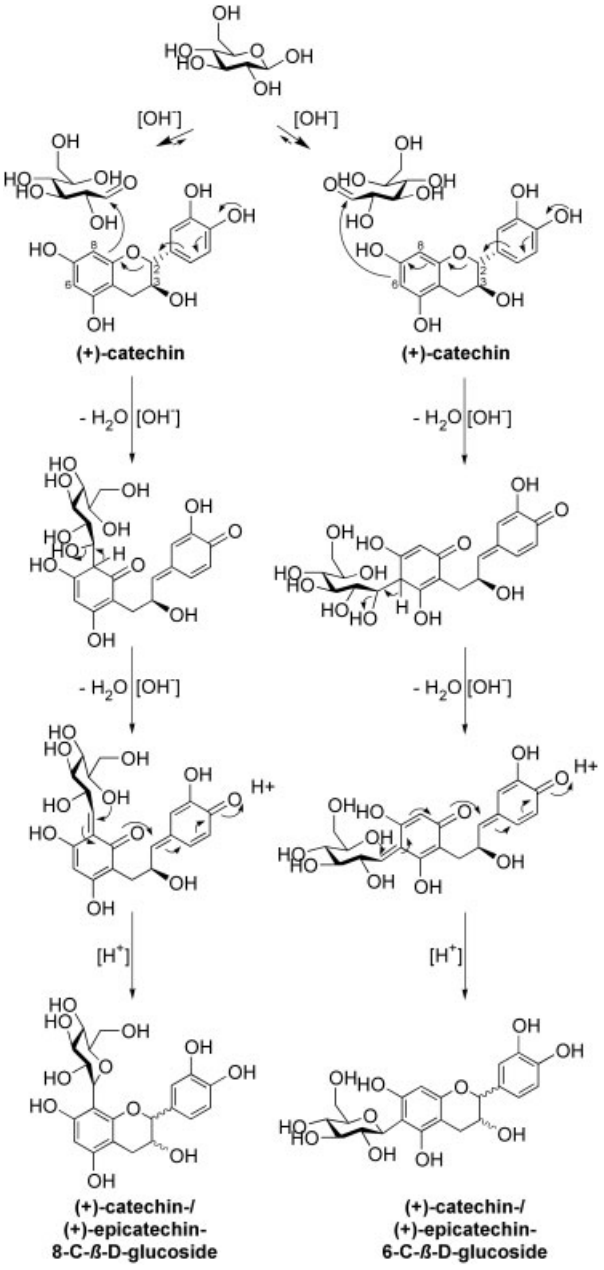

Figure 2. Proposed mechanism for the formation of (+)-catechin-8-*C*-β-D-glucosid, (+)-epicatechin-8-*C*-β-D-glucosid, (+)-catechin-6-*C*-β-D-glucosid, (+)-epicatechin-6-*C*-β-D-glucosid by reaction of (+)-catechin and glucose.

shown). In previous studies, it has been shown that most of the flavonoid *O*-glycosides resist acid degradation in the stomach [18]. In comparison, *C*-linked glycosides are even more resistant to cleavage under acidic conditions [1]. Thus, in accordance with our results, they are supposed to definitely pass the stomach undegraded. This finding is consistent with literature data showing that flavan-3-ol monomers and polymers also resist degradation in the acidic conditions of the stomach [18, 32].

3.2.3 *Ex vivo* incubations in ileostomy fluid

The *C*-glycosides **1a**, **1d**, **2b** and **3b** were incubated in ileostomy fluid diluted with carbonate-phosphate buffer (pH 6.3) at 37°C for 0, 0.5, 1, 2, 5, 7 and 10 h. With recovery rates found between 98–115% no significant degradation of **1d** and **3b** during 10 h of incubation in ileostomy fluid was detected (data not shown). Due to coelution of matrix compounds the content of *C*-glycosides **1a** and **2b** could not be determined in the incubation samples. In contrast to these results obtained with *C*-glycosides, it is known that many of the orally ingested polyphenol *O*-glycosides are metabolized in the small intestine [13, 18]. Due to their β-glycosidic structure they are not cleaved by pancreatic enzymes; hydrolysis of polyphenol *O*-glycosides is primarily attributed to the enzymatic activity of the ileal microflora. Furthermore, lactase phlorizin hydrolase, a membrane-bound β-glycosidase, and cytosolic β-glucosidase are involved in ileal flavonoid hydrolysis [13, 18]. The rate of hydrolysis is strongly influenced by the type of sugar moiety [33]. In comparison to glucosidase, expressed by almost all bacteria species, the activity of specific enzymes hydrolysing other glycosidic bondings is reduced or even completely absent in the ileum. As during our experiments no degradation of *C*-glycoside substrates occurred, we suppose that in the used ileostomy fluid there are no intestinal bacteria species expressing enzymes able to cleave *C*-glycosidic bonds.

3.2.4 *Ex vivo* incubations in colostomy fluid

In addition, the *C*-glycosides **1d** and **2b** were incubated in colostomy fluid diluted with carbonate-phosphate-buffer (pH 7.4) at 37°C for 0, 2, 4, 6, 8, 10 and 24 h. Figure 3 shows the mass recoveries of *C*-glycosides in the incubation samples. In contrast to the results obtained in our experiments with saliva, simulated gastric juice and ileostomy fluid, the substrates were not stable in colostomy fluid. (+)-Epicatechin 6-*C*-β-D-glucopyranoside (**1d**) was completely metabolized between 2 and 4 h. Degradation of (+)-catechin 6-*C*-β-D-galactopyranoside (**2b**), however, reached only 16 ± 2 % within 4 h and the compound was detectable up to 8 h of incubation. Linear degradation rates of the flavan-3-ol *C*-glycosides (+)-epicatechin 6-*C*-β-D-glucopyranoside (**1d**) and (+)-catechin 6-*C*-β-D-galactopyranoside (**2b**) in colostomy fluid differed significantly. According to the literature [17] microbial metabolism of flavan-3-ols is not influenced by the stereochemistry of the aglycon. Thus, varying degradation rates can be ascribed to the effect of sugar moiety. Human colonic content contains 10^{11} to 10^{12} bacteria *per* gram consisting mainly of anaerobics as *Bacteroides, Eubacterium* and *Clostridium* species [34]. Due to its microflora in comparison to the other parts of the gastrointestinal tract, the colon has the highest catalytic and hydrolytic potential and exhibits extensive enzymatic

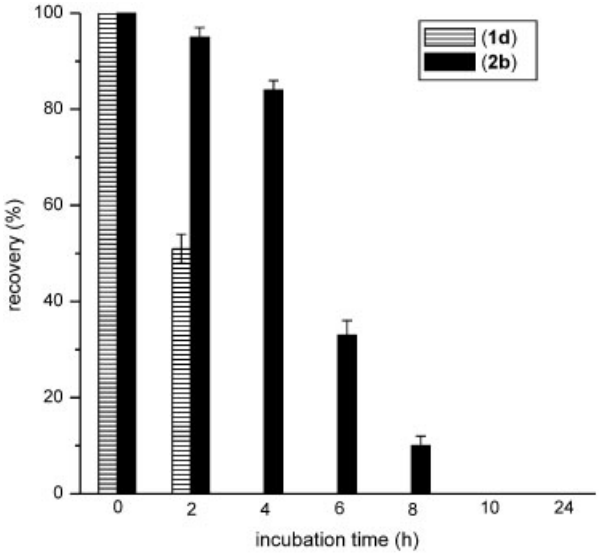

Figure 3. Recovery of flavan-3-ol *C*-glycosides (+)-epicatechin-6-*C*-β-D-glucoside (**1d**) and (+)-catechin-6-*C*-β-D-galactoside (**2b**) during incubation in colostomy fluid for 24 h. The amount detected at $t = 0$ h was set at 100%. Data expressed as mean ± SD ($n = 2$).

activity, *e.g.*, leading to hydrolysis of glycosides, esters, lactones, as well as ring-cleavage, reduction and demethylation reactions [13, 35]. Specific *C*-glycosidase activity of intestinal bacteria isolated from human feces was described by Jin *et al.* [36] Thus, the detected degradation of flavan-3-ol *C*-glycosides in colostomy fluid is ascribed to the enzymatic activity of the microflora. Recently, one of our group has studied the *ex vivo* stability of the flavone *C*-glucoside vitexin in pig cecum being a suitable model system for intestinal metabolism in humans. In accordance with our current data degradation of the compound was observed [14].

4 Concluding remarks

We prepared and characterized flavan-3-ol *C*-glycosides from the reaction of (+)-catechin and various sugars, proposing a novel reaction mechanism for their formation. Our work revealed that the examined flavan-3-ol *C*-glycosides are stable in saliva, simulated gastric juice as well as ileostomy fluid and are degraded in colostomy fluid.

Zhang *et al.* have studied the metabolization of orientin, homoorientin, vitexin and isovitexin in rats. In agreement with the results obtained in our study, these flavone *C*-glycosides largely reached the colon unmetabolized where they were degraded into small phenolic molecules following a metabolic pathway that consists of deglycosylation and opening of the heterocyclic C-ring [20]. In contrast, many flavonoid *O*-glycosides are already hydrolyzed in the small intestine by enzymatic activity of intestinal microbiota, lactase phlorizin hydrolase or cytosolic β-glucosidase, allowing absorption of the liberated aglycons by passive diffusion through the intestinal epithelium [13, 18]. As flavan-3-ol *C*-glycosides pass the small intestine unmodified, absorption of the intact glycosides must occur if the substances are bioavailable. Studies with the *C*-linked dihydrochalcone aspalathin from rooibos revealed that it is absorbed as intact *C*-glucoside from the small intestine in humans and pigs [6, 37, 38]. Liang *et al.* identified the intact vitexin rhamnoside in plasma after its oral administration to rats [39]. Thus, the intestinal absorption of flavan-3-ol *C*-glycosides is as well considered to be possible. Due to their hydrophilic nature, flavonoid *C*-glycosides are unlikely absorbed by passive diffusion. Instead, the action of membrane carriers, *e.g.* sodium-dependent glucose transporter 1 is more feasible.

Up to date there are no studies on the biological properties of flavan-3-ol *C*-glycosides, but various flavone-, isoflavone- and chalcone *C*-glycosides have already been examined [40–42]. Based on their polyphenolic structure these *C*-glycosyl flavonoids are potent antioxidants. Furthermore, anti-inflammatory, antiviral, antibacterial, anxiolytic and hypoglycemic effects have been found in various *in vitro* test systems [43]. Due to their structural similarity the flavan-3-ol derivatives may exhibit related properties.

Our studies revealed the long-lasting presence of flavan-3-ol *C*-glycosides in the gastrointestinal tract, and we assume an intestinal absorption of the substances. Thus, local effects of flavan-3-ol *C*-glycosides in the gut as well as systemic effects in tissues and cells should be considered. To clarify the biological activity of flavan-3-ol *C*-glycosides their biological properties as well as their absorption characteristics have to be studied.

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