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# Soy Isoflavones and Other Isoflavonoids Activate the Human Bitter Taste Receptors hTAS2R14 and hTAS2R39

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Supporting Information

**ABSTRACT:** The aim of this study was to identify the bitter receptor(s) that recognize the bitter taste of the soy isoflavone genistein. Screening of all 25 human bitter receptors revealed genistein as agonist of hTAS2R14 and hTAS2R39. Genistein displayed threshold values of 4 and 8 $\mu$M on hTAS2R14 and hTAS2R39 and $EC_{50}$ values of 29 and 49 $\mu$M, respectively. In addition, the behavior of structurally similar isoflavonoids was investigated. Although the two receptors are not closely related, the results for hTAS2R14 and hTAS2R39 were similar toward most isoflavonoid aglycones. By trend, threshold values were slightly lower on hTAS2R14. Glucosylation of isoflavones seemed to inhibit activation of hTAS2R14, whereas four of five glucosylated isoflavones were agonists of hTAS2R39, namely, glycitin, genistin, acetylgenistin, and malonylgenistin. A total of three hydroxyl substitutions of the A- and B-rings of the isoflavonoids seemed to be more favorable for receptor activation than fewer hydroxyl groups. The concentration of the trihydroxylated genistein in several soy foods exceeds the determined bitter receptor threshold values, whereas those of other soy isoflavones are around or below their respective threshold value. Despite its low concentration, genistein might be one of the main contributors to the bitterness of soy products. Furthermore, the bioactive isoflavonoids equol and coumestrol activated both receptors, indicating that their sensory impact should be considered when used as food ingredients.

**KEYWORDS:** bitterness, T2R, TAS2R, hTAS2R, genistein, daidzein, equol, coumestrol, soy

## INTRODUCTION

Soybeans (*Glycine max* L. Merrill) are consumed by Asian populations on a regular basis. Growing evidence of the positive health effects of soy compounds[1] and the need for alternatives to dairy products, due to food-related allergies and intolerances, increase the interest in soybean products in North America and Europe. An important group of health-beneficial compounds from soybean are isoflavones, of which genistein is the predominant representative.[2] Soybeans contain mainly 12 isoflavones, the aglycones genistein, daidzein, and glycitein and their respective malonylglucosides, acetylglucosides, and unsubstituted glucosides.[3] The quantities of isoflavones in soybeans depend on cultivar and year of cultivation.[4] Commonly, the malonylglucosides and unsubstituted glucosides dominate in soybeans, whereas acetylglucosides and aglycones are present in very small amounts.[3] Processing can lead to the conversion of malonylglucosides into acetylglucosides (decarboxylation due to dry heating) and unsubstituted glucosides (mainly due to moist heating).[5,6] Fermentation strongly increases the amount of aglycones due to the action of $\beta$-glucosidases.[5] In the whole bean, genistein forms are most abundant, followed by daidzein and glycitein forms.[6] Unfortunately, soybean isoflavones have undesirable sensory properties, for example, bitterness and astringency. Interestingly, the outcomes of sensory studies are not consistent, and the undesirable sensory properties are not ascribed to the same isoflavones.[7−11] The aglycone genistein is frequently reported as being bitter.[8−11]

Bitter taste is perceived by bitter taste receptors, referred to as T2Rs or TAS2Rs, which belong to the family of G-protein coupled receptors.[12] For almost all 25 human bitter receptors (hTAS2Rs) agonists have been identified.[13] The remaining orphan receptors are hTAS2R41, hTAS2R42, hTAS2R45, hTAS2R48, and hTAS2R60. Despite the occurrence of many bitter compounds in food, research has mainly been focused on toxic compounds, and only a few studies have been carried out on dietary compounds.[13−19] Bitter taste receptor activation by isoflavonoids has not been studied yet. The umbrella term "isoflavonoids" incorporates, among others, isoflavones, isoflavans, and coumestans.

The objective of the present study was to identify the bitter receptors activated by the soy isoflavone genistein. Our second objective was to map the structural requirements for receptor activation, using a variety of structurally similar isoflavonoids.

## MATERIALS AND METHODS

**Materials.** Isoflavonoids were purchased from Indofine Chemical Co. (Hillsborough, NJ), Extrasynthese (Genay, France), Sigma-Aldrich (Steinheim, Germany), Brunschwig (Amsterdam, The Netherlands), Bioconnect (Huissen, The Netherlands), and WAKO (Neuss, Germany). All compounds were dissolved in DMSO (Sigma-Aldrich) at a 100 mM stock concentration. Trypan blue solution (0.4%) was purchased from Sigma-Aldrich.

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Tyrode's buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$, and 20 mM Hepes, pH 7.4) was used for dilution of compound−DMSO stock solutions and for calcium imaging assays. If not mentioned otherwise, it contained 0.5 mM probenecid (Sigma-Aldrich).

**Expression of hTAS2Rs in HEK293 Cells.** For functional expression of the human bitter taste receptors, HEK293 T-Rex Flp-In cells (Invitrogen, San Diego, CA) stably expressing the chimeric G-protein $\alpha$-subunit G$\alpha$16-gust44 (cloned into pcDNA4 (Invitrogen))[20] and 1 of the 25 human bitter receptor genes (cloned into pcDNA5/FRT (Invitrogen)) were used. To improve membrane targeting of the receptor protein, each bitter receptor gene contained a DNA sequence encoding the first 45 amino acids of rat somatostatin receptor type 3 at its 5′ end (the receptor expression was achieved according to ref 21 with exception of the HSV-tag). The nomenclature of bitter receptors is identical to that of Meyerhof et al.[13] Cells were maintained in DMEM and 10% (v/v) tetracycline-free FBS (both Lonza, Verviers, Belgium) supplemented with blasticidin (5 $\mu$g/mL), geneticin (400 $\mu$g/mL), and hygromycin (100 $\mu$g/mL) (all from Invitrogen). All cells were grown and maintained at 37 °C and 5% (v/v) $CO_2$.

**Monitoring hTAS2Rs Activation by Intracellular Calcium Release.** Activation of human TAS2 receptors releases intracellular $Ca^{2+}$, which can be measured by a fluorescent calcium dye.[22] Variations in intracellular $Ca^{2+}$ concentrations were monitored with a FlexStation II 384 (Molecular Devices Corp., Sunnyvale, CA). hTAS2R-expressing cells were seeded into poly-L-lysine-coated (Sigma-Aldrich) 96-well plates (black wall, clear bottom, Greiner bio-one, Frickenhausen, Germany) at a density of $10^4$ cells in 100 $\mu$L/well and cultured for 24 h. Transcription of the receptors was induced by adding 0.25 $\mu$g/mL doxycycline (Sigma-Aldrich). Cells were induced for 24 h and then loaded with the calcium-sensitive fluorescent dye Fluo-4-AM (2.5 $\mu$M, Molecular Probes, Eugene, OR), which was dissolved in Tyrode's buffer containing 5% (v/v) tetracycline-free FBS (Lonza). One hour after loading, cells were washed with Tyrode's buffer and taken up in Tyrode's buffer. Stock solutions of test compounds were prepared in DMSO and diluted to the appropriate concentration in Tyrode's buffer, not exceeding a DMSO concentration of 1% (v/v). Screening of the 25 bitter receptors was done at 400 $\mu$M for genistein and genistin. For acetylgenistin and malonylgenistin, this was 100 $\mu$M due to limited availability of the compounds. Both hTAS2R14 and hTAS2R39 were screened for activation by the other isoflavonoids at 500 $\mu$M. In case of activation they were measured at different concentrations up to 1 mM to establish dose−response curves. For hTAS2R16, the applied concentrations were 100 $\mu$M acetylgenistin and malonylgenistin and 1 mM genistin, daidzin, and glycitin. Calcium responses of induced cells upon test compound addition were measured using a Flexstation II 384 for 90 s as described elsewhere.[23] The first 17 s before compound addition was used for baseline determination. After compound addition, the fluorescence signals (excitation 485 nm/emission 520 nm) were measured for an additional 70 s at 37 °C. Maximum fluorescence was reached within the measuring time of 90 s, but for some compounds the decrease of fluorescence back to baseline levels lasted longer than the time frame (for example, see Figure S1 in the Supporting Information). As negative control, noninduced cells were always measured in parallel. As positive controls, two wells/plate were measured with epicatechin gallate[18] for hTAS2R39 and with naphthoic acid[21] (or genistein) for hTAS2R14. All experiments were conducted in duplicate on two or more different days.

**Tests for Toxicity and Autofluorescence.** To investigate the effect of the isoflavonoids on the viability of the cells, dye exclusion tests were conducted after 2 min of incubation with each isoflavonoid (1 mM). The number of viable cells within one well was quantified using trypan blue (0.1%) and a cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA). Per compound, six cell counts were carried out, and an average number of cells was calculated. Viability of cells was determined by dividing the viable cell count by the total cell count. Furthermore, all isoflavonoids were tested for autofluorescence at their highest concentration (1 mM). For this, they were measured in a FlexStation II 384 under the conditions applied during the bitter receptor assay, with the exception that no cells were seeded in the 96-well plate. As positive control, the autofluorescent compound riboflavin was used.

**Data Processing and Statistical Analysis.** SoftMax Pro 4.8 software (Molecular Devices Corp.) was used to plot the fluorescence signals. The fluorescence values ($\Delta F/F_0$) were calculated by subtracting the baseline fluorescence ($F_0$) prior to loading from the maximum fluorescence ($F$) after addition of the bitter compounds, divided by the signals of the baseline to normalize to background fluorescence.[19] Besides the response of induced cells, also the response of noninduced cells was measured as negative control for every compound at every concentration on the same plate. In cases that a nonspecific signal occurred, the respective compound was considered as active when the signal of the induced cells was significantly higher than that of the negative control cells ($P = 0.05$). The signal intensity of noninduced cells was taken at the reading point at which the signal of the induced cells was maximal. Threshold values of the agonists toward receptor activation were determined as first concentration showing significant difference from the baseline and from the response of noninduced cells. Differences were considered to be significant at $P < 0.05$, using a $t$ test (two sided, nonpaired) (SAS 9.2, SAS Institute Inc., Cary, NC). Between 4 and 12 concentrations were used to fit nonlinear regression curves using Graph Pad Prism (version 4 for Windows, Graph Pad Software, San Diego, CA). The error bars reflect the standard error of the mean (SEM). For all compounds that reached a maximum in the concentration−response curve, the $EC_{50}$ values were calculated (after subtraction of the response of noninduced cells, if applicable). For compounds that evoked nonspecific signals at higher concentrations, only the data for the appropriate concentrations are shown in the figures. Some compounds were not completely soluble and, therefore, their real potency might be underestimated, leading to lower values than determined.

## RESULTS

**Screening for Bitter Receptor Activation by Various Forms of Genistein.** Genistein, one of the compounds strongly associated with bitterness of soybeans and soy products, and its glucoside forms, genistin, acetylgenistin, and malonylgenistin, were tested using HEK 293 cell lines, each cell line expressing 1 of the 25 different human bitter receptors. This was done to determine whether they activated one or more bitter receptors. At screening concentrations, two receptors were unambiguously activated by the aglycone genistein, hTAS2R14 and hTAS2R39 (Figure 1), whereas no bitter receptor was clearly activated by any of the three glucosides genistin, acetylgenistin, and malonylgenistin (data not shown). The activation of hTAS2R14 was stronger than that of hTAS2R39.

Screening for agonists was done with hTAS2Rs containing the most frequent single-nucleotide polymorphisms (SNPs). For hTAS2R38 the taster haplotype PAV[24] was used. As there are no agonists for the five orphan bitter receptors identified yet, we cannot be sure that they are functional. Therefore, we cannot exclude that receptors other than hTAS2R14 and hTAS2R39 might be activated by genistein and its glucoside forms.

hTAS2R14 is a well-known bitter receptor, which is activated by many, structurally diverse, bitter compounds (see, e.g., refs 13, 15, and 21). hTAS2R39 was just recently deorphanized, and it was stated that this receptor belonged to a group of bitter

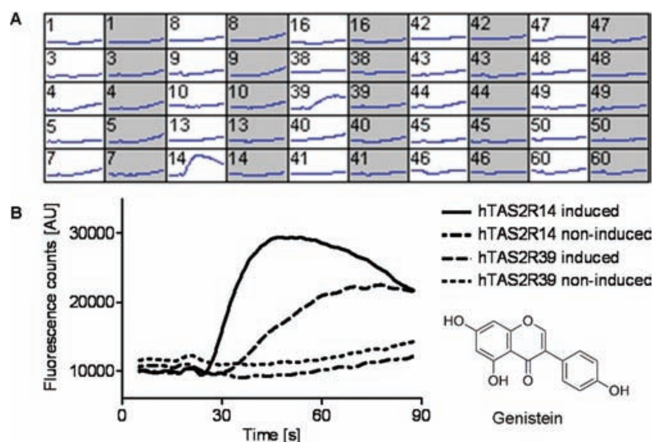


**Figure 1.** Bitter receptor activation by genistein. (A) Screening of HEK 293 cells, each stably expressing one of the 25 hTAS2Rs, toward activation by genistein (400 $\mu$M). The signals of induced cells are depicted with white background. Noninduced cells were used as negative controls (gray background). (B) Calcium traces of hTAS2R14 and hTAS2R39 and their respective negative controls.

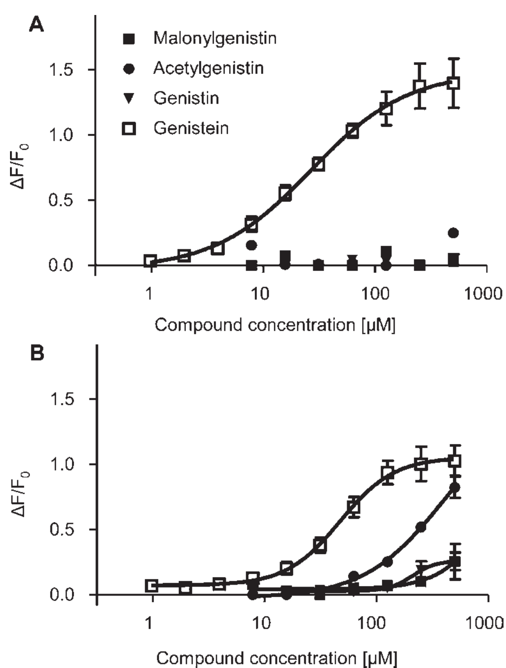


**Figure 2.** Normalized concentration−response curves of (A) hTAS2R14 and (B) hTAS2R39 containing HEK 293 cells stimulated with malonylgenistin, acetylgenistin, genistin, and genistein.

receptors that has a penchant for natural compounds.[13] Its agonist spectrum was smaller than that of the broadly tuned receptor hTAS2R14.

**Dose−Response Behavior of Genistein and Its Glucoside Forms on hTAS2R14 and hTAS2R39.** To compare the activations of hTAS2R14 and hTAS2R39 by genistein accurately, responses were recorded in the concentration range of 0.5−500 $\mu$M genistein. Genistein displayed threshold values of 4 and 8 $\mu$M on hTAS2R14 and hTAS2R39, respectively (dose−response curves shown in Figure 2). The $EC_{50}$ values after correction for nonspecific signals were calculated to be 28.9 ± 8.2 $\mu$M for hTAS2R14 and 49.4 ± 8.9 $\mu$M for hTAS2R39. Additionally, the three glucoside forms of genistein were investigated. None of them clearly activated hTAS2R14, whereas acetylgenistin activated hTAS2R39 from 125 $\mu$M onward and genistin and malonylgenistin activated hTAS2R39 at 500 $\mu$M. $EC_{50}$ values of the glucoside forms could not be determined because no maximum was reached in the concentration−response curves.

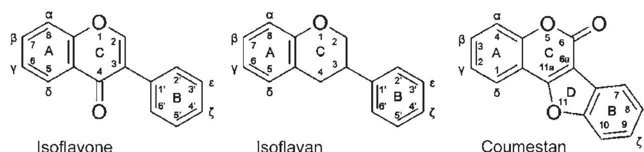


**Figure 3.** Chemical structures of the isoflavone, isoflavan, and coumestan skeletons. A−D denote the different rings of the isoflavonoids, 1−6′ and 1−11a the commonly used carbon positions (inside), and $\alpha$−$\zeta$ the positions used in this study (outside). The carbon positions can be substituted with −OH, $-OCH_3$, −*O*-Glc, −*O*-Glc-6″-*O*-acetyl, or −*O*-Glc-6″-*O*-malonyl.

**Structure−Activity Relationship of Selected Isoflavonoids on hTAS2R14 and hTAS2R39.** To understand the molecular signature involved in bitter receptor activation of genistein, 15 additional compounds were selected besides the 4 genistein forms, based on the structure of the soy isoflavone genistein: 13 isoflavones with different substitution pattern of the skeleton, 1 isoflavan, and 1 coumestan. To simplify comparisons between molecules, the common position numbering was substituted by Greek letter labeling as shown in Figure 3. For structural variation of the A- and B-rings, molecules with different hydroxyl and *O*-methyl substitutions were selected (summarized in Tables 1 and 2). Not all compounds were known as bitter, and the selection was purely based on their structural similarity to the bitter compound genistein.

These structurally similar isoflavonoids were screened for their ability to activate hTAS2R14 and hTAS2R39. When activation occurred at 500 $\mu$M, different concentrations were measured to map their dose−response behaviors. Thresholds values and, when appropriate, $EC_{50}$ values are given in Table 1 for hTAS2R14 and in Table 2 for hTAS2R39. In total, 13 compounds activated hTAS2R14, and 15 compounds were active on hTAS2R39. On the basis of the threshold values for each of the two receptors, the compounds were classified into four groups: I ≤ 32 $\mu$M (low); 32 $\mu$M < II ≤ 125 $\mu$M (medium); 125 $\mu$M < III ≤ 500 $\mu$M (high); IV, no activation up to 500 $\mu$M (not active, (na)). hTAS2R14 was activated by four compounds at low threshold, by two compounds at medium threshold, and by seven compounds at high threshold. Six compounds were considered to be inactive on hTAS2R14. For hTAS2R39, fewer compounds belonged to the low (2×) and the medium threshold groups (2×), but more compounds belonged to the high threshold group (11×). Four compounds were inactive on hTAS2R39. In a number of cases it was not possible to determine whether a compound activated the receptor or not, due to nonspecific signals in the negative controls, which were not significantly lower than those in the induced cells. Those compounds were considered as not active. Dye exclusion tests were conducted to investigate whether the applied isoflavonoid concentrations influenced the viability of the cells. Even at highest concentration (1 mM), no toxic effect on the cells was observed (see the Supporting Information, Figure S2). Furthermore, all compounds were tested for autofluorescence. None of the isoflavonoids in

Table 1. Summary of Threshold and $EC_{50}$ Values of Various Isoflavonoids on hTAS2R14

| | | positions | | | | | | $EC_{50}$ | threshold[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| compound name (code) | subgroup | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ | $\varepsilon$ | $\zeta$ | $\mu$M | $\mu$M | group |
| genistein (**Gein**) [b,c] | isoflavone | H | OH | H | OH | H | OH | 28.9 | 4 | I |
| (±)-equol (**10**) [d] | isoflavan | H | OH | H | H | H | OH | 47.2 | 8 | |
| prunetin (**6**) | isoflavone | H | $OCH_3$ | H | OH | H | OH | nd[e] | 16 | |
| 7-hydroxyisoflavone (**3**) | isoflavone | H | OH | H | H | H | H | nd | 32 | |
| biochanin A (**1**) [b] | isoflavone | H | OH | H | OH | H | $OCH_3$ | nd | 63 | II |
| 7,8,4′-trihydroxyisoflavone (**8**) | isoflavone | OH | OH | H | H | H | OH | 124 | 63 | |
| isoflavone (**5**) | isoflavone | H | H | H | H | H | H | nd | 250 | III |
| 6,7,4′-trihydroxyisoflavone (**7**) | isoflavone | H | OH | OH | H | H | OH | 378 | 250 | |
| 7,3′,4′-trihydroxyisoflavone (**9**) | isoflavone | H | OH | H | H | OH | OH | 264 | 250 | |
| coumestrol (**11**) [b] | coumestan | H | OH | H | H | H | OH | 358 | 250 | |
| formononetin (**2**) [b] | isoflavone | H | OH | H | H | H | $OCH_3$ | nd | 500 | |
| daidzein (**Dein**) [b,c] | isoflavone | H | OH | H | H | H | OH | nd | 500 | |
| glycitein (**Glyein**) [b] | isoflavone | H | OH | $OCH_3$ | H | H | OH | nd | 500 | |
| 7-hydroxy-6-methoxyisoflavone (**4**) | isoflavone | H | OH | $OCH_3$ | H | H | H | nd | nsp[f] | IV |
| acetylgenistin (**AGe**) [b] | isoflavone glucoside | H | *O*-Glc-6″-*O*-acetyl | H | OH | H | OH | nd | na | |
| daidzin (**D**) [b,c] | isoflavone glucoside | H | *O*-Glc | H | H | H | OH | nd | na | |
| genistin (**Ge**) [b,c] | isoflavone glucoside | H | *O*-Glc | H | OH | H | OH | nd | na | |
| glycitin (**Gly**) [b] | isoflavone glucoside | H | *O*-Glc | $OCH_3$ | H | H | OH | nd | na | |
| malonylgenistin (**MGe**) [b,c] | isoflavone glucoside | H | *O*-Glc-6″-*O*-malonyl | H | OH | H | OH | nd | na | |

[a] Four groups of thresholds for activation of hTAS2R14 correspond to I (low) (≤ 32 $\mu$M), II (medium) (>32−125 $\mu$M), III (high) (>125−500 $\mu$M), and IV (not active up to 500 $\mu$M, na). [b] Occurring in soybean (*Glycine max* L. Merrill).[3,42] [c] Compound frequently reported as bitter.[7−11] [d] *S*-(−)Equol is an intestinal metabolite of daidzein.[40] [e] nd, not determined. [f] nsp, not specific, activity could not be determined due to nonspecific signals in the negative control.

this study showed fluorescent behavior under the conditions used in the assay.

**Effect of Glucosylation.** Aglycones showed similar effects on hTAS2R14 and hTAS2R39, whereas glucosides showed different effects. Thirteen of the 14 aglycones activated hTAS2R14, whereas there were 11 for hTAS2R39. Most of them activated hTAS2R14 at lower concentrations than hTAS2R39. By contrast, glucosylated isoflavones did not show any activity on hTAS2R14. Although thresholds were generally high, hTAS2R39 could be activated by four of five isoflavone glucosides, namely, glycitin, genistin, acetylgenistin, and malonylgenistin (Figure 2). This finding suggested that glucosylation inhibited activation of hTAS2R14.

As $\beta$-glucopyranosides are reported to activate hTAS2R16,[25] the five glucosides daidzin, glycitin, genistin, acetylgenistin, and malonylgenistin were additionally tested on hTAS2R16. None of them activated this receptor. A very recent publication reported the inhibition of hTAS2R16 by probenecide,[26] a compound commonly used in G-protein coupled receptor assays to prevent the efflux of calcium-sensitive dyes from the cells.[27] Therefore, experiments on hTAS2R16 were repeated without probenecide in the buffer. Also without probenecide, no activation of hTAS2R16 by soy isoflavone glucosides occurred.

**Influence of C-Ring Configuration.** To investigate the influence of the C-ring of isoflavonoids on bitter receptor activation, the isoflavone daidzein (**Dein**), the isoflavan equol (**10**), and the coumestan coumestrol (**11**) were compared. They all have two hydroxyl groups (on positions $\beta$ and $\zeta$) in common, whereas the C-ring is different (position labeling illustrated on skeletons in Figure 3). All three compounds were able to activate hTAS2R14 and hTAS2R39, which is illustrated in Figure 4A for hTAS2R14 (equol ≫ daidzein, coumestrol). The results suggested that bitter receptor activation is not hindered by variation in C-ring structure, although potency and efficacy might be affected. Although only three compounds were compared, our data might suggest that planarity of the C-ring is less favorable for binding to the bitter receptors.

**Substitutions of A- and B-Rings.** In Figure 4B, the activation of hTAS2R14 by three isoflavones is compared. These three isoflavones (**7**−**9**) are all substituted by three hydroxyl groups. **7** and **8** have two hydroxyl groups on the A-ring and one hydroxyl group on the B-ring, whereas **9** has vice versa. They all activated the bitter receptor, but with differences in potency and efficacy. The exact location of hydroxylation is of importance, as, for example, two hydroxyl groups on positions $\alpha$ and $\beta$ (compound **8**) led to higher efficacy and potency than two hydroxyl groups on positions $\beta$ and $\gamma$ (compound **7**). Also, the number of substitutions matters; in most cases, three substitutions were more favorable for activation than two, which can be seen (Table 1) when **Glyein** is compared to **4**, **1** is compared to **2**, or **Gein** is compared to **Dein**. For the activation of hTAS2R39, substitution of the A-ring is important, as the unsubstituted compound (**5**) was inactive. The absence of substitutions had less influence on hTAS2R14 activation. For both receptors, an *O*-methyl group instead of a hydroxyl group negatively influenced receptor activation (with the

Table 2. Summary of Threshold and $EC_{50}$ Values of Various Isoflavonoids on hTAS2R39

| | | positions | | | | | | $EC_{50}$ | threshold[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| compound name (code) | subgroup | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ | $\varepsilon$ | $\zeta$ | $\mu$M | $\mu$M | group |
| genistein (**Gein**)[b,c] | isoflavone | H | OH | H | OH | H | OH | 49.4 | 8 | I |
| (±)-equol (**10**)[d] | isoflavan | H | OH | H | H | H | OH | 55.8 | 32 | |
| 7,8,4′-trihydroxyisoflavone (**8**) | isoflavone | OH | OH | H | H | H | OH | 184 | 63 | II |
| acetylgenistin (**AGe**)[b] | isoflavone glucoside | H | *O*-Glc-6″-*O*-acetyl | H | OH | H | OH | nd[e] | 125 | |
| 7-hydroxyisoflavone (**3**) | isoflavone | H | OH | H | H | H | H | 315 | 250 | III |
| 6,7,4′-trihydroxyisoflavone (**7**) | isoflavone | H | OH | OH | H | H | OH | nd | 250 | |
| 7,3′,4′-trihydroxyisoflavone (**9**) | isoflavone | H | OH | H | H | OH | OH | nd | 250 | |
| coumestrol (**11**)[b] | coumestan | H | OH | H | H | H | OH | nd | 250 | |
| biochanin A (**1**)[b] | isoflavone | H | OH | H | OH | H | $OCH_3$ | nd | 500 | |
| formononetin (**2**)[b] | isoflavone | H | OH | H | H | H | $OCH_3$ | nd | 500 | |
| daidzein (**Dein**)[b,c] | isoflavone | H | OH | H | H | H | OH | nd | 500 | |
| genistin (**Ge**)[b,c] | isoflavone glucoside | H | *O*-Glc | H | OH | H | OH | nd | 500 | |
| glycitein (**Glyein**)[b] | isoflavone | H | OH | $OCH_3$ | H | H | OH | nd | 500 | |
| glycitin (**Gly**)[b] | isoflavone glucoside | H | *O*-Glc | $OCH_3$ | H | H | OH | nd | 500 | |
| malonylgenistin (**MGe**)[b,c] | isoflavone glucoside | H | *O*-Glc-6″-*O*-malonyl | H | OH | H | OH | nd | 500 | |
| prunetin (**6**) | isoflavone | H | $OCH_3$ | H | OH | H | OH | nd | nsp[f] | IV |
| 7-hydroxy-6-methoxyisoflavone (**4**) | isoflavone | H | OH | $OCH_3$ | H | H | H | nd | nsp | |
| isoflavone (**5**) | isoflavone | H | H | H | H | H | H | nd | na | |
| daidzin (**D**)[b,c] | isoflavone glucoside | H | *O*-Glc | H | H | H | OH | nd | na | |

[a] Four groups of thresholds for activation of hTAS2R39 correspond to I (low) (≤ 32 $\mu$M), II (medium) (>32−125 $\mu$M), III (high) (>125−500 $\mu$M), and IV (not active up to 500 $\mu$M, na). [b] Occurring in soybean (*Glycine max* L. Merrill).[3,42] [c] Compound frequently reported as bitter.[7−11] [d] *S*-(−)equol is an intestinal metabolite of daidzein.[40] [e] nd, not determined. [f] nsp, not specific, activity could not be determined due to nonspecific signals in the negative control.

exception of prunetin (**6**) on hTAS2R14). Hydroxylation of $\beta$ and $\zeta$ and one additional position seemed to be most favorable for receptor activation, as all compounds containing these features were able to activate hTAS2R14 and hTAS2R39.

## DISCUSSION

**Common Agonists on hTAS2R14 and hTAS2R39.** The identity between amino acid sequences of hTAS2R14 and hTAS2R39 is only 25%.[28] Despite this, they are both activated by a largely overlapping group of isoflavonoids. We found 11 isoflavonoids that were able to activate both bitter receptors (compounds **Gein**, **Dein**, **Glyein**, **1**−**3**, and **7**−**11**). Until now, only four compounds were described that stimulated both hTAS2R14 and hTAS2R39, which are azathioprine, chlorpheniramine, diphenidol, and quinine[13] (see the Supporting Information). These four compounds and the newly identified isoflavonoid agonists do not share structural characteristics with regard to charge, hydrophobicity, or the ability to act as proton donor or acceptor. In Figure 5A, the threshold values of the agonists on hTAS2R14 are displayed against the threshold values on hTAS2R39. It was remarkable that the compounds with low threshold at one receptor also demonstrated low threshold at the other receptor. The dashed line in the figure depicts the correlation. A similar trend was observed for $EC_{50}$ values on hTAS2R14 and hTAS2R39, as illustrated in Figure 5B. Aglycones showed similar effects on both receptors, but the agonists were by trend less active on hTAS2R39 than on hTAS2R14.

The question remains as to which of the two bitter receptors is more important for recognition of isoflavones. In situ hybridization experiments indicated that the expression frequencies of hTAS2R14 and hTA2R39 in taste receptor cells of human circumvallate papillae were similar.[29] They were reported to be 5.4% for hTAS2R14 and 4.2% hTA2R39. It might be concluded from our results that hTAS2R14 is slightly more important for the recognition of isoflavone aglycones, as the determined bitterness threshold values were lower for hTAS2R14 than for hTAS2R39. In contrast, hTAS2R39 was activated by isoflavone glucosides, and, therefore, might be seen as more important bitter receptor for the recognition of soy isoflavone glucosides, as glucosides are more abundant in most soy products. For fermented soy products, which are richer in aglycones, hTAS2R14 gains importance.

**New Dietary Agonists of Receptor hTAS2R39.** So far, 17 agonists had been described for hTAS2R39[13,18,30] (see the Supporting Information). Of these agonists, four are flavonoids and therefore structurally similar to isoflavonoids. The other 13 agonists belong to different chemical classes, and they are structurally very different from isoflavonoids. Six of the 17 known agonists are regularly consumed in our diet as tea catechins, vitamin B1, and quinine in bitter tonic. In our study, 15 new ligands of hTAS2R39 were identified, namely, **Gein**, **Dein**, **Glyein**, **Ge**, **Gly**, **AGe**, **MGe**, **1**−**3**, and **7**−**11**. Most of the new agonists are dietary compounds. In conclusion, it can be expected that hTAS2R39 plays a key role in the detection of dietary compounds.

It was reported[25] that $\beta$-glucopyranosides with a hydrophobic group attached to C1 of the pyranose activated the human bitter receptor hTAS2R16. The $\beta$-configuration of the glycosidic bond and the steric position of the hydroxyl group at C4 of the pyranose were crucial for activation of hTAS2R16, whereas the hydrophobicity of the C1 substituent was reported to be important but not essential for activation of hTAS2R16. As the glucosylated soy isoflavones in this study also belong to the class of $\beta$-glucopyranosides, we speculated that they might also be agonists of hTAS2R16. Under the conditions used by us, we did not find activation of hTAS2R16 by isoflavone glucosides. It cannot be excluded that higher concentrations would lead to activation, but such high concentrations are not expected in most soy products. More likely, the aglycone part of the isoflavone glucosides did not match the size and hydrophobicity requirements of hTAS2R16. It was surprising to identify isoflavone glucosides as agonists of hTAS2R39 instead.

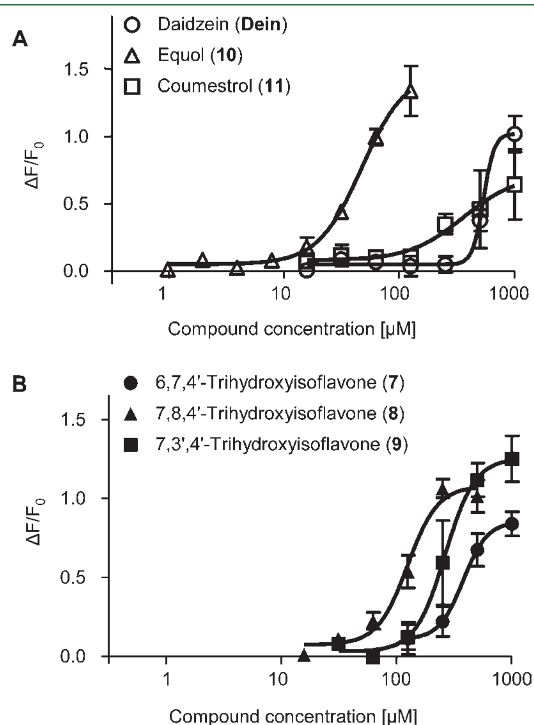


**Figure 4.** Concentration−response curves of hTAS2R14 for (A) the isoflavone daidzein (**Dein**), the isoflavan equol (**10**), and the coumestan coumestrol (**11**) and (B) the three isoflavones 6,7,4′-trihydroxyisoflavone (**7**), 7,8,4′-trihydroxyisoflavone (**8**), and 7,3′,4′-trihydroxyisoflavone (**9**).

**Relevance for Sensory Perception.** Okubo et al.[9] described the threshold values for bitterness and astringency together to be in the nanomolar to micromolar range (from $10^{-6}$ to $10^{-7}$ M for genistein, from $5 \times 10^{-7}$ to $5 \times 10^{-8}$ M for daidzein). Kudou et al.[10] determined threshold values for undesirable taste and determined $10^{-5}$ M for genistein, $10^{-6}$ M for daidzein, and $10^{-5}$ M for the three glucoside forms of genistin. Another study[8] investigated daidzein and genistein regarding bitterness alone, but applied them in starch solutions. Reported bitter threshold values ($4 \times 10^{-3}$ M for genistein, $2.9 \times 10^{-3}$ M for daidzein) are in the millimolar range, whereas the bitter receptor threshold values determined in our study are in the micromolar range. This observation is in line with the investigations of Intelmann et al.,[15] comparing results of taste receptor assays with those of human psychophysical experiments. From that study, it was concluded that the difference between receptor threshold values (low) and sensory threshold values (higher) was caused by interaction of bitter compounds with the oral mucosa. It is remarkable that the results from sensory studies on soy isoflavones differ so much. This might have been caused by genetic variation of sensory panelists. Different SNPs in hTAS2R genes might lead to differences in the perception of soy products. In our study, screening for agonists was done with hTAS2Rs that contained the most frequently occurring SNPs and the taster haplotype PAV of hTAS2R38. The receptors hTAS2R14 and hTAS2R39, identified for soy isoflavones in our study, contained the SNPs that occur with a frequency of 99% (GPCR Natural Variance database, http://nava.liacs.nl[31]). Therefore, we assume that the various outcomes of the sensory studies described above were more likely caused by different experimental setups and the fact that subjects can perform differently than by genetic variation. Besides interaction of bitter tastants with oral mucosa, also changes in salivary flow and composition,[32] adaptational states, and hormone levels can cause variable taste responses.[33] With the bitter receptor activation assay, we have now measured the intrinsic bitterness of a number of (dietary) isoflavonoids in

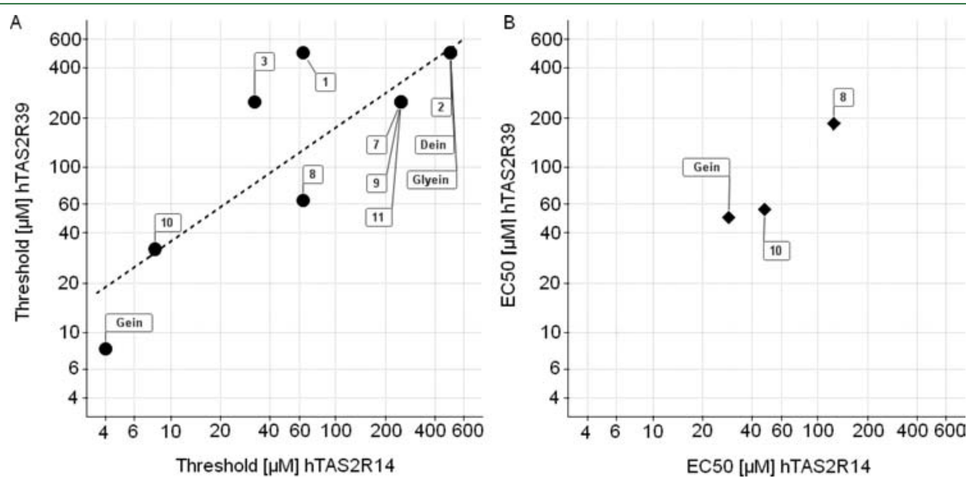


**Figure 5.** Correlation of (A) threshold values and (B) $EC_{50}$ values of hTAS2R14 and hTAS2R39. The dashed line in panel A represents the linear regression line ($R^2$ = 0.75).

which the actual taste characteristic is uncoupled from the other influences mentioned above. Confirmation of the bitterness of newly identified bitter receptor agonists in a sensory study remains to be established.

The concentrations of isoflavones in soy foods can be variable. For example, the concentration of genistein reported by Prabhakaran[34] varied between 0.4 and 14.3 $\mu$g/g in soy milk and between 2.4 and 60.4 $\mu$g/g in tofu. This is approximately 2−225 $\mu$M, which exceeds the bitterness threshold values determined. Despite its low concentrations in relation to other soy isoflavonoids, the presence of genistein can have a significant impact on the bitter taste of soy food. During processing, malonyl forms decrease drastically in concentration, whereas the amounts of the other three forms increase.[35−37] Malonyl forms might therefore be less important for the perception of the final products. Acetyl forms are generated during dry heating, so they occur in a subset of soy products only. Their concentration in common soy products ranges from 0 to 110 $\mu$g/g (soy milk[38] and fried tofu,[39] respectively), which is approximately 0−250 $\mu$M. Therefore, depending on the soy product, it is below or just above the determined threshold values on hTAS2R39 and will probably not lead to strong bitter perception. The concentration of genistin in soy products is between 23 $\mu$g/g (soy milk[38]) and 562 $\mu$g/g (tofu[39]), which is approximately 50 $\mu$M−1.3 mM, so it can be above the threshold values determined for hTAS2R39. In soy products, the bitter taste of isoflavones might be masked by other constituents present in the product.

Irrespective the amounts of isoflavones in different soy products, most of the investigated isoflavonoids belonged to the high threshold group III. From a nutritional perspective, this is probably advantageous, as strong bitterness would prevent consumption of soy products, which can provide multiple health benefits. Today's attempts to make healthier food include the incorporation of more bioactive isoflavonoids as ingredients. For example, equol, which is originally an intestinal metabolite formed from daidzein, and now considered to be a neutraceutical, is more estrogenic than its precursor.[40] Also, coumestrol, which is formed from daidzein by combined malting by a food-grade fungus, is more estrogenic than its precursor.[41] The results of our study show that equol and coumestrol are intrinsically more bitter than most of the common soybean isoflavones, indicating that their sensory impact should be considered when food products enriched in these compounds are formulated.

## ■ ASSOCIATED CONTENT

**S Supporting Information.** Overview of agonists of hTAS2R14 and hTAS2R39, outcomes of viability tests, and an example for curve shape after prolonged measuring time. This material is available free of charge via the Internet at http://pubs.acs.org.

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