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Bitter Taste Impact and Thermal Conversion of a Naringenin Glycoside from *Cyclopia genistoides*

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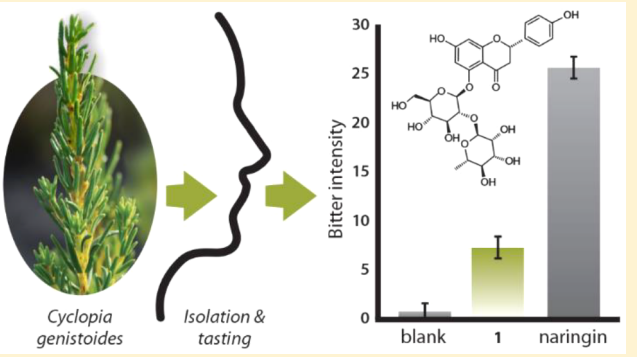
Supporting Information

ABSTRACT: A naringenin derivative, isolated from *Cyclopia genistoides*, a bitter tasting herbal tea, especially when in green (unoxidized) form, was identified as (2*S*)-5-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]naringenin (**1**). The compound partially epimerizes to (2*R*)-5-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]naringenin (**2**) when heated at different temperatures (80, 90, 100, 110, and 120 °C) for a prolonged period in a phosphate buffer at pH 5. The fractional conversion model predicted the decrease in the concentration of compound **1** the best. The activation energy of the conversion reaction was calculated as 99.16 kJ mol^{-1}. Prolonged heating resulted not only in formation of compound **2** but eventually a decrease in its concentration and the formation of another conversion product, (*E*)-2′-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]-4′,6′,4-trihydroxychalcone (**3**). In contrast, naringin, glycosylated at C-7, remained stable when heated under the same conditions (100 °C for 6 h at pH 5). The bitter intensity of compound **1** was substantially less than that of naringin, both tested at 0.04 mM, a concentration typical of compound **1** in an herbal tea infusion of green *C. genistoides*. This comparison indicates that the position of the sugar moiety plays an important role in determining both bitter intensity and heat stability of naringenin glycosides.



Bitter taste plays an important role in the consumer acceptance of food and beverages. When inherent to the sensory quality of the product, it could be one of the drivers of liking,[1] but mostly bitterness influences consumer response negatively.[2] The aversion to bitter taste is considered to be a defense mechanism against the ingestion of potential poisons.[3] The presence of bitter taste receptors in the gut and their role in the release of gut hormones involved in the control of food intake, however, emphasize a new role for bitter compounds in the fight against obesity.[4]

Cyclopia genistoides Vent. is one of several *Cyclopia* species used to produce the herbal tea honeybush. This herbal tea has a notable bitter taste, contrary to the slightly sweet tasting herbal teas prepared from other commercially important *Cyclopia* species. The bitter taste is especially prominent in infusions prepared from green *C. genistoides*, a product produced by excluding the high-temperature oxidation step in the manufacture of traditional honeybush tea.[5] Insight into the phenolic composition of *C. genistoides* and the stability of its phenolic constituents during thermal processing may help to elucidate their role in the bitter taste of this herbal tea. Phenolic compounds elicit a bitter taste in many foods and beverages. One such compound is naringin, a potent bitter naringenin derivative present in grapefruit.[2] Beelders et al.[6] tentatively identified a naringenin-hexosyloxy-deoxyhexosyloxy derivative (compound **1**) with the same molecular mass as naringin in *C. genistoides*. Different elution times on a reversed-phase HPLC column indicated structural differences between naringin and compound **1**. Furthermore, high-temperature oxidation, performed at 90 °C for 16 h according to recommended conditions for optimum aroma and flavor development of traditional honeybush tea,[5] lowered the concentration of **1** in the plant material and also led to formation of an isomer (compound **2**).[6] The objectives of the present study were therefore to unambiguously elucidate the structure of the two compounds, herewith identified as the diastereomers (2*S*)-5-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]naringenin (**1**) and (2*R*)-5-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]naringenin (**2**). Another conversion product, *E*-2′-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]-4′,6′,4-trihydroxychalcone (**3**), is reported here for the first time. Additionally, the bitter intensity

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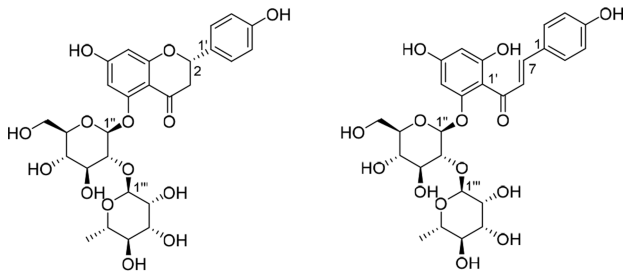
of compound **1** was determined to assess its potential impact on the taste of honeybush tea. Heating experiments provided kinetic data for calculation of the activation energy required for conversion of compound **1** to **2** in a phosphate buffer at pH 5, a pH relevant for a "natural" iced tea beverage (no acids added). Naringin was included in sensory analysis and heat experiments to enhance current knowledge of the effect of the position of the sugar moiety on taste and thermal stability of naringenin derivatives.

RESULTS AND DISCUSSION

Structure Elucidation. Compound **1** was heated in phosphate buffer (pH 5.0) at 90 °C for 4 h. Conversion products were separated by semipreparative RP-HPLC to yield compounds **2** and **3** as major and minor products, respectively. ^{1}H and 2D NMR spectra, GC-MS sugar analysis, and HR-ESIMS spectra of compounds **1**−**3** as well as UV spectra of compound **1** are supplied in the Supporting Information (Figures S1 to S19).



Compound **1** was assigned a molecular formula of $C_{27}H_{32}O_{14}$, according to HR-ESIMS analysis (m/z 581.1865 $[M + H]^+$, calcd 581.1865). ^{1}H and 2D NMR data (Table 1) indicated naringenin bearing a disaccharide moiety attached to C-5 via an *O*-glycosidic linkage. The sugars were identified by GC-MS analysis after derivatization with L-cysteine methyl ester and subsequent silylation as L-rhamnose and D-glucose[7] (Figures S13 and S15, Supporting Information). The coupling constants of the anomeric protons at δ_H 5.16 (1H, d, J = 6.9 Hz, H-1″) and 5.19 (1H, br s, H1‴) led to assignment of β- and α-configurations for the D-glucopyranose and L-rhamnopyranose moieties, respectively. HMBC correlations (Figure 1) between δ_H 5.19 (1H, br s, H1‴) and δ_C 76.6 (C-2″) and between δ_H 5.16 (1H, d, J = 6.9 Hz, H-1″) and δ_C 158.8 (C-5) established the attachment positions of the two sugars, and **1** was thus identified as 5-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]naringenin.

Compound **2** was assigned a molecular formula of $C_{27}H_{32}O_{14}$ (HR-ESIMS m/z 581.1865 $[M + H]^+$, calcd 581.1865). ^{1}H and 2D NMR data (Table 1) closely resembled those of compound **1**. Given that the same COSY, HMBC, and NOESY correlations were observed, compound **2** had to be a diastereoisomer of **1**.

The absolute configuration of compounds **1** and **2** was determined by electronic circular dichroism (ECD), since those compounds possess a carbonyl group and two aromatic rings close to the C-2 stereocenter. Both compounds exhibited the same UV spectrum with maxima at 227 and 281−282 nm (Figure S19, Supporting Information). For compound **1**, the experimental ECD spectrum (Figure 2) exhibited positive Cotton effects (CEs) at 216 and 331 nm along with a negative CE at 286 nm, the latter being indicative of the (2*S*) absolute configuration of the flavanone unit.[8,9]

The experimental ECD spectrum of compound **2** (Figure 2) showed two high-amplitude negative CEs at 217 and 235 nm, a low-amplitude negative CE at 331 nm, and a low-amplitude positive CE at 283 nm. The latter Cotton effect is reminiscent of a (2*R*) absolute configuration of the flavanone moiety.[8,9] In conclusion, compound **1** was assigned as (2*S*)-5-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]naringenin, and compound **2** as (2*R*)-5-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]naringenin. Previously, only 5-[α-L-rhamnopyranosyl-(1→2)-α-D-glucopyranosyloxy]naringenin isolated from *Cyclopia intermedia* has been published,[10] albeit under the incorrect name of 5-*O*-α-D-rutinosylnaringenin, and identified from the peracetate derivative. Therefore, NMR spectroscopic data of 5-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]naringenin and the absolute configuration of both diastereoisomers are reported here for the first time.

Compound **3** also had a molecular formula of $C_{27}H_{32}O_{14}$, as deduced from its HR-ESIMS data (m/z 581.1879 $[M + H]^+$, calcd 581.1865). ^{1}H and HSQC NMR data were similar to those of compound **1**. The difference was in the presence of signals for a 1,2-disubstituted vinylic moiety [δ_C 125.1, δ_H 7.92 (d, J = 15.6) and δ_C 142.1, δ_H 7.55 (d, J = 15.6)] instead of the methylene and methine signals of the flavanone. An *E*-configuration was indicated by the vicinal coupling of 15.6 Hz and a correlation in the HMBC spectrum with the resonance at δ_C 192.2 (Figure 1) signifying an (*E*)-α,β-unsaturated carbonyl group. Since the molecular formula and, thus, the index of hydrogen deficiency were the same as for compound **1**, these data indicated that the C-ring of **1** was cleaved during the heating to produce naringenin chalcone glycoside **3**. GC-MS analysis confirmed the presence of D-glucose and L-rhamnose as sugar constituents (Figures S14 and S15, Supporting Information). Naringin chalcone, a regioisomer of **3**, has been previously isolated from grapefruit,[11] but (*E*)-2′-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]-4′,6′,4-trihydroxychalcone (**3**) has not been reported before.

Thermal Conversion of Compound 1. The temperature range 80 to 120 °C used to test conversion was governed by likely conditions encountered when a *Cyclopia* extract is used as a functional ingredient in a variety of food and beverage products, such as food bars, bread, and iced tea. This will expose the extract to high temperatures during food processing, i.e., heat sterilization (121 °C) of "natural" honeybush ready-to-drink iced tea (pH ∼5) to ensure stability against microbial spoilage.

Heating of dilute solutions of compound **1** in a 0.1 M phosphate buffer (pH 5) at the various temperatures decreased its concentration, with increasing temperature accelerating the process (Figure 3). Fitting of the data to different kinetic models revealed that the decrease in the concentration of compound **1** could be best predicted by the fractional conversion model ($R^2 \geq 0.97$). The first-order kinetic model, commonly used to predict changes in quality parameters of food products during thermal processing,[12] gave a good fit only when the solution was heated at 80 °C. The reaction rate constants (k) varied from 0.36 to 6.64 h^{-1} (Table 2), and their temperature-dependence complied with the Arrhenius equation. The activation energy of the reaction was calculated as 99.16 kJ mol^{-1} (R^2 = 0.9704), which is slightly lower than that of mangiferin (107 kJ mol^{-1}),[13] the major phenolic compound of *C. genistoides* and determined under the same conditions.

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Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectroscopic Data for Compounds 1−3 (DMSO-d_6; δ in ppm, J in Hz)

	1		2		3	
position	δ_C, type[b]	δ_H (J in Hz)	δ_C, type[b]	δ_H (J in Hz)	δ_C, type[b]	δ_H (J in Hz)
1					126.6, C	
2	78.0, CH	5.32, dd (12.7, 2.6)	78.5, CH	5.30, dd (12.4, 2.9)	130.9, CH	7.59, d (8.5)
3	44.7, CH	2.97, dd (15.2, 12.8)	45.2, CH	2.91, dd (16.8, 12.5)	116.5, CH	6.81, d (8.5)
		2.51, dd (15.7, 2.6)		2.58, dd (16.6, 2.9)		
4	187.5, C		187.9, C		160.5, C	
5	158.8, C		159.9, C		116.5, CH	6.81, d (8.5)
6	95.2, CH	6.19, s	96.0, CH	6.22, s	130.9, CH	7.59, d (8.5)
7	163.7, C		165.0, C		142.1, CH	7.55, d (15.6)
8	96.3, CH	5.98, s	97.0, CH	6.01, s	125.1, CH	7.92, d (15.6)
9	163.4, C		164.5, C		192.2, C	
10	104.6, C		105.6, C			
1′	129.2, C		129.6, C		106.7, C	
2′	127.7, CH	7.28, d (8.2)	128.4, CH	7.27, d (8.5)	160.3, C	
3′	115.0, CH	6.79, d (7.9)	115.7, CH	6.79, d (8.2)	95.5, CH	6.21, d (1.5)
4′	157.2, C		158.1, C		166.0, C	
5′	115.0, CH	6.79, d (7.9)	115.7, CH	6.79, d (8.2)	97.9, CH	5.95, d (1.8)
6′	127.7, CH	7.28, d (8.2)	128.4, CH	7.27, d (8.5)	166.0, C	
1″	97.5, CH	5.16, d (7.0)	98.3, CH	5.14, d (7.3)	98.5, CH	5.34, d (7.3)
2″	76.6, CH	3.60, dd (7.5, 7.5)	76.7, CH	3.55−3.65[a]	78.9, CH	3.45−3.55[a]
3″	76.8, CH	3.43−3.56[a]	77.8, CH	3.45−3.54[a]	77.4, CH	3.45−3.55[a]
4″	69.5, CH	3.32, dd (9.0, 9.0)	70.2, CH	3.32, dd (9.2, 9.2)	70.4, CH	3.25, dd (9.0, 9.0)
5″	76.6, CH	3.35−3.41[a]	77.3, CH	3.34−3.41[a]	77.7, CH	3.33−3.38[a]
6″	60.4, CH_2	3.65−3.73[a]	61.1, CH_2	3.65−3.70[a]	60.9, CH_2	3.68, m
		3.43−3.56[a]		3.45−3.54[a]		3.45−3.55[a]
1‴	99.4, CH	5.19, br s	99.9, CH	5.23, br s	101.3, CH	5.03, br s
2‴	70.2, CH	3.65−3.73[a]	71.0, CH	3.65−3.70[a]	70.9, CH	3.77, m
3‴	70.1, CH	3.35−3.41[a]	70.9, CH	3.34−3.41[a]	71.0, CH	3.33−3.38[a]
4‴	71.9, CH	3.12, dd (9.3, 9.3)	72.6, CH	3.17, dd (9.5, 9.5)	72.4, CH	3.09, dd (9.3, 9.3)
5‴	68.2, CH	3.43−3.56[a]	68.8, CH	3.55−3.65[a]	68.9, CH	3.41, m
6‴	17.5, CH_3	0.99, d (6.1)	18.4, CH_3	1.05, d (6.1)	17.8, CH_3	0.81, d (6.1)

[a]Overlapping signals. [b]From inverse detected HSQC and HMBC experiments.

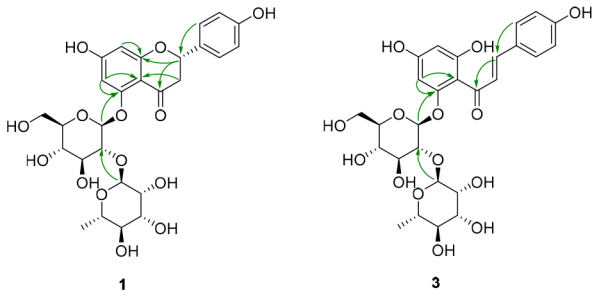

Figure 1. Key HMBC correlations (green arrows) of compounds 1 and 3.

Formation of compound 2 at the various temperatures showed a progressive shift in formation/degradation (Figure 3). After 6 h at 80 °C the concentration of compound 2 was still increasing, at 90 °C the maximum was reached and degradation had started, and at 100, 110, and 120 °C, the optimum was reached earlier with each higher temperature. Degradation also became more pronounced as temperature increased. Naringin remained stable over 6 h when heated at 100 °C, indicating that glycosylation at C-7 instead of C-5 conferred increased thermal stability. Heating of an aqueous naringin solution at ca. pH 6.6 at 100 °C also showed little degradation and k values of 0.0004 and 0.0016 min^{-1} at 110 and 120 °C, respectively.[14]

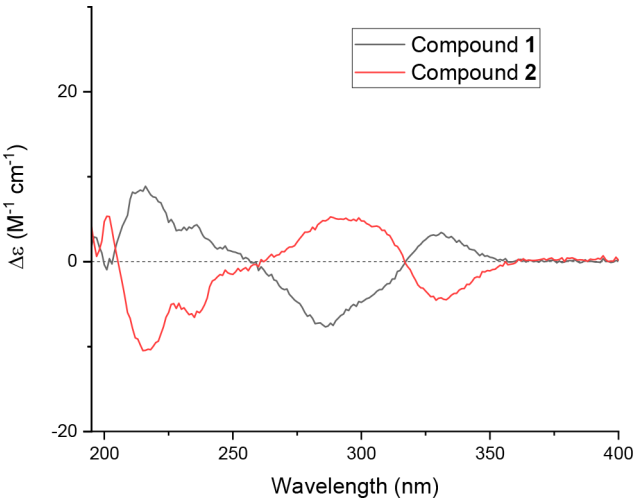

Figure 2. Experimental ECD spectra of compounds 1 and 2 in MeOH (0.1 mg/mL).

The appearance of compound 3 and other minor compounds in the reaction mixtures (Figure S20, Supporting Information) indicates conversion of the flavanone to a chalcone and degradation of 2 and/or 3. Flavanones are mostly stable in acidic and neutral solutions of protic solvents, conditions favoring cyclization.[15,16] At pH 5 the reaction

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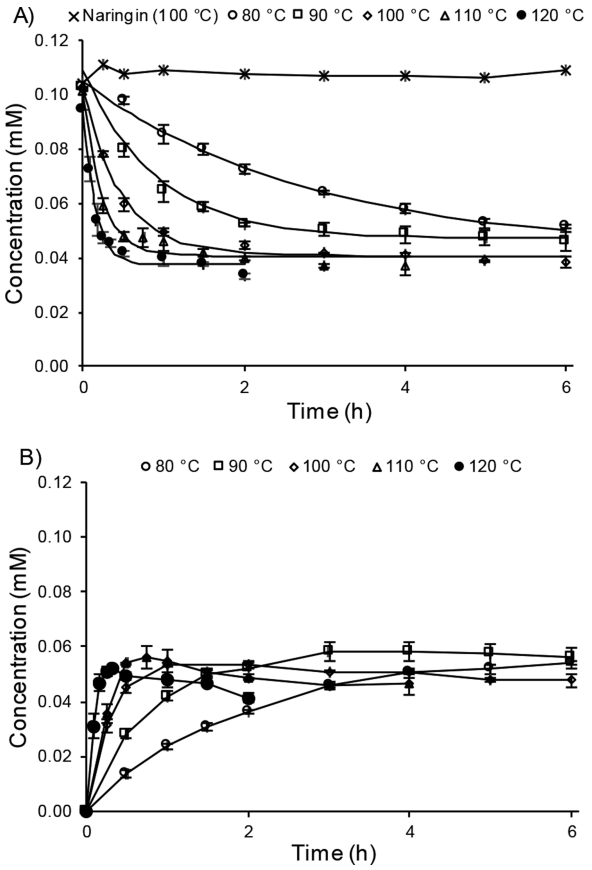

Figure 3. Change in the concentration of compounds **1** (A) and **2** (B) when **1** was heated in an aqueous phosphate buffer at pH 5 at temperatures ranging from 80 to 120 °C. Data points for **1** were fitted to the fractional conversion model (smooth lines). Naringin (A) was heated under the same conditions at 100 °C. The initial concentration of the compounds was 0.1 mM. Data indicated by markers are means ± standard deviation.

equilibrium would thus be toward cyclization and not ring opening, explaining the limited formation of compound **3**.

Sensory Analysis. Descriptive sensory analysis confirmed the discrimination in bitter taste intensity between compound **1** and naringin (Figure 4). Compound **1**, tested at a concentration similar to what could be expected in a *C. genistoides* infusion, was only slightly bitter, whereas naringin, at the same concentration, was considerably more bitter. Their bitterness was rated 7 and 26, respectively, on a 100-point scale. A bitter intensity of 7 is just perceptible. During panel training panelists noted a difference in mouthfeel between compound **1** and naringin. Compound **1** appeared "smooth", while naringin had a delayed bitter taste, only reaching maximum bitterness after ~2 s in the mouth and lingering on

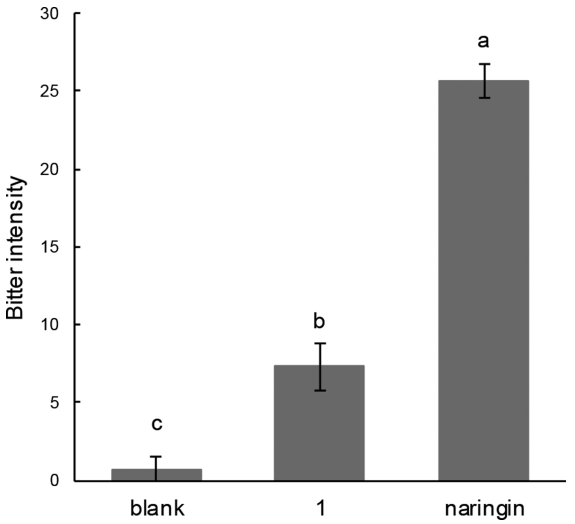

Figure 4. Bitter intensity of compound **1** compared to naringin at the same concentration (24 mg/L; 0.04 mM). Samples were prepared in hot water (blank) and served hot (60 °C) to a trained sensory panel (n = 8). Different letters indicate a significant difference ($p < 0.05$) in mean values. Error bars indicate standard deviation.

the tongue for several minutes after tasting. Many flavanones and flavanone glycosides are known to affect bitter taste, either acting as agonists of bitter receptors or by masking the bitter taste of other compounds.[17,18] Molecular structure is important, especially the substitution pattern as indicated by Roland et al.[19] for (iso)flavonoid aglycones. In this case compound **1** and naringin differ only with regard to the location of the sugar moiety on the aglycone. Interestingly, a (1→6) linkage of the rhamnose to the glucose moiety instead of a (1→2) linkage (rutinose instead of neohesperidose) also eliminates the bitter taste.[20] The C-2 configuration of the flavanone may also play a role in the interaction with bitter receptors and, hence, in the sensory quality of the compounds. For example, L-enantiomers of tryptophan and phenylalanine elicit a bitter taste and even a cellular TAS2R response,[21] while the D-enantiomers have a distinct sweet taste.[22] However, the effect of the conversion of compound **1** to **2** on taste is not known at present, due to limited availability of **2** for sensory analysis. Results obtained by Gaffield et al.[20] for the 2*S* and 2*R* stereoisomers of naringin were inconclusive, but suggest the possibility that conversion of **1** to **2** could have little effect on bitterness.

EXPERIMENTAL SECTION

General Experimental Procedures. HPLC-grade solvents were obtained from Macron Fine Chemicals (Avantor Performance Materials, Phillipsburg, NJ, USA) and Merck (Darmstadt, Germany).

Table 2. Kinetic Parameters for the Degradation of Compound 1 Determined by Fitting Data to the Fractional Conversion Model[a]

temperature	equilibrium concentration (C_∞, mM)	initial concentration (C_0, mM)	reaction rate constant (k, h^{-1})	R^2
80 °C	0.0430 ± 0.0046 b	0.1047 ± 0.0002 a	0.36 ± 0.06 e	0.9914
90 °C	0.0471 ± 0.0006 a	0.1027 ± 0.0008 b	1.11 ± 0.07 d	0.9845
100 °C	0.0407 ± 0.0006 bc	0.1018 ± 0.0001 b	2.11 ± 0.14 c	0.9920
110 °C	0.0406 ± 0.0009 bc	0.1005 ± 0.0002 c	4.26 ± 0.39 b	0.9716
120 °C	0.0374 ± 0.0002 c	0.0951 ± 0.0008 d	6.64 ± 0.27 a	0.9781

[a]Values are mean ± standard error. Different letters indicate a significant difference at $p > 0.05$.

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DMSO-d_6 was purchased from Armar Chemicals (Döttingen, Switzerland). Analytical-grade formic acid, XAD porous resin (Amberlite, 20−60 mesh), analytical-grade EtOH, MeOH, DMSO, anhydrous pyridine, caffeine for sensory analysis, L-ascorbic acid, L-cysteine methyl ester hydrochloride, and hexamethyldisilazane/trimethylchlorosilane/pyridine (3:1:9) were purchased from Merck. L-Glucose and D-glucose were obtained from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA), while L-rhamnose and D-rhamnose were sourced from Carbosynth (Compton, UK). HCl and analytical-grade EtOAc were purchased from Scharlau (Scharlab S. L.) (Barcelona, Spain). Deionized water and ultrapure water were prepared using an Elix and Milli-Q water purification system (Merck).

Optical rotations were measured in MeOH on a Jasco P-2000 digital polarimeter (Tokyo, Japan) equipped with a sodium lamp (589 nm) and a 10 cm length temperature-controlled microcell. UV and ECD spectra were recorded in MeOH on a Chirascan spectrometer (Applied Photophysics, Leatherhead, UK) with 1 mm path precision cells 110 QS (HellmaAnalytics, Müllheim, Germany). NMR spectra were recorded in DMSO-d_6 on a Bruker AVANCE III 500 MHz spectrometer (Billerica, CA, USA) operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. Measurements were performed with a 1 mm TXI probe at 18 °C. Data were processed with Bruker TopSpin 3.5 software. HR-ESIMS data were recorded in positive ion mode on a Shimadzu LC-20A system (Kyoto, Japan) with a Thermo Scientific Orbitrap LTQ XL detector (Waltham, CA, USA) and a Waters SunFire C_{18} column (5 μm, 150 × 10 mm i.d.). Data acquisition was recorded using Xcalibur 2.1 software. Fragmentation data (MS^E using a collision energy ramp from 20 to 60 V) were obtained by analysis in the negative ion mode on an Acquity UPLC system coupled to a Synapt G2 Q-TOF equipped with an electrospray ionization source (Waters, Milford, MA, USA). The LC inlet method used a Kinetex C_{18} column (2.6 μm, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA) as described by Beelders et al.[6] Preparative HPLC was performed on a Waters preparative LC, with autosampler, variable UV−visible detector, and fraction collector, equipped with a Gemini-NX C_{18} preparative column (5 μm; 150 × 21.2 mm, 100 Å; Phenomenex), protected by a guard column with the same stationary phase. Semipreparative HPLC was performed on an Agilent 1100 Series instrument with a DAD detector (Santa Clara, CA, USA) and equipped with a Waters SunFire C_{18} column (5 μm, 150 × 10 mm i.d.), protected by a guard column (10 × 10 mm i.d.; Waters). Data acquisition and processing were performed using ChemStation software. GC-MS analysis was performed on an Agilent G1530A equipped with an RXI 5 MS column (25 m × 0.2 mm i.d., 0.3 μm).

Plant Material. Shoots of *C. genistoides* Vent. (genotype GK5 of the ARC Honeybush Plant Breeding Programme) were harvested at an evaluation site (GPS coord. −34.702, 19.618), shredded, dried (40 °C/16 h), and sieved to obtain the tea bag fraction (<1.68 mm, >0.42 mm; retention sample CGN_L0152).

Isolation of Compound 1. A freeze-dried, hot water extract (120 g), prepared from the plant material (600 g) as described by Beelders et al.,[23] was sonicated in EtOH (1:10 m/v) for 60 min. The EtOH-soluble fraction was recovered and freeze-dried, and 15 g of the fraction, suspended in 50 mL of HPLC-grade water, was further fractionated on a dechlorinated XAD (Amberlite, 20−60 mesh) column (68 × 500 mm). An EtOH/water gradient at a flow rate of 38 mL/min was employed: 0% EtOH (4.5 L), 5% EtOH (4.5 L), 10% EtOH (6 L), 20% EtOH (4.5 L), 30% EtOH (4.5 L), 100% EtOH (4.5 L). Column fractions (750 mL) were monitored by HPLC-DAD, and those containing flavanones (fractions 29 to 38) were pooled, vacuum-evaporated, and freeze-dried, yielding a 4 g fraction. An aliquot of the XAD fraction (2 g) was dissolved at 5 mg/mL (as limited by solubility) in a 23% MeOH/water solution containing 10% DMSO (v/v), and 5000 μL (maximum capacity of injection loop) was repeatedly injected on preparative HPLC, employing a gradient solvent program (solvent A: 0.1% formic acid(aq); solvent B: 100% MeOH): 0−2 min (23% B), 2−15 min (23−24% B), 15−16 min (24−90% B), 16−17 min (90% B), 17−18 min (90−23% B), and 18−25 min (23% B); 35 °C; flow rate 21.2 mL/min) to yield compound **1** (80 mg, t_R = 13.3 min; 97% purity based on LC-MS).

Isolation of Compounds after Heat Treatment. Compound **1** (15.1 mg) was dissolved in water (10 mg/mL) and heated for 4 h at 90 °C. The resulting mixture was purified by semipreparative RP-HPLC [0.1% aq. formic acid (A), 0.1% formic acid in MeCN (B); 0−5 min (5−15% B), 5−20 min (15% B), 20−40 min (15−60% B); flow rate 4 mL/min; injection volume 4 × 250 μL, room temperature] to yield compound **2** (3.1 mg, t_R 14.4 min), compound **1** (5.3 mg, t_R 15.9 min) and compound **3** (0.6 mg, t_R = 28.1 min).

*(2S)-5-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]-naringenin (**1**):* yellow, amorphous powder; $[\alpha]^{25}_D$ −68.0 (*c* 0.17, MeOH); UV λ_{max} (MeOH) (log ε) 227 (3.08), 281 (2.81) nm; ECD (MeOH, *c* 1.8 × 10^{-4} M, 1 mm path length) $\lambda_{max}(\Delta\varepsilon)$ 216 (+8.88), 286 (−7.67), 331 (+3.42) nm; ^{1}H and ^{13}C NMR, see Table 1; HR-ESIMS *m/z* 581.1865 [M + H]$^+$ (calcd for $C_{27}H_{32}O_{14}^+$, 581.1865); MS^E fragment ions (negative ionization) *m/z* 459, 433, 313, 271*, 209, 169, 151, 145, 125.

*(2R)-5-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]-naringenin (**2**):* yellow, amorphous powder; $[\alpha]^{25}_D$ −72.3 (*c* 0.25, MeOH); UV λ_{max} (MeOH) (log ε) 227 (2.91), 282 (2.63) nm; ECD (MeOH, *c* 1.7 × 10^{-4} M, 1 mm path length) λ_{max} ($\Delta\varepsilon$) 216 (−10.48), 235 (−6.57), 294 (+5.11), 329 (−4.55) nm; ^{1}H and ^{13}C NMR, see Table 1; HR-ESIMS *m/z* 581.1865 [M + H]$^+$ (calcd for $C_{27}H_{32}O_{14}^+$, 581.1865); MS^E fragment ions (negative ionization) *m/z* 433, 271*, 227, 151, 145, 125, 119, 107.

*E-2′-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]-4′,6′,4-trihydroxychalcone (**3**):* ^{1}H and ^{13}C NMR, see Table 1; HR-ESIMS *m/z* 581.1879 [M + H]$^+$ (calcd for $C_{27}H_{32}O_{14}^+$, 581.1865); MS^E fragment ions (negative ionization) *m/z* 271*, 169, 151, 145, 125, 107.

Sugar Analysis. Compound **1**, **2**, or **3** (0.5 mg) was heated at 105 °C for 1 h in 1 mL of 2 M HCl. After extraction with EtOAc, the aqueous phase was lyophilized and resolubilized in 1 mL of anhydrous pyridine. Derivatization with L-cysteine methyl ester hydrochloride (200 μL, 60 °C, 1 h) and subsequently silylation with hexamethyldisilazane and Me_3SiH in pyridine (3:1:9; 200 μL; 60 °C, 30 min) were performed. Pyridine was evaporated prior to GC-MS analysis. The column temperature was kept at 60 °C for 1 min and then increased at 10 °C/min until 300 °C; L-rhamnose (t_R 21.29 min), D-rhamnose (t_R 21.39 min), D-glucose (t_R 22.59 min), L-glucose (t_R 22.73 min).

Thermal Conversion of Compound 1. The thermal degradation of **1** in 0.1 M phosphate buffer solution (pH 5) was assessed at five temperatures (80, 90, 100, 110, and 120 °C). Similarly, naringin was heated at 100 °C. The experiments were performed as previously described.[13] Aliquots (800 μL; *n* = 25) of the working solution of each compound (58 μg/mL = 0.1 mM, dissolved 0.1 M phosphate buffer solution) were transferred into 5 mL glass reaction vials (Merck). One aliquot served as unheated control while the remaining vials were heated in a preheated Stuart heating block with glycerin added to the cavities to improve heat transfer. Replicate samples (*n* = 3) were randomly removed at predetermined time points (*n* = 8), cooled, filtered (0.22 μm pore size, 4 mm diameter Millex-GV syringe filters; Merck), and analyzed using UHPLC-DAD.[13] The content of **1**, **2**, and naringin was quantified using the peak area at 288 nm.

Univariate analysis of variance (ANOVA) was performed on the data sets for each temperature (SAS, version 9.4; SAS Institute, Cary, NC, USA). The Shapiro−Wilk test was performed to test for normality. Fisher's least significant difference was calculated at the 5% level ($p < 0.05$) to compare means across treatment times. The kinetic data for compound **1** were fitted to zero-, first-, and second-order and fractional conversion models by nonlinear regression, using SAS. The fractional conversion model (eq 1) was selected based on goodness-of-fit of predicted and actual data (R^2).

$$C = C_\infty + (C_0 - C_\infty)\exp^{-kt} \quad (1)$$

where *C*, C_0, and C_∞ are the concentration of compound **1** (mM) at time *t*, time 0, and equilibrium conditions, respectively, *t* is the time in h, and *k* is the reaction rate constant (h^{-1}).

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The values of k, C_0, and C_∞ were compared over the different treatment temperatures (ANOVA) to establish significant differences at the 5% significance level. By fitting a linear regression for $\ln(k)$ on $1/T$ (T = absolute temperature in K) it was confirmed ($R^2 = 0.9768$, $p < 0.05$) that k was related to T according to the Arrhenius law (eq 2).

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (2)$$

where A is the pre-exponential factor, E_a is the activation energy, and R is the universal gas constant (8.314 J mol^{-1} K^{-1}).

To estimate E_a, a global model that includes both time and temperature effects, combining eq 1 with the reparametrized form of eq 2, was used:[24]

$$C = C_\infty + (C_0 - C_\infty)e^{[-k_{ref}e^{[-E_a/R(1/T-1/T_{ref})]t}]} \quad (3)$$

where k_{ref} is the reaction rate at the reference temperature (T_{ref}). T_{ref} was the average value of the temperature range evaluated (i.e., T_{ref} = 100 °C or 373.15 K).

The model parameters were estimated by nonlinear regression analysis using the NLIN procedure of SAS, solving the nonlinear least-squares problem, using the Marquart algorithm. The parameter's precision was evaluated by standard errors, and the quality of regression was assessed by the coefficient of determination (R^2) and randomness and normality of residuals, thus allowing best fit model parameters.

Sensory Analysis. The bitter taste of compound **1** was compared to that of naringin at a concentration similar to what could be expected in a *C. genistoides* infusion, by taking into account its concentration in the hot water extract (1.21 g/100 g) and the typical soluble solids content of a hot water infusion (2 g/L).[25] Samples (0.04 mM) were dissolved in hot water by stirring the solution for ca. 20 min in a jacketed flask connected to a circulating bath controlled at 70 °C. The three samples (hot water blank, compound **1**, and naringin) were presented to each panelist in three-digit blind coded 40 mL screw-cap amber vials (ca. 10 mL/serving). The vials were placed in metal racks and kept in a heated water bath (60 °C) during tasting to prevent precipitation. Samples were randomized in each session for each panelist. Panelists were also supplied with two marked reference samples, namely, a blank (hot water, 0 bitter intensity) and a bitter sample (caffeine, 0.4 g/L, bitter intensity of 45). Descriptive sensory analysis (DSA) was conducted under standardized conditions by a panel of eight judges experienced in the sensory analysis of green *C. genistoides* honeybush tea, specifically bitter taste. Panelists were assigned individual booths, and each sample was scored for bitter taste intensity on an anchored, unstructured 100-point line scale (0 = not bitter, 100 = extremely bitter), using Compusense Five software (Compusense, Guelph, Canada). Each sample set was presented to each panelist four times during four 20 min sessions with 10 min breaks scheduled between sessions to prevent panel fatigue. Distilled water, water biscuits, and dried apple pieces were provided between samples as palate cleansers, and a 2 min delay was scheduled between samples to minimize bitter taste carryover. Panel performance was monitored by applying Panelcheck software (version 1.4.0; Nofima, Ås, Norway) to the DSA data. Panel reliability was tested using an ANOVA model that included panelist, replicate, sample, and interaction effects.[26] The residuals were tested for non-normality, using the Shapiro–Wilk test, and outliers were identified and removed in the event of deviation from normality ($p \leq 0.05$). Subsequent ANOVA was conducted on means over judges, and Fisher's LSDs were calculated to compare treatment means ($p \leq 0.05$).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00710.

^{1}H and 2D NMR spectra of compounds **1**–**3**; GC-MS sugar analysis of compounds **1**–**3**; HR-ESIMS spectra of compounds **1**–**3**; UV spectra of compound **1**; HPLC chromatograms of compound **1** heated at 80, 90, 100, 110, and 120 °C for 2 h (PDF)

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