JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Human Psychometric and Taste Receptor Responses to Steviol Glycosides

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ABSTRACT: Steviol glycosides, the sweet principle of *Stevia Rebaudiana* (Bertoni) Bertoni, have recently been approved as a food additive in the EU. The herbal non-nutritive high-potency sweeteners perfectly meet the rising consumer demand for natural food ingredients in Europe. We have characterized the organoleptic properties of the most common steviol glycosides by an experimental approach combining human sensory studies and cell-based functional taste receptor expression assays. On the basis of their potency to elicit sweet and bitter taste sensations, we identified glycone chain length, pyranose substitution, and the C16 double bond as the structural features giving distinction to the gustatory profile of steviol glycosides. A comprehensive screening of 25 human bitter taste receptors revealed that two receptors, hTAS2R4 and hTAS2R14, mediate the bitter off-taste of steviol glycosides. For some test substances, e.g., stevioside, we observed a decline in sweet intensity at supra-maximum concentrations. This effect did not arise from allosteric modulation of the hTAS1R2/R3 sweet taste receptor but might be explained by intramolecular cross-modal suppression between the sweet and bitter taste component of steviol glycosides. These results might contribute to the production of preferentially sweet and least bitter tasting *Stevia* extracts by an optimization of breeding and postharvest downstream processing.

KEYWORDS: *stevia, steviol glycosides, taste receptors, sweetener, taste modulation*

INTRODUCTION

The attraction to sweet taste is innate and might have enabled our early ancestors to identify carbohydrate rich food to cover their demand for energy. For centuries honey and sucrose extracted from sugar cane (*Saccharum officinale* L.) have been the primary source of sweetness in many parts of the world. However, the prevalence of undesired health effects such as dental caries[1−3] or cardiovascular diseases, obesity, and type-2 diabetes[4−6] has been associated with the increased consumption of sugar and required the development of low-calorie, high-impact sweeteners worldwide. For decades, synthetic non-nutritive sweeteners like saccharin and aspartame have been used to reduce nutritive sugar load. To meet the increasing consumer demand for natural food ingredients, much effort directed toward the development of nonnutritive sweeteners from herbal sources has been made in the recent years.

The most prominent example of a natural source for nonnutritive sweeteners is the South American shrub *Stevia rebaudiana* Bertoni (Bertoni). Its leaves have been used for centuries by the native population in Paraguay and Brazil to sweeten bitter herbal teas.[7,8] Since December 2011, the sale and use of steviol glycosides as a food additive have also been permitted in the European Union.[9] The key flavor principle of the so-called "sweet herb" *Stevia rebaudiana* is constituted by a number of diterpenic *ent*-kaurene glycosides (Figure 1 and Table 1), all of which exhibit 13-hydroxykaur-16-en-18-oic acid (steviol) as the common aglycone. Among these steviol glycosides, stevioside (**1**) was found to be most abundant,

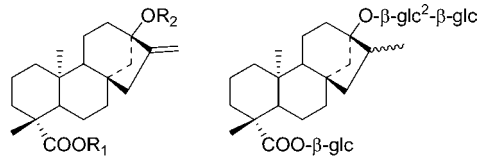

Figure 1. Chemical structure of steviol glycosides **1**−**10** (R_1 and R_2 refer to Table 1) and 16,17-dihydrostevioside (**11**).

followed by the rebaudiosides A-F (**2**−**7**), steviolbioside (**8**), and dulcoside A (**9**) besides some additional derivatives present in trace amounts.[10−16] Another member of the class of *ent*-kaurene glycosides is rubusoside (**10**), isolated from the sweet tasting leaves of the Chinese herb *Rubus suavissimus* S. Lee.[17]

Steviol glycosides are potent sweeteners; however, systematic and comparative studies on the organoleptic properties of the individual substances are lacking. The main constituent of *Stevia*, stevioside (**1**), was reported to be 210 to 300 times sweeter than sucrose, depending on the sensory protocol applied.[18,19] The most potent sweetner among the steviol glycosides is reported to be rebaudioside A (**2**) exhibiting a 9-fold higher sweetness impact when compared to those of

Received: March 26, 2012
Revised: May 21, 2012
Accepted: May 22, 2012
Published: May 22, 2012

Table 1. Chemical Structure of Steviolglycosides[a]

Compound	Abbr.	R_1	R_2
Stevioside (**1**)	Stev	ß-glc	ß-glc-ß-glc (2-1)
Rebaudioside A (**2**)	RebA	ß-glc	ß-glc-ß-glc (2-1) | ß-glc (3-1)
Rebaudioside B (**3**)	RebB	H	ß-glc-ß-glc (2-1) | ß-glc (3-1)
Rebaudioside C (**4**)	RebC	ß-glc	ß-glc-α-rha (2-1) | ß-glc (3-1)
Rebaudioside D (**5**)	RebD	ß-glc-ß-glc (2-1)	ß-glc-ß-glc (2-1) | ß-glc (3-1)
Rebaudioside E (**6**)	RebE	ß-glc-ß-glc (2-1)	ß-glc-ß-glc (2-1)
Rebaudioside F (**7**)	RebF	ß-glc	ß-glc-ß-xyl (2-1) | ß-glc (3-1)
Steviolbioside (**8**)	Stbs	H	ß-glc-ß-glc (2-1)
Dulcoside A (**9**)	DulcA	ß-glc	ß-glc-α-rha (2-1)
Rubusoside (**10**)	Rub	ß-glc	ß-glc

[a] R_1 and R_2 refer to Figure 1. glc, D-glucopyranosyl; rha, L-rhamnopyranosyl; xyl, D-xylopyranosyl.

rebaudioside C (**4**) and dulcoside A (**9**).[19,20] For many steviol glycosides such as rubusoside (**10**) and rebaudioside C (**4**), a lingering bitter aftertaste along with the characteristic sweet sensation has been described.[19,21] Moreover, the bitter off-taste was hypothesized to be related to hydrophilicity in a congeneric series of synthetic analogues of stevioside (**1**) and rebaudioside A (**2**).[22]

Gustatory responses to sweet and bitter compounds are mediated by G protein-coupled receptors expressed by taste receptor cells (TRCs) that assemble into groups of ~100 cells referred to as taste buds.[23−28] On the tongue, taste buds are located in the gustatory papillae. Functional in vitro expression assays demonstrated that the heteromeric sweet taste receptor, made up by a combination of the hTAS1R2 and the hTAS1R3 protein,[25,27] is tuned to detect the broad range of chemically diverse sweet tasting compounds, such as mono- and disaccharides, sweet D-amino acids, sweet proteins, and synthetic non-nutritive sweeteners.[25,27,29−35] The receptive range of hTAS1R2/hTAS1R3 also comprises steviol glycosides as inferred from activation of the heterologously expressed receptor by stevioside (**1**) and rebaudioside A (**2**).[35−37] Human gustatory responses to sweet compounds can be attenuated by 2-(*p*-methoxy)propionic acid sodium salt, coined lactisole, which binds and selectively blocks the activation of hTAS1R2/hTAS1R3.[35,38−42]

In contrast to the single receptor-based detection of sweet taste, transduction of bitter taste in humans is mediated by ~25 receptors of the *hTAS2R* gene family.[23,24,26] TRCs dedicated to bitter taste form a heterogeneous cell population coexpressing multiple but not all hTAS2R genes at once.[43] In vitro, cell-based functional expression assays that allow for the characterization of single bitter taste receptors demonstrated substantial differences in their breadth of tuning ranging from single compounds for some TAS2Rs to numerous substances for others.[44,45] Moreover, they exhibit unique but partially overlapping molecular receptive ranges.[45] Artificial sweeteners like saccharin and acesulfame K are known to interact with both, hTAS1R2/hTAS1R3 and bitter taste receptors, thus eliciting sweetness and bitterness simultaneously.[27,46,47] Like saccharin and acesulfame K, steviol glycosides evoke sweetness that is accompanied by a bitter off-taste at high concentrations.[19,21] However, the hTAS2 bitter taste receptors accounting for this effect still need to be identified.

In order to investigate the molecular determinants of their sweetness, we recorded the psychometric functions of the most important steviol glycosides, as well as their potency at the functionally expressed hTAS1R2/hTAS1R3 sweet taste receptor. We also identified the bitter receptors mediating the off-taste of steviol glycosides by challenging the 25 hTAS2Rs expressed in cell lines with the same compounds. Our findings could help to effectively navigate breeding of *Stevia rebaudiana* and improve postharvest downstream processing toward the production of sweet taste-optimized and bitter taste-minimized *Stevia* extracts. Finally, understanding the mechanism underlying the bitter taste of steviol glycosides could facilitate the identification of TAS2R-selective antagonists.[48,49]

MATERIALS AND METHODS

Chemicals. Sucralose (Merck, Darmstadt, Germany), colchicine, and aristolochic acid (Sigma-Aldrich, Steinheim, Germany) were purchased. 2-(*p*-Methoxy)propionic acid sodium salt (lactisole) was provided by Cargill (Minneapolis, USA). Stevioside (**1**), rebaudioside A (**2**), rebaudioside C (**4**), rebaudioside D (**5**), and dulcoside A (**9**) were isolated and purified from commercial *Stevia* extracts (Cargill, Minneapolis, USA) following literature procedures.[10,11,13,14] Rebaudioside B (**3**) and steviolbioside (**8**) were prepared from rebaudioside A (**2**) and stevioside (**1**), respectively, by alkaline hydrolysis and purified as reported earlier.[11,50] Rubusoside (**10**) was isolated from a commercial extract of *Rubus suavissimus* (MedHerbs, Wiesbaden, Germany) and purified as reported earlier.[17] Dihydrostevioside (2H-Stev, **11**) was synthesized by heterogeneous hydrogenation of **1** using catalytic amounts of palladium (5%) on charcoal in methanol under atmospheric pressure and was then purified by means of preparative high-performance liquid chromatography.[51] Spectroscopic data ($^1H/^{13}C$ NMR, LC-MS, and LC-TOF-MS) of the individual steviol derivatives **1**−**5** and **8**−**11** were in good agreement with those published in the literature. A final cleanup by means of HPLC afforded each individual glycoside in a purity of >98% (HPLC-ELSD and ^1H NMR). As rebaudioside E (**6**) and F (**7**) could not be isolated in sufficient purity, these derivatives were omitted from the study. Prior to sensory studies and cell culture assays, trace amounts of solvents and buffers were removed in high vacuum (<5 mPa), followed by freeze-drying twice. Each test substance was analytically confirmed to be essentially free from solvent and buffer compounds by means of GC-FID, HPLC-ELSD, and ^1H NMR spectroscopies.

Psychophysical Studies. *General Conditions and Sensory Training.* Fifteen healthy nonsmokers (10 women and 5 men, age 25−38), who had given informed consent to participate in the sensory tests of the present investigation and who had no history of known taste disorders, were trained for at least one year in weekly sessions in recognizing and distinguishing different qualities and intensities of oral sensations in analytical sensory experiments. Subjects were trained for sensory evaluation of aqueous solutions (5 mL each, pH 6.0) of the following reference compounds and binary combinations: sucrose (10−1000 mM) and sucralose (5.5−5620 μM) for sweet taste, lactic acid (2−20 mM) for sour taste, NaCl (5−100 mM) for salty taste, caffeine (0.5−5.0 mM), salicin (0.5−5.0 mM), and rubusoside (33.6−4300 μM) for bitter taste, monosodium L-glutamate (1−10 mM) for umami taste, and quercetin-3-*O*-β-D-glucopyranoside (0.1−100 μM) for astringency. On the basis of their capability to identify the quality of reference taste solutions and to consistently rate the elicited taste intensity, 10 trained subjects (seven women and three men, age 25−35) were selected for the sensory experiments of this study.

Participants did not eat or drink (except water) for at least 60 min prior to each test session. Nose clips were used to prevent cross-modal interactions from olfactory inputs. Experiments were performed in three independent sessions at 22 °C in an air-conditioned sensory laboratory with separated booths. Test substances were dissolved in water (Evian; low mineralization: 500 mg/L). Trace amounts of formic acid, which is GRAS listed as a flavoring agent for food and feed applications, was used to adjust the pH value of the test solutions to 6.0. In order to minimize the uptake of test compounds, sensory analyses were performed by using the sip-and-spit method, according to which test materials are not swallowed but expectorated.

Taste Recognition Threshold Concentrations. Taste recognition threshold concentrations of purified steviol glycosides were determined by the trained panel by means of a triangle test according to the protocol detailed in ISO 4120.[52] Using bottled water (pH 6.0) as the solvent and an interlevel interval length of 5 min, linear dilutions of the samples (5−50 μM; for sweetness) and 1 + 1 dilutions (v/v; for bitterness), respectively, were presented to the panel in ascending concentrations in three independent sessions. The panelists were asked to swirl around the solution in the oral cavity for 10 s prior to expectoration. The individual threshold concentration of each panelist corresponds to the geometric mean of the last incorrectly and the first correctly identified test solution. The geometric mean of all individual threshold concentrations was defined as the sweet recognition threshold of the panel. The arithmetic mean and standard deviation of the sweet recognition thresholds from three different sessions were calculated. For statistical analysis of the sweet recognition threshold concentrations, a two-way repeated measurement analysis of variance (ANOVA) was applied, using the individual threshold concentrations of each subject from three different sessions. Results with p values <0.05 were considered as statistically significant. A pairwise multiple comparison procedure (Holm−Sidak method) was used for multiple comparison.

Psychometric Concentration−Response Curves. The concentration range of each test compound presented to the sensory panel for concentration−response recordings was defined by its taste threshold values on the lower end and its maximum solubility on the higher end. Concentrations of 4.1−8392 μM (**1**, **2**), 4.1−2098 μM (**3**, **4**), 4.1−1049 μM (**5**, **8**, **11**), 16.4−2098 μM (**9**), and 16.4−4196 μM (**10**) were presented for the sweet concentration−response recordings, and concentrations of 4.1−8392 μM (**1**, **2**), 8.2−2098 μM (**3**, **4**, **9**), 4.1−1049 μM (**5**, **8**, **11**), and 16.4−4196 μM (**10**) were used for the determination of bitter concentration−response functions. Experiments on sweet and bitter taste were performed in independent sessions.

In a first set of experiments, panelists evaluated the sweet or bitter taste intensity of a series of 11 dilutions of the reference compound sucralose (5.5−5620 μM; 1:1 dilutions) or 9 dilutions of rubusoside (33.6−4300 μM; 1:1 dilutions), each on a free scale. Individual sweet or bitter taste intensities were normalized (highest sweet or bitter intensity was set to 10), and average taste intensities for each sucralose or rubusoside concentration over the whole panel were calculated as relative sweet or bitter taste intensities, respectively. Using this procedure, the following concentrations of sucralose (sweet reference) and rubusoside (bitter reference) were assigned to the relative sweet or bitter taste intensities given in parentheses: for sucralose, 5.5 μM (0.09), 11.0 μM (0.20), 22.0 μM (0.36), 43.9 μM (0.68), 87.8 μM (1.44), 175.6 μM (2.36), 351.2 μM (3.58), 702.5 μM (5.13), 1405.0 μM (6.82), 2810.0 μM (8.15), and 5620.0 μM (10.0); for rubusoside, 33.6 μM (0.17), 67.2 μM (0.26), 134.4 μM (0.39), 268.8 μM (0.62), 537.5 μM (1.24), 1075.0 μM (2.57), 2150.0 μM (5.12), 3230.0 μM (7.56), and 4300.0 μM (10).

In a second set of experiments, panelists ought to judge the sweet or bitter taste intensity of increasing test compound concentrations in aqueous solutions (pH 6.0) by cross-checking to reference compounds (sweet reference, sucralose, 5.5−5620 μM; bitter reference, rubusoside, 33.6−4300 μM). Concentration−response functions were generated for each test compound by plotting the sensory data half-logarithmically against the glycoside concentration. The shape of the curve was extrapolated by applying nonlinear regression to the sigmoidal function $f(x) = \max/(1 + [EC_{50}/x]^{Hillslope})$. Concentration ranges at which relative sweetness declined were not used for regression.

Characterization of in Vitro Taste Receptor Responses to Steviol Glycosides. *Functional Expression of Taste Receptors.* To investigate the potency of individual steviol glycosides to stimulate the human sweet taste receptor in vitro, we used HEK293 FlpIn T-Rex cells (Invitrogen) constitutively expressing human TAS1R2 and the chimeric G protein-α-subunit $G_{\alpha 15}G_{i3}$ ($G\alpha_{15}$ with five C-terminal aa residues exchanged to $G\alpha_{i3}$). Cells were cultured under regular conditions: Dulbecco's modified Eagle's medium (D-MEM), 10% FCS-To, 1% penicillin/streptomycin, 37 °C, 5% CO_2, and 95% humidity. The expression of the functional sweet taste receptor heteromer was enabled by induction of hTAS1R3 expression via a tetracycline-responsive element. Noninduced cells lacking a functional sweet taste receptor heteromer were employed to control for unspecific reactions of the cellular background.

The functional expression of human bitter taste receptors was carried out as described earlier with those *hTAS2R* gene variants used previously.[46,48,49,53,54] Briefly, hTAS2R cDNA harboring an amino-terminal sst-tag to improve plasma membrane-targeting and a carboxyterminal hsv-tag for immunological detection in pcDNA5/FRT (Invitrogen, San Diego, CA) or pEAK-10 (Edge BioSystems, Gaithersburg, MD) vector was transiently transfected into HEK293T Gα16gust44 cells.[55] Cells transfected with empty vector were used as negative control.

For functional experiments, cells were seeded into poly-D-lysine-coated (10 μg/mL) 96well plates (Greiner Bio-One, Frickenhausen, Germany). Twenty-four hours prior to the experiment, hTAS1R3-expression in HEK293 $G\alpha_{15}G_{i3}$/hTAS1R2 cells was induced by addition of tetracycline (0.5 μg/mL in D-MEM Glutamax low glucose with 1% penicillin/streptomycin and 10% FCS-To dialyzed). Constructs encoding for human bitter taste receptors were transfected using Lipofectamine2000 (Invitrogen, San Diego, CA, in D-MEM high glucose) according to the manufacturer's instructions. Twenty-four hours after induction and transfection, respectively, cells were loaded with the calcium indicator dye Fluo-4AM (Molecular Probes, Karlsruhe, Germany; 2 μM in D-MEM Glutamax low glucose or D-MEM high glucose with 2.5 mM probenicid) for 1 h at 37 °C. Excess dye was washed off twice with C1 buffer solution (130 mM NaCl, 5 mM KCl, 10 mM HEPES, and 2 mM $CaCl_2$, pH 7.4), containing 10 mM glucose (5 mM for HEK293 $G\alpha_{15}G_{i3}$/hTAS1R2 cells). In the mean time, incubation at room temperature for 40 min allowed for complete de-esterification of the dye. Test compounds were dissolved in C1 buffer. For coapplication experiments, individual substances were mixed prior to the experiment. Maximum applicable compound concentrations were limited by fluorescence signals in mock cells. Intracellular calcium transients during automated application of test compound were recorded at λ = 515−575 nm after excitation of the fluorescence dye at λ = 477−495 nm using a fluorometric imaging plate reader (FLIPR Tetra, Molecular Devices, Munich, Germany). To control for cell vitality and integrity of the cellular signal transduction cascade, the β-adrenergic receptor agonist isoproterenol (10 μM, Sigma-Aldrich, Steinheim, Germany) was applied after the taste stimuli. Experiments were performed in duplicate and repeated at least twice. The signal amplitudes of receptor-expressing cells were reduced by fluorescence signals of mock cells and normalized to background fluorescence ($\Delta F/F = (F-F_0)/F_0$).

Analysis of Taste Receptor Functional Expression Experiments. For the calculation of concentration−response curves, mean $\Delta F/F$ values of wells receiving the same stimulus were plotted half-logarithmically against test compound concentrations. Half-maximal effective concentrations (EC_{50}) and maximal signal amplitudes (max) were calculated using nonlinear regression to the sigmoidal function $f(x) = \min + (\max-\min)/(1 + [x/EC_{50}]^{Hillslope})$ (SigmaPlot 9.01, Systat Software GmbH, Erkrath, Germany) with x representing the agonist concentration.

Threshold values for activation of hTAS1R2/hTAS1R3-expressing cells were defined as the lowest concentration of steviol glycosides which led to a fluorescence signal significantly higher compared to that

Table 2. Properties of Steviol Glycosides in Human Sensory Studies and in Functional Taste Receptor Expression Assays

	human sensory test				cells expressing			
	sweet		bitter		hTAS1R2/hTAS1R3		hTAS2R4	hTAS2R14
no[a]	TC (μM)[b]	MRL[c]	TC (μM)[d]	RB[e]	TC (μM)[f]	MA[g]	TC (μM)[h]	TC (μM)[h]
1	11.1 (±2.3)	2.7	112	1.4	4.3	1.6	200	600
2	8.3 (±2.2)	3.7	194	1.3	4.3	1.7	200	600
3	18.1 (±1.3)	3.4	137	1.1	12.9	1.9	200	1000
4	27.8 (±4.0)	1.5	49	1.4	38.8	1.4	400	400
5	5.3 (±0.3)	4.8	162	0.6	2.2	1.7	n.s.[I]	n.s.[I]
8	26.8 (±1.3)	2.0	84	0.9	12.9	1.8	400	n.s.[j]
9	32.9 (±5.3)	1.1	49	1.7	38.8	1.5	200	50
10	27.3 (±2.5)	1.8	61	2.7	25.9	1.9	50	400
11	28.1 (±6.0)	1.6	23	2.7	38.8	1.1	n.d.	n.d.

[a]Substance number refers to structures given in Figure 1 and Table 1. [b]The geometric mean over all panelists is defined as the panel threshold for sweetness. The threshold values of the sensory group are approximated by averaging the threshold value of the group in three independent sessions. Standard deviation (±s.d.) was calculated from the arithmetic means of the panel threshold concentrations from three different sessions. [c]Maximum rel. sweetness. [d]The geometric mean over all panelists is defined as the panel threshold for bitter taste. [e]Relative bitterness at a concentration of 1 mM. [f]Threshold concentrations are defined as the lowest concentration of steviol glycosides, which led to a fluorescence signal significantly higher compared to that of the bath application. [g]Maximum signal amplitudes were calculated using nonlinear regression (see text for details). [h]Threshold concentration is defined as the lowest compound concentration leading to a significant fluorescence signal compared to that of the buffer application (see text for details). [I]No response to the test compound up to the maximal soluble concentration of 400 μM. [j]No response to the test compound up to the maximal applicable concentration of 800 μM. n.d. Not determined due to receptor-independent fluorescence signal in control cells.

of the bath application. In order to determine threshold concentrations for bitter taste receptor activation, test compounds were applied at concentrations of 10, 50, 100, 200, 400, 600, 800, 1000, and 1200 μM. Threshold value was defined as the lowest test compound concentration resulting in a significantly higher fluorescence signal (Mann−Whitney U Statistic) compared to that of the bath application.

RESULTS

The organoleptic properties of some steviol glycosides have been subject of investigation of few pilot experiments in the past,[18−22] but systematic comparative sensory analyses of individual steviol glycosides including human psychometric concentration responses are not available. Attempts to correlate in vivo data obtained from human psychophysical experiments with in vitro data from cell-based taste receptor functional expression assays are particularly limited.[35−37] With the present study, we provide comprehensive data on the structure−function relationship of highly pure (>98%) steviol glycosides on sweet and bitter taste responses determined in human psychophysical experiments and cell-based receptor assays.

Human Taste Recognition Threshold Concentrations. At first, the taste recognition thresholds of steviol glycosides **1**−**5** and **8**−**11** were determined by means of a triangle test. The test substances showed sweet threshold values in the range of 5.3 to 32.9 μM and slightly higher bitter threshold levels between 23 and 194 μM (Table 2). The lowest sweet threshold concentrations were found for the rebaudiosides D (**5**; 5.3 ± 0.3 μM) and A (**2**; 8.3 ± 2.2 μM) as well as for stevioside (**1**; 11.1 ± 2.3 μM). Hydrogenation of the exocyclic double bond of stevioside (**1**) yielding 2*H*-stevioside (**11**) increased the sweet taste threshold significantly from 11.1 to 28.1 μM. The highest sweet threshold values were observed for the two rhamnose-containing steviol glycosides, namely, rebaudioside C (**4**; 27.8 ± 4.0 μM) and dulcoside A (**9**; 32.9 ± 5.3 μM), as well as for the least hydrophilic glycosides, namely, rubusoside (**10**; 27.3 ± 2.5 μM) and steviolbioside (**8**; 26.8 ± 1.3 μM). A two-way repeated measurement of variance (ANOVA), followed by a multiple comparison procedure (Holm-Sidak method) with an overall significance level of 0.05 revealed significant differences among the individual glycosides [$F(8, 112) = 44.8$, $P < 0.001$]. The sweet taste recognition threshold determined for rebaudioside A (**2**) did not differ significantly from that of rebaudioside D (**5**) and stevioside (**1**) (Figure 2).

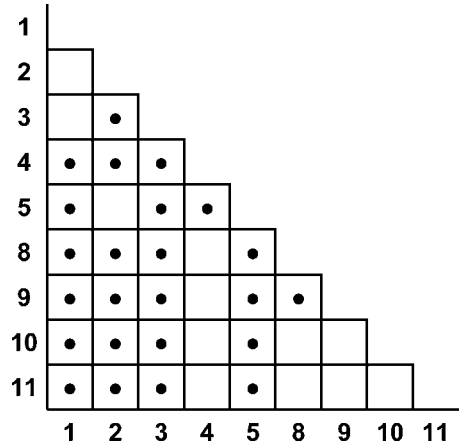

Figure 2. Multiple comparison graph of sweet recognition thresholds of steviol glycosides **1**−**5**, **8**−**10**, and **11** for two-way repeated measurement of variance (ANOVA), followed by multiple comparison procedures (Holm−Sidak method) with overall significance level = 0.05. Symbol indicates significant differences.

Whereas stevioside (**1**) and rebaudioside B (**3**), both exhibiting three β-glucose moieties, did not differ significantly in their threshold concentration, the threshold found for **3** (18.1 μM) was significantly above that of rebaudioside A (**2**) (8.3 μM), containing four glucose moieties. No significant differences were found between the rhamnose-containing glycosides rebaudioside C (**4**) and dulcoside A (**9**), and steviolbioside (**8**) and rubusoside (**10**), both bearing two glucose moieties.

Intriguingly, steviol glycosides that showed the highest sweet threshold values in the human sensory test, dulcoside A (**9**), rebaudioside C (**4**), rubusoside (**10**), 2*H*-stevioside (**11**), and steviolbioside (**8**) turned out to exhibit the lowest bitter threshold values ranging from 23 μM (**11**) to 84 μM (**8**). Thus, the bitter threshold of the rhamnose-containing glycosides,

dx.doi.org/10.1021/jf301297n | J. Agric. Food Chem. 2012, 60, 6782−6793

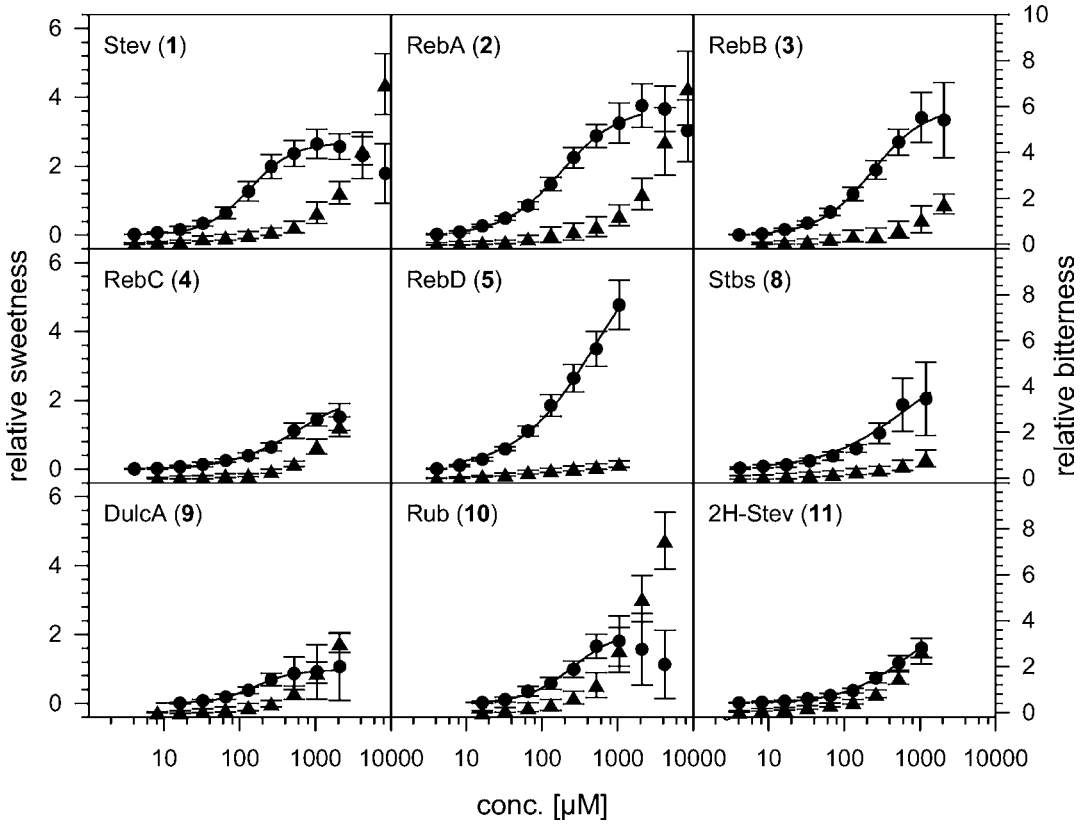

Figure 3. Concentration−responses of steviol glycosides (**1**−**5** and **8**−**10**) and 16,17-dihydro stevioside (**11**) on perceived sweet taste intensity (left axis, circles) and on perceived bitter taste intensity in human volunteers (right axis, triangles). For a reference to determine the relative sweetness, serial dilutions of sucralose (5.5−5620 μM) were presented to the panel in each session. For a reference to determine the relative bitterness, serial dilutions of rubusoside (**10**) (33.6−4300 μM) were presented to the panel in each session. Relative sweetness and relative bitterness were determined in independent sessions. Substance numbers refer to Table 1. Error bars represent the confidence interval ($p < 0.05$).

dulcoside A (**9**) and rebaudioside C (**4**), is only a factor of ∼1.5 higher compared to their sweet taste threshold. Panelists described the bitter sensation of both compounds as long-lasting even at low concentrations. For 2*H*-stevioside (**11**), a substantially lower bitter threshold value was found when compared to that of stevioside (**1**) (23 vs 112 μM), thus indicating that saturation of the exocyclic double bond at C16 does moderately impair sweet taste but considerably promote bitter taste elicited by steviol glycosides.

Human Concentration−Response Functions. Since supra-threshold measures have been reported to be more suitable to evaluate the sensory activity of taste compounds when compared to threshold values,[56,57] concentration−response functions were recorded to obtain information on the sweet and bitter intensities elicited by the steviol glycosides **1**−**5** and **8**−**11**. Defined dilution series of sucralose and rubusoside were applied as references for sweet and bitter taste scoring (Figure 3).

With a maximum intensity of 4.8, the glycoside containing the most β-glycosyl moieties, rebaudioside D (**5**), turned out to be most sweet among the tested compounds (Table 2). However, the concentration−response function for the sweet taste elicited by rebaudioside D (**5**) did not reach saturation up to the maximum solubility of 1 mM suggesting an advanced sweet intensity at higher substance concentrations. Like already observed for the sweet threshold values, hydrogenation of the exocyclic double bond of stevioside (**1**) leads to a reduced maximum sweetness of 2*H*-stevioside (**11**) by a factor of nearly 2 (2.7 vs 1.6), implying a moderate impact of the double bond on both, sweet threshold and maximum sweetness of steviol glycosides. The maximum sweetness of steviol compounds is also restricted by the total number of β-glycosyl moieties (glc), as demonstrated by comparing rubusoside (**10**; 2 glc; 1.8 max sweet), stevioside (**1**; 3 glc; 2.7 max sweet), and rebaudioside A (**3**; 4 glc; 3.4 max sweet). The α-rhamnosyl-containing glycoside dulcoside A (**9**) exhibited the lowest maximum sweetness detected with a value of 1.1. However, the detrimental effect of the α-rhamnose moiety on the maximum sweetness can be partially compensated by an additional β-glycosyl residue at the C13 glycosylation site indicated by the increased maximum sweetness of rebaudioside C (**4**; 1.5).

Intriguingly, the sweet taste sensation elicited by stevioside (**1**), rebaudioside A (**2**), and rubusoside (**10**) started to decrease at concentrations >1−2 mM after passing through the maximum. This effect is reminiscent of the sweet taste inhibition caused by high concentrations of the synthetic sweeteners saccharin and acesulfame K.[58] Both compounds are supposed to bind to a low-affinity allosteric site at the sweet taste receptor heteromer, thus shifting the receptor equilibrium toward the inactive state and inhibiting sweet taste. The question as to whether this scenario also applies to the decrease in maximum sweetness observed for stevioside (**1**), rebaudioside A (**2**), and rubusoside (**10**) will be subsequently investigated in the cell-based sweet taste receptor assay.

Unlike their sweetness concentration-intensities, the bitter taste sensation elicited by steviol glycosides **1**−**5** and **8**−**11** did not reach saturation within the maximum solubility implying an exponential rise of bitterness above the tested concentrations

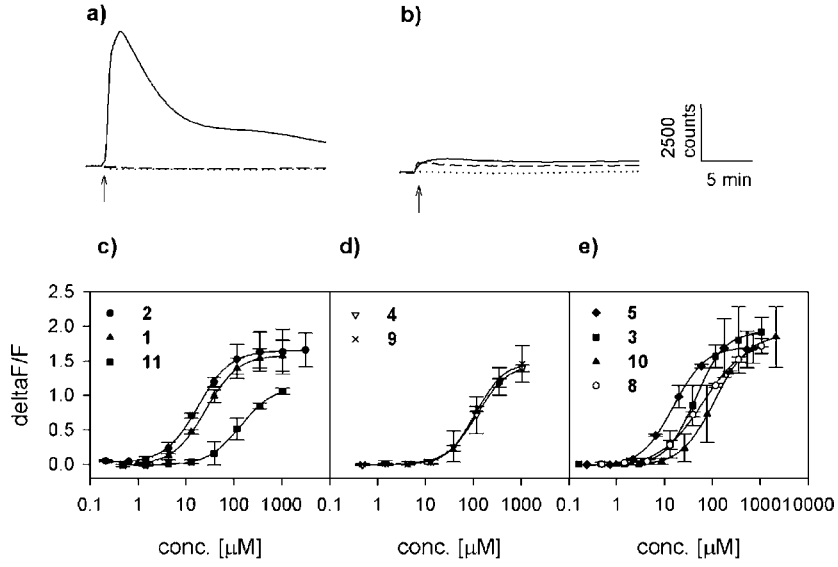

Figure 4. Representative calcium traces of cells expressing the human sweet taste receptor hTAS1R2/R3 (a) and of mock cells (b) upon stimulation (↑) with stevioside (**1**; 700 μM, solid line). Specificity of the fluorescence signals was controlled by coapplication of the selective hTAS1R2/R3-antagonist lactisole (1 mM, dashed line) and sole application of lactisole (dotted line). Concentration−responses of human sweet taste receptor-expressing cells exposed to steviol glycosides (c−e). Net fluorescence changes were plotted vs log agonist concentration. Error bars represent standard deviation (n = 2).

(Figure 3). At 1 mM, the strongest bitter impression was observed for rubusoside (**10**) with a score of 2.7 which further increased to 7.5 at the highest tested concentration of 4.2 mM. However, the glycosylation site rather than the low total number of two β-glycosyl residues in rubusoside (**10**) seems to account for its high bitter intensity since steviolbioside (**8**; 2 glc) was rated with a significantly lower relative bitterness of 0.9 at 1 mM. The presence of an α-rhamnosyl moiety resulted in a low-concentration onset of bitter taste for rebaudioside C (**4**) and dulcoside A (**9**), but not a strong slope of their bitter intensity. Both compounds revealed only medium bitter intensities of 1.4 (**4**) and 1.7 (**9**) at a concentration of 1 mM.

Potency of Steviol Glycosides on the Functionally Expressed Human Sweet Taste Receptor. In order to investigate the structure−function relationships of steviol glycosides on the human TAS1R2/TAS1R3 sweet taste receptor, functional calcium imaging experiments were performed in HEK293 FlpIn T-Rex $G\alpha_{15}G_{i3}$/hTAS1R2 cells. This cell line stably expresses the chimeric G protein $G_{\alpha 15}G_{i3}$ to couple activation of the sweet taste receptor to cytosolic calcium levels that can be monitored via a calcium-sensitive fluorescence dye. The functional sweet taste receptor heteromer is implemented by stable expression of the subunit hTAS1R2, and inducible expression of the second subunit, hTAS1R3, through a tetracycline-responsive element.[58,59]

Upon stimulation with steviol glycosides, hTAS1R2/R3-expressing cells responded with an immediate strong transient increase of calcium fluorescence as exemplified by the application of stevioside (**1**; 700 μM) shown in Figure 4a-b. All fluorescence signals induced by the tested steviol glycosides (**1**−**5**, **8**−**11**) were blocked completely in the presence of the selective sweet taste receptor antagonist lactisole (1 mM), as exemplified for stevioside in Figure 4. In conclusion, the observed signals are mediated by activation of the hTAS1R2/R3-sweet taste receptor.

Concentration−response analyses revealed that all tested steviol glycosides (**1**−**5**, **8**−**11**) stimulate the functionally expressed sweet taste receptor in a concentration-dependent manner (Figure 4c-e). In contrast to the psychophysical tests, all compounds reached saturation within the applied concentration range. The onset of responses from hTAS1R2/R3-expressing cells is observed at concentrations comparable to those of the sensory study (Table 2). Also in vitro, rebaudioside D (**5**) was found to be the most sweet potent steviol glycoside with a threshold value of 2.2 μM, which is in reasonable agreement with the value of 5.3 μM found in the in vivo experiment. The rank order of potency of functionally expressed hTAS1R2/R3 for the individual glycosides is similar to the sensory ranking, e.g., high threshold values of rhamnose-containing glycosides and low threshold values of rebaudioside D and A and stevioside, exhibiting the most β-glycosyl residues. However, noteworthy are the differences in potency between stevioside (**1**) and its derivative 2*H*-stevioside (**11**), which are much more pronounced in vitro than in vivo and which are apparent by the 9-fold increased threshold value of 2*H*-stevioside (**11**) compared to stevioside (**1**).

In contrast to the mostly comparable potencies of steviol glycosides to induce sweet taste in vivo and activation of hTAS1R2/R3 in vitro, their efficacies expressed as the maximal sweet intensity and the maximal fluorescence ratio in the functional assay differ largely. Whereas compounds differed in their maximal sweetness by more than a factor of 5 in the sensory study, they stimulated hTAS1R2/R3-expressing cells almost equally to reach a maximal fluorescence ratio between 1.4 and 1.9. Only 2*H*-stevioside (**11**; $\Delta F/F$ = 1.1) induced weaker calcium responses (Table 2). This finding suggests that the different sweet intensities perceived from individual steviol glycosides were not mediated at the level of hTAS1R2/R3 activation.

The perceived sweet taste intensity from steviol glycosides did not reach saturation for all tested compounds in the sensory study. The concentration−responses of rebaudioside B (**3**) and dulcoside A (**9**) reached a constant maximum level with the two highest concentrations tasted by the sensory panel (Figure

3). In contrast, the sweetness intensity of stevioside (**1**), rebaudioside A (**2**), and rubusoside (**10**) started to decline after passing through the maximum. As an example, the sweetness of stevioside (**1**) was rated with a maximum intensity of 2.7 at 1 mM, while subsequent test concentrations, 2.1 mM, 4.2 mM, and 8.4 mM, were scored with a relative sweetness of 2.6, 2.3, and 1.8, respectively. When we subjected the steviol glycosides **1**−**5** and **8**−**11** to characterization in the functional sweet taste receptor expression assay, we observed saturation of receptor activity within the applicable concentration range only for stevioside (**1**), rebaudioside A (**2**), and rebaudioside D (**5**) (Figure 4). In remarkable contrast to the perceived sweetness in vivo, the sweet receptor responses evoked by stevioside (**1**) and rebaudioside A (**2**) did not decline beyond the maximum. For example, rebaudioside A (**2**) induced constant maximum fluorescence signals over more than 1 order of magnitude (Figure 4). Thus, other than the sulfonylamide sweeteners saccharin and acesulfame K, rebaudioside A (**2**) and stevioside (**1**) do not block the hTAS1R2/R3 active state at supra-maximum concentrations.

Identification of Bitter Taste Receptors Responding to Steviol Glycosides. In order to identify the candidate taste receptors mediating the bitter after-taste of the steviol glycosides **1**−**5** and **8**−**11**, we transiently expressed 25 hTAS2Rs individually in HEK293T Gα16gust44 cells. Human *TAS2R* genes contain numerous single nucleotide polymorphisms (SNP) which can influence the function of the encoded receptors.[47,60−65] For the experiments described below, we used the receptor variants of our previous publications.[48,49]

At a concentration of 1 mM, stevioside (**1**) showed pronounced bitter taste in the preceding human sensory experiments and hence was chosen for the initial test of bitter taste receptor activation by steviol glycosides. Upon bath application of stevioside (**1**), exclusively cells expressing hTAS2R4 or hTAS2R14 showed a robust fluorescence signal (arrows, Figure 5a). Also rebaudioside A (**2**; 1 mM) evoked responses only in cells expressing hTAS2R4 or hTAS2R14 (arrows, Figure 5b). Together, the data indicate that only two of the 25 bitter taste receptors are sensitive to the selected steviol glycosides. Moreover, in consideration of the structural similarities between steviol glycosides, we conclude that hTAS2R4 and hTAS2R14 are general sensors of this class of compounds. Thus, we performed the subsequent in vitro analyses of steviol glycosides with hTAS2R4 and hTAS2R14 only.

In the next experiment, we challenged hTAS2R4- and hTAS2R14-expressing cells with the other steviol glycosides **3**−**5** and **8**−**10** at a concentration of 1 mM or at their highest applicable concentration, respectively (Figure 5c). Because of hTAS2R-independent fluorescence signals in mock cells, the effect of 2*H*-stevioside (**11**) on bitter taste receptor-expressing cells could not be evaluated. The functional integrity of the test system was controlled by application of cognate agonists for both bitter receptors, i.e., colchicine for hTAS2R4 and aristolochic acid for hTAS2R14.[45,66] As expected, both bitter taste receptors responded to most of the steviol glycosides. Cells expressing hTAS2R4 were in particular effectively activated by rebaudioside C (**4**), dulcoside A (**9**), and rubusoside (**10**). Similarly, rebaudioside C (**4**) and dulcoside A (**9**) induced the highest fluorescence signals from hTAS2R14-expressing cells.

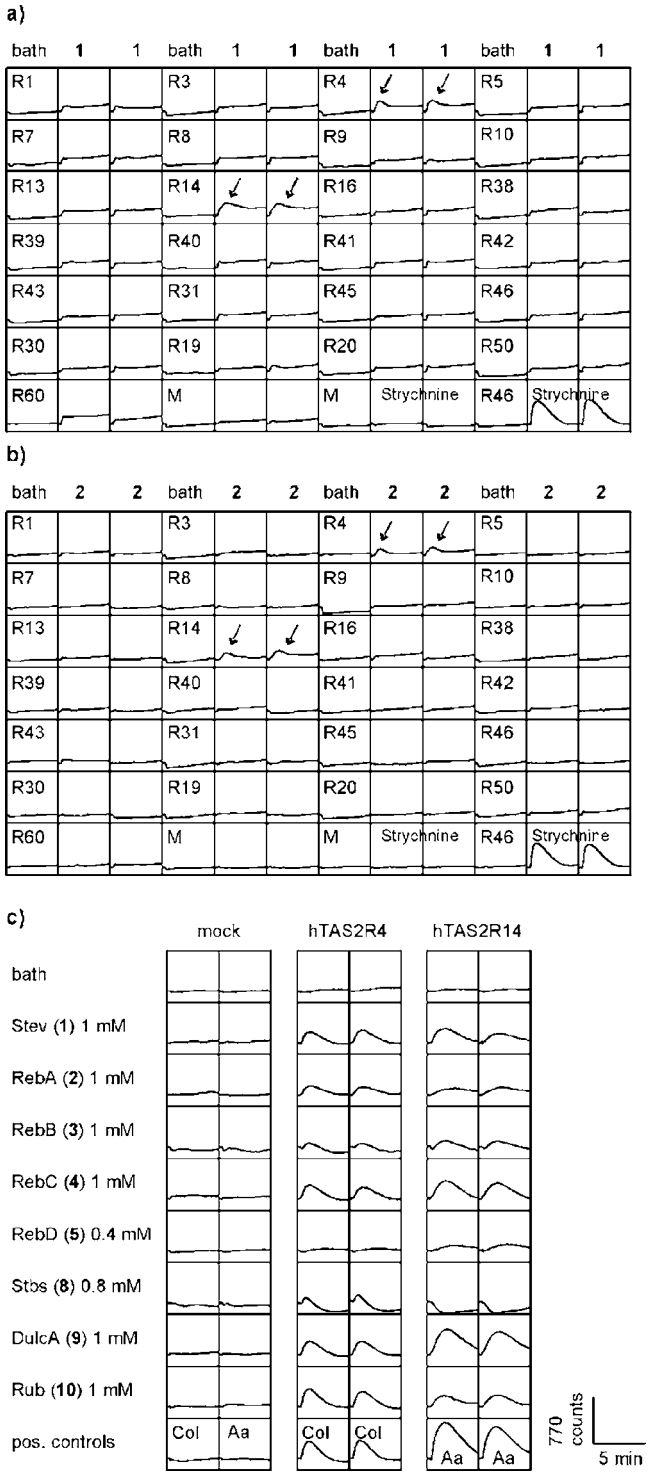

Figure 5. Calcium responses of human bitter taste receptor-expressing cells and mock (M) to bath application of 1 mM (a) stevioside (**1**) and (b) rebaudioside A (**2**). Arrows point to fluorescence signals in hTAS2R4- and hTAS2R14-expressing cells. Response of hTAS2R46 to strychnine (10 μM) was used as the positive control. Scale/well: *y*, 590 counts; *x*, 300 s. (c) Calcium traces of HEK293T Gα16gust44-cells expressing hTAS2R4 or hTAS2R14 or empty vector (mock) following administration of steviol glycosides. Colchicine (Col; 3 mM) and aristolochic acid (Aa; 10 μM) were used as positive controls.

In marked contrast, some steviol glycosides did not elicit robust responses in hTAS2R4 and/or hTAS2R14 cells. Rebaudioside D (**5**) failed to stimulate both, hTAS2R4 and

hTAS2R14, and steviolbioside (**8**) did not activate hTAS2R14. Although they were rather small, signals evoked by rebaudioside A (**2**) in hTAS2R14-cells were reproducible and differed significantly ($\Delta F/F$ = 0.09 ± 0.03 at 0.6 mM) from those of mock-transfected cells ($\Delta F/F$ = 0.01 ± 0.01 at 0.6 mM). It is important to note that, due to its limited solubility, rebaudioside D was tested at a lower concentration (0.4 mM) than all other glycosides. Although it cannot be excluded that rebaudioside D is capable of stimulating bitter receptor responses at higher concentrations, its high bitter threshold and low bitter intensity rating in vivo points to a limited potency of compound **5** to evoke strong hTAS2R responses in vitro. Along these lines, steviolbioside (**8**), the other substance that did not stimulate hTAS2R14, showed the second lowest bitter intensity rating in the psychophysical test.

In order to compare the potency of the individual steviol glycosides to stimulate hTAS2R4 and hTAS2R14, we determined their threshold concentrations in the functional assays. We challenged receptor-expressing cells with increasing concentrations up to 1.2 mM of the test compounds and determined the lowest concentration leading to a significant fluorescence signal (Figure 6 and Table 2). Stevioside (**1**)

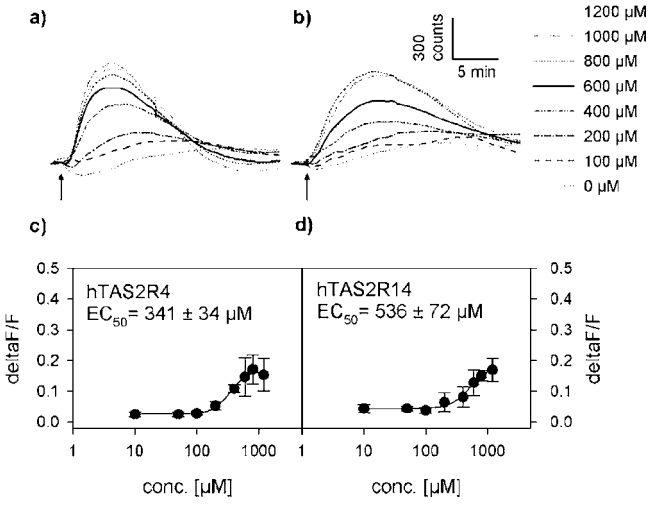

Figure 6. Calcium traces of cells expressing human TAS2R4 (a) or TAS2R14 (b) upon administration (↑) of increasing concentrations of stevioside (**1**). Concentration−response curves of cells expressing hTAS2R4 (c) or hTAS2R14 (d) challenged with stevioside (**1**). Net fluorescence signals are plotted vs log agonist concentration. Error bars represent standard deviation.

evoked dose-dependent calcium responses in hTAS2R4- and hTAS2R14-expressing cells with EC_{50} values of 341 ± 34 μM and 536 ± 72 μM, respectively (Figure 6). As the cellular signals for the other test compounds did not saturate at their solubility maximum and, therefore, complete dose−response curves and EC_{50} values could not be recorded, only threshold data are given in Table 2.

Remarkably, the data show that the two responsive hTAS2Rs were neither equally sensitive to the test compounds nor showed the same rank order of potency for the various steviol glycosides. In most cases, hTAS2R4 was activated at lower test substance concentrations compared with hTAS2R14, except for dulcoside A (**9**). In fact, hTAS2R14 seems to be better tuned to short-chained and rhamnose-containing steviol glycosides since dulcoside A (**9**), rebaudioside C (**4**), and rubusoside (**10**) activate this receptor at lower concentrations than the other compounds. For glycosides with a higher number of β-glycosyl residues, hTAS2R14 is less sensitive as exemplified by the concentration−responses of stevioside (**1**) (Figure 6c, d) and its high threshold values for rebaudiosides A (**2**) and B (**3**) (Table 2).

In general, the activation thresholds determined in the receptor assays correspond reasonably well to the bitter taste thresholds derived in the psychophysical tests. However, this did not apply to rebaudiosides C (**4**) and D (**5**) as well as steviolbioside (**8**), which elicited stronger responses in vivo than in vitro (Table 2).

DISCUSSION

The detrimental health effects of sucrose overconsumption typical for the common Western diet in developed countries necessitate the use of low-caloric sweeteners. However, besides their more or less pronounced organoleptic drawbacks compared to sucrose, the synthetic origin of low-calorie sweetener delimitates the acceptance by the consumer. Thus, the herbal non-nutritive sweet steviol glycosides of *Stevia rebaudiana* that have recently been approved in the EU meet the rising consumer demand for natural food ingredients. Despite few publications indicating sensory differences between single steviol glycosides, comprehensive data on the organoleptic properties and relation to the molecular structure of the individual steviol compounds were lacking. In the present study, we determined psychometric functions for the sweet and the bitter taste elicited by the most prominent steviol glycosides isolated from *Stevia rebaudiana* and of rubusoside isolated from *Rubus suavissimus*. In order to investigate the molecular principles underlying the gustatory perception of steviol glycosides, we correlated our in vivo data to the properties of steviol glycosides in a cell-based taste receptor expression assay.

The in vivo analysis of taste thresholds and post-threshold taste intensities clearly demonstrated the crucial influence of the number of β-glycosyl residues on the sweet taste elicited by individual steviol glycosides. Both R1 and R2 separate glycone length, and the total number of β-glycosyl moieties in the molecule are related to lower sweet thresholds and higher maximum sweet intensity. For example, rubusoside (**10**), stevioside (**1**), and rebaudioside A (**2**), which exhibit one β-glycosyl residue at R1, show lower sweet threshold values and higher sweetness intensity as a function of the number of β-glycosyl residues at R2 (Table 2). This trend is paralleled by the left-shifted concentration−response functions of these compounds as determined in the receptor assay (Figure 4). Similarly, sweetness also increases with the total number of β-glycosyl residues as evidenced by the data for rubusoside (**10**; 2 glc), stevioside (**1**; 3 glc), rebaudioside A (**2**; 4 glc), and rebaudioside D (**5**; 5 glc) (Table 2). Moreover, the low sweetness and potency in the receptor assay of rubusoside (**10**) bearing only one β-glycosyl residue in position 13 supports the hypothesis that a disaccharide is required in this position for high-impact sweetness of steviol glycosides.[67] Substitution of β-glycosyl residues by α-rhamnose diminishes the sweet taste of steviol glycosides. This is demonstrated by the reduced sweetness and potency in the receptor assay of dulcoside A (**9**) in comparison with stevioside (**1**) (Table 2). Thus, the data confirm previous observations about the effects on sweetness reduction of replacing glucose by rhamnose.[19] Finally, the C16 double bond of the steviol scaffold also impacts sweet taste. Saturation of the double bond of stevioside (**1**) yielding 2*H*-stevioside (**11**) resulted in a 3-fold increased sweet taste

dx.doi.org/10.1021/jf301297n | J. Agric. Food Chem. 2012, 60, 6782−6793

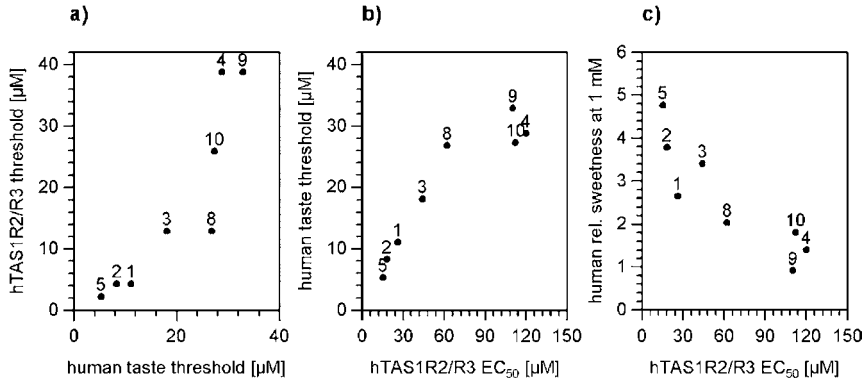

Figure 7. Correlation of human sensory studies with data obtained by using a cell based receptor assay: Correlation of (a) threshold for activation of hTAS1R2-hTAS1R3 with human taste thresholds for sweet taste (b) human taste thresholds with in vitro human TAS1R2-TAS1R3 assay derived EC_{50} values (c) relative sweetness at a concentration of 1 mM in human dose–response behavior with hTAS1R2-hTAS1R3 EC_{50} values. Substance numbers refer to Table 1.

threshold and a 2-fold reduced maximal relative sweetness (Table 2).

The response thresholds of the heterologously expressed human sweet taste receptor hTAS1R2/R3 to steviol glycosides correlated well with the values obtained in our psychophysical tests (Figure 7). In marked contrast, the efficacy of the test compounds in the cell-based assay was rather constant, whereas the maximal relative sweet intensities in vivo varied substantially. Hence, the distinct sweet intensities elicited by the various steviol glycosides seem not to be mediated at the level of the sweet taste receptor. Similarly, sucrose and the artificial sweeteners, aspartame and cyclamate, were shown to activate the recombinant hTAS1R2/R3 equally robustly but elicit distinct sweet taste intensity in human subjects.[42,68] Another unanticipated difference between our human sensory experiments and the functional receptor assay concerns the decreased sweetness of stevioside (**1**), rebaudioside A (**2**), and rubusoside (**10**) at supra-maximal concentrations (Figure 3). This attenuating effect is not due to the inhibition of hTAS1R2/R3 since the corresponding concentration–response functions deduced from the transfected cells clearly display constant signal maxima (Figure 4). It is known that perceived tastes of different qualities are prone to mutual influence when the stimuli were presented at the same time.[69] Hence, the reduced sweet intensity of the three steviol glycosides could be caused by a cross-modal suppressing effect of the associated intrinsic bitter taste. This assumption is supported by the fact that sweet intensities decreased below maximal levels of those compounds that are associated with strongest bitterness, i.e., compounds **1**, **2**, and **10** (Table 2). Although mixture suppression in the gustatory system is not well investigated, there is a finding that supports the aforementioned conclusion. It has been shown that the bitter substance phenylthiocarbamide (PTC) suppresses the sweetness of sucrose in people who can taste PTC bitter, so-called PTC-tasters. This effect was absent in people who inherited the inability to taste this bitter substance, the so-called PTC-nontasters. These observations strongly suggest that bitter perception is a prerequisite for suppressing sweetness in binary mixtures.[70] In order to further elucidate the molecular and physiological principle underlying the decreasing sweet taste of some steviol glycosides, psychophysical experiments under the elimination of the bitter component would be required, e.g., by a panel which is insensitive to the bitter taste of steviol glycosides or by the use of selective bitter blockers.[48,49]

Like other class C GPCRs, hTAS1Rs possess an orthosteric ligand binding site in the venus flytrap module (VFD) of the large N-terminal domain and in addition an allosteric binding site constituted by the heptahelical domain. Mutational analysis and docking to an in silico receptor model suggested binding of stevioside to the orthosteric binding site of T1R2.[37] Given the structural similarity of steviol glycosides, it can be assumed that they all interact with the same binding site. We observed an increasing potency of steviol glycosides with increasing glycone length on the functionally expressed sweet taste receptor suggesting a major influence of polarity on receptor activation. This idea is supported by literature observations on the correlation of the number of potential hydrogen donor/acceptor sites in a sweetener molecule with its sweet potency.[71,72] The conversion of class C GPCRs from resting into the active state involves the binding of a ligand to the orthosteric binding site and closure of the VFD.[73] In hTAS1Rs, sweet compounds are thought to stabilize the closed VFD conformation, i.e., by building hydrogen bonds with receptor residues.[37] On the basis of that hypothesis, it is tempting to speculate that steviol glycosides with several glucose moieties stabilize the closed conformation of VFD more efficiently than those with a lower number of glucose residues and hence less hydrogen bond donor/acceptor sites.

The bitter taste of steviol glycosides seems to be promoted by structural features that impair sweet taste, but the correlation between structure and bitterness is less evident. Only marked differences in the total number of β-glycosyl moieties cause differences in bitter taste characteristics. Steviol glycosides bearing few β-glycosyl residues, such as rubusoside (**10**; 2 glc), showed lower bitter threshold values in vivo and higher bitter intensities compared to glycosides with many β-glycosyl residues, such as rebaudioside D (**5**; 5 glc) (Table 2). Substitution of β-glycosyl moieties by α-rhamnose leads to a low-concentration onset of bitter taste but not to an increased bitter intensity at 1 mM (Table 2). The bitter taste of stevioside (**1**) and rebaudioside A (**2**) is mediated by two bitter receptors, hTAS2R4 and hTAS2R14. We further demonstrated their sensitivity also to other steviol glycosides, with the exception of rebaudioside D (**5**). This compound elicited the weakest bitter taste in the preceding sensory experiments and thus might induce a bitter receptor response off-detection limit in vitro. Both bitter taste receptors differed in their rank order of potency for the various steviol glycosides. Human TAS2R4 tended to be more sensitive to most of the test compounds

with the highest potency for rubusoside (**10**) (Table 2). Threshold values of hTAS2R14 for the test substances varied considerably, yet were low for the rhamnose-containing steviol glycosides dulcoside A, rebaudioside C, and rubusoside (Table 2). The agonist profile of both receptors has been well characterized in recent publications.[45,53] Although hTAS2R4 is activated by a limited set of bitter compounds, its activation profile substantially overlaps with that of the very broadly tuned receptor, hTAS2R14. This functional similarity is further supported by our present data. The impact of glycone chain length on bitter taste is reflected by the molecular receptive ranges of both bitter receptors. For example, rubusoside (**10**) bearing two β-glycosyl residues is detected at 50 μM by hTAS2R4 and at 400 μM by hTAS2R14. Rebaudioside A (**2**), with four β-glycosyl residues, showed higher threshold values of 200 μM at hTAS2R4 and of 600 μM at hTAS2R14. These differences match exactly the bitter threshold differences seen in vivo. In contrast to the sweet taste receptor, hTAS2Rs possess only a single binding pocket which harbors the cognate bitter ligands.[74−77] This binding cavity is mainly constituted by the receptor transmembrane regions with moderate participation of the extracellular regions. The precise dimensions of this cavity have not yet been determined, but it is obviously limited in space. Hence, one possible scenario to explain the decreased bitter potency of steviol glycosides with increasing glycone length is the size of the molecules which might become too bulky to fit into the receptor's binding cavity. This information on structure/activity relationship might support the manufacturing of preferentially sweet and the least bitter tasting *Stevia* extracts based on the proper selection the best target molecules and optimization of breeding and postharvest downstream processing.

Human bitter taste receptors are known to exhibit considerable coding sequence diversity.[60] This has been linked to functional differences in receptor responses to bitter compounds as well as to individual differences in bitter taste perception.[47,61,62,64,65,78] Both hTAS2Rs identified in the present study as sensors for the bitter taste of steviol glycosides contain several single nucleotide polymorphisms (SNP), e.g., 8 cSNPs (7 nonsynonymous) for hTAS2R4 and 4 cSNPs (2 nonsynonymous) for hTAS2R14.[60] Our sensory panel was biased by selecting subjects who were sensitive to the bitter taste of rubusoside (**10**) and excluding those who were not. This perceptual difference could be explained by sequence variation in the genes encoding for hTAS2R4 and hTAS2R14. An integrative experimental approach combining genetic analysis, human sensory studies, and functional characterization of receptor variants would be required to prove or disprove this conjecture.

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Funding

This work was supported by funds from the German Research Foundation (DFG) to WM (Me1024/2-3; Me1024/8-1). We are also grateful to Cargill, Inc. for partially funding this project.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Cargill, Incorporated for providing Stevia samples and, in particular, to Brian Guthrie, Ph.D., for his continuous support.

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