Intrinsic bitterness of flavonoids and isoflavonoids and masking of their taste activity

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ABSTRACT

The presence of flavonoids and isoflavonoids in foods and their addition as bioactives to food products can impart unpleasant bitterness. Therefore, debittering strategies are demanded. The aim of this research was to identify human bitter receptors (hTAS2Rs) sensing (iso)flavonoids and to determine the intrinsic bitterness and structure-activity relationships of soy isoflavones, tea catechins and a large set of structurally similar (iso)flavonoids by testing for activation of hTAS2Rs *in vitro*. A subsequent aim was to use the bitter receptor assay for investigation of different debittering strategies.

Out of all 25 human bitter taste receptors, hTAS2R14 and hTAS2R39, were activated by soy isoflavones. hTAS2R14 was only activated by isoflavone aglycones, whereas hTAS2R39 was activated by isoflavone glucosides as well. Investigation of almost 100 (iso)flavonoid aglycones for activation of hTAS2R14 and hTAS2R39 revealed that many (iso)flavonoids activated these receptors. The structural characteristics for an (iso)flavonoid to activate hTAS2R14 and hTAS2R39 were determined by 3D-pharmacophore models to be composed of two (for hTAS2R14) or three (for hTAS2R39) hydrogen bond donor sites, one hydrogen bond acceptor site, and two aromatic ring structures, of which one had to be hydrophobic. Three 6-methoxyflavanones were identified which reduced activation of hTAS2R39 by epicatechin gallate (ECG). These bitter receptor blockers were characterized as reversible insurmountable antagonists. Furthermore, complexation of epigallocatechin gallate (EGCG) with food proteins (mainly β-casein and Na-caseinates) reduced hTAS2R39 activation. A trained sensory panel confirmed reduced bitterness perception.

The systematic investigation of (iso)flavonoid aglycones indicated that the substitution pattern of (iso)flavonoids is of higher importance for bitter receptor activation than the backbone structure. In case of bitter receptor antagonists, the substitution pattern as well as backbone structure revealed to be crucial for functionality. The bitter receptor assay was shown to be an appropriate tool not only for identification of bitter receptor agonists and antagonists, but also for identification of reduced receptor activation by complexing agents.

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Chapter 1

General introduction

BITTER TASTE IN RELATION TO DIETARY COMPOUNDS

Bitter taste in food is mostly disliked by consumers. On the other hand, bitter taste in food can be appreciated, as long as it is moderate, in products such as bitter lemon, coffee, beer, and dark chocolate *(1)*. Bitter tasting compounds in food are mainly derived from plants (*e.g.* caffeine in coffee, naringin in grapefruit, sinigrin in cabbage, hop bitter acids in beer), but they can also occur in products from animal origin (*e.g.* bitter peptides in cheese), due to processing (*e.g.* the Maillard reaction products such as quinizolate or (bis)pyrrolidinohexose reductones) and upon storage (*e.g.* oxidized lipids) *(2-4)*. In this thesis, dietary compounds are defined as compounds naturally present in food raw materials, food products, and compounds (natural or synthetic) added to food products. Drugs, herbal medicine, and synthetic or natural non-consumable bitter substances are considered as non-dietary.

The biological function of bitter compounds in plants is to protect the plant against pathogens, parasites and predators. Especially toxins (*e.g.* strychnine) are often bitter. Therefore, it has been assumed that the ability to taste bitter compounds and rejection of bitter taste has long been crucial to survival. However, a correlation between toxicity and bitter taste thresholds has not been found *(1)*.

Numerous natural bitter compounds have been associated with putative health effects. Amongst them, isoflavonoids and flavonoids play a central role. Therefore, a trend in food product development is the design of functional foods, enriched in (iso)flavonoids. However, their incorporation in, or fortification of, foods can introduce or enhance bitterness, and, therefore, debittering strategies are demanded. Investigation of bitter taste and reduction of bitter taste by sensory tests is laborious and can be influenced by many factors. A fast and objective way of investigating bitterness is the measurement of intrinsic bitterness on bitter taste receptors by the use of a cell-based assay system. This method constitutes an important part of this thesis.

Intrinsic bitterness is the capacity of a compound to activate a bitter taste receptor, uncoupled from other taste qualities (*e.g.* sweet), senses (*e.g.* smell), influences (*e.g.* hormone levels) and interactions (*e.g.* with saliva). Due to its high throughput character, many (potentially) bitter compounds (even of non-food quality) can be examined in a bitter receptor assay. In this way, sensory panels can be disburdened from tasting many aversive compounds, and their services can be minimized to confirm the impact of compounds of high interest.

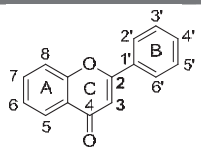
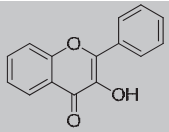
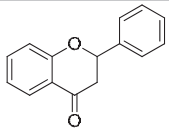
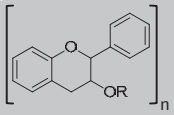
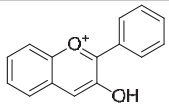
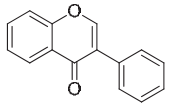
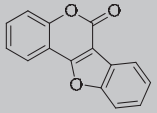
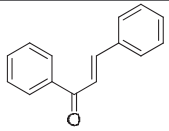
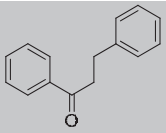
STRUCTURAL CLASSIFICATION OF FLAVONOIDS AND ISOFLAVONOIDS

Flavonoids are ubiquitously occurring secondary plant metabolites that play, besides their physiological role in plants as pigment and defence compounds, a role in the human diet *(5)*. Isoflavonoids occur almost exclusively in the *Leguminosae* family *(6)*. Flavonoids and isoflavonoids are classified as members of phenolic compounds. The non-(iso)flavonoid phenolics are *e.g.* hydroxycinnamic acids, coumarins, xanthones, stilbenes, ellagitannins, and lignans.

(Iso)flavonoids consist of two aromatic rings, connected via a three-carbon bridge (C_6-C_3-C_6). In most cases, the three carbon bridge is part of a six member heterocyclic ring, including one oxygen atom (pyran ring) *(7)*. In **Table 1**, an overview of the most important dietary (iso)flavonoid subclasses is shown, including their generic numbering pattern. (Iso)flavonoids belong to the super family of phenyl benzopyrans *(6)*, and their nomenclature is based on the variation of the C-ring, and the connection between B- and C-rings. The linkage of the B-ring to position 2 of the benzopyran describes the class of flavonoids (2-phenyl benzopyrans), whereas a linkage to position 3 describes the class of isoflavonoids (3-phenyl benzopyrans). The positions 2 and 3 are accentuated in the first structure of **Table 1**. In literature, isoflavonoids are often referred to as flavonoids, but due to their different substitution of the C-rings compared to flavonoids, this classification is controversial. In this thesis, a distinction between flavonoids and isoflavonoids is, therefore, made. Besides attachment to position 2 or 3, the B-ring can be connected to position 4, a class of compounds referred to as neoflavonoids (4-phenyl benzopyrans). Neoflavonoids hardly occur in the human diet. Furthermore, there are C_6-C_3-C_6 compounds not containing the pyran C-ring, *e.g.* chalcones and aurones, which are referred to as minor or miscellaneous flavonoids.

The backbone structures are mostly substituted by –OH, -OCH_3 and O-glycoside groups, but also C-glycoside, prenylation and sulfonation are possible substitutions *(5, 8)*. In foods, (iso)flavonoids occur as glycosides (*e.g.* in many fruits, seeds, and vegetables), as aglycones (*e.g.* in tea and in fermented soy products), or as oligomers (such as proanthocyanidins, *e.g.* in grapes and peanuts). This thesis focusses on the aglycones and takes two food products as examples: soybean-derived (*Glycine max*) products and tea (*Camellia sinensis*).

Table 1. Mostly described dietary (iso)flavonoid subclasses, with examples of compounds and their sensorial characteristics (combined from ***(1, 9)*** and adapted according to ***(3, 10-20)***). n.i. no information found ***(21)***.

Subclass	Backbone structure	Example	Typical food source	Sensorial characteristics
Flavonoids				
Flavone		Tangeretin Nobiletin Luteolin Apigenin	orange juice orange juice green spices green spices	bitter bitter n.i. n.i.
Flavonol		Quercetin Kaempferol Myricetin	wine, onion capers wine	bitter n.i. bitter
Flavanone		Naringin Eriodictyol	grapefruit juice oregano	bitter tasteless
Flavanol		Catechin (n=1, R=H) Procyanidin B2 (n=2, R=H) Epigallocatechin gallate (n=1, R=gallic acid)	wine, chocolate wine, chocolate tea	bitter, astringent astringent, bitter bitter, astringent
Antho-cyanidin		Pelargonidin Cyanidin-3-O-glycoside	strawberry blackberry	n.i. n.i.
Isoflavonoids				
Isoflavone		Genistein Daidzein Biochanin A	soy products soy products peanut	bitter, astringent bitter, astringent n.i.
Coumestan		Coumestrol	soy products	n.i.
Miscellaneous flavonoids				
Chalcone		Xanthohumol Isoliquritigenin	beer licorice	bitter n.i.
Dihydro-chalcone		Phloretin Neohesperidin-dihydrochalcone	apple juice artificial sweetener in chewing gum	neutral - bitter sweet

PROPERTIES OF (ISO)FLAVONOIDS

Sensorial effects

(Iso)flavonoids can produce several sensorial responses, from sweet (dihydrochalcones) to astringent (proanthocyanidins, flavanols), and bitter (most subclasses) *(9)*. For flavanols, it has been found that their degree of polymerization determines their sensorial properties. Monomeric flavanols (catechins) tend to be more bitter than astringent, whereas bitterness decreases and astringency increases upon increasing degree of polymerization (proanthocyanidins) *(12)*. Sweet and bitter taste result from the activation of taste receptors on the tongue, whereas astringency is a puckering, rough or dry sensation in the mouth *(22)*, caused by the interaction between phenolic compounds and proline-rich salivary proteins. This interaction leads to precipitation of salivary proteins and a loss of lubrication in the mouth *(23)*. The mechanism underlying astringency is not fully understood yet. Sensory impressions by astringency and bitterness are easily confused and require training to be reliably distinguished. The majority of flavonoids with known sensorial properties is reported as tasting bitter. Examples of taste properties are given in **Table 1**. The following subchapters will go more into detail about taste, especially bitter taste.

Effects on color and stability

The name "flavonoids" is derived from the Latin word "*flavus*", meaning yellow, due to the fact that many flavonoids are yellow. Additionally, flavonoids can be whitish, brownish, greenish, orange, red, purple, and blue. The latter three colors are the typical anthocyanin colors, which are responsible for the appearance of *e.g.* red wine and berries.

Although of minor importance compared to taste, flavonoids can also be involved *(24)* in the stability of a food product, *e.g.* beer and wine, by changes of color (oxidation) or haze formation (interaction of haze-forming proteins and phenolics).

Effects on health

A large number of publications reports on potential health benefits of flavonoids and isoflavonoids. These are *e.g.* prevention of some cancers, cardiovascular disease, menopausal complaints and osteoporosis, and are ascribed to properties such as antioxidant, anti-inflammatory, anti-carcinogenic, anti-thrombotic, and binding to the human estrogen receptor *(5, 9, 25)*. It should be mentioned that there are also contradictory studies on health effects of (iso)flavonoids, and the real effect on health has often not been established yet *(26)*. Nevertheless, as the large majority of reports has proposed beneficial health effects of (iso)flavonoids, a trend in food product development is the design of functional foods, enriched in (iso)flavonoids.

DIETARY OCCURRENCE OF (ISO)FLAVONOIDS

Consumption and content in food

The (iso)flavonoid consumption in several Western countries has been calculated to vary between 20 mg / day and 220 mg / day *(7, 27-30)*. Flavanols are often reported to be the most consumed flavonoids. The mean dietary intake of isoflavonoids accounts for a few mg / day only in non-Asian countries *(7, 31)*, whereas it can add up to 25-100 mg / day in Asian countries *(31)*.

Three databases list contents of (iso)flavonoids reported for various food sources: (i) USDA database for the flavonoid content of selected foods *(32)*, (ii) USDA database for the isoflavonoid content of selected foods *(33)*, and (iii) Phenol-Explorer *(18-20)*. Flavonoid and isoflavonoid contents in food can vary largely. A complicating factor is the common practice to hydrolyze glycosides into aglycones before quantitative analysis *(34)*. Therefore, the exact contents of individual (iso)flavonoid glycosides and aglycones are often not known.

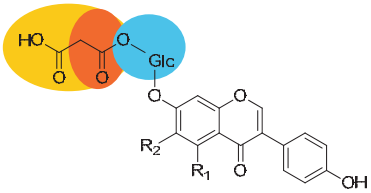
Soy products and tea are given as examples for the dietary occurrence of (iso)flavonoids in food products rich in aglycones.

Soy

In Western markets, soybean products have, despite their claimed health-promoting effects *(35)*, limited acceptance due to undesirable tastes *(1)*. Besides astringency and beany flavor, bitterness is regarded as one of the key off-tastes, probably evoked by the presence of isoflavones and saponins *(36)*. It is unknown which of the two classes has the highest contribution to soy off-taste. Nevertheless, this thesis focusses on isoflavones. Furthermore, several soybean-derived peptides and oxidized fatty acids can taste bitter, but as they are usually removed during soy food production they are not considered important for bitter taste in soy products *(1)*. Twelve different isoflavones (**Table 2**) commonly occur in soybeans and soybean-derived products *(37)*. The composition of isoflavone forms depends on cultivar, growth conditions, and processing method *(38-40)*. Examples of isoflavone compositions in unprocessed soybeans and soy products are shown in **Figures 1 and 2**. Commonly, genistein forms are most abundant in soybeans, followed by daidzein and glycitein forms *(37, 40)*. Malonyl glucosides and unsubstituted glucosides are the predominant form in unprocessed soybeans, whereas acetyl glucosides and aglycones are present in very small amounts *(37)* (**Figure 1)**. Processing can lead to the conversion of malonyl glucosides into acetyl glucosides (decarboxylation upon dry heating) and unsubstituted glucosides (mainly due to deesterification upon moist heating) *(40, 41)*. During soaking of soybeans, aglycone formation is started due to action of β-glucosidases (prior to heating). Fermentation strongly increases the amount of aglycones *(41)*. The

effects of processing are reflected in the change of isoflavone composition, as shown for selected soybean products in **Figure 2**. In literature, isoflavone aglycones *(1, 36, 42)*, glucosides *(36, 42)*, and malonyl glucosides *(43)* have been reported as bitter. Contradictory observations have been made about the taste of the aglycones genistein and daidzein and their glucosides genistin and daidzin. Whereas converting isoflavones enzymatically into their aglycones has been suggested as method to reduce bitterness and astringency by one author *(44)*, another author reported an increased objectionable taste after β-glucosidase treatment, caused by aglycones *(45)*. An objective tool to determine the intrinsic bitterness of isoflavones is needed to identify the target molecules for modifying bitter taste of soy products.

Table 2. Generic structure of soy isoflavones.



		Isoflavone aglycones	Unsubstituted isoflavone glucosides	Acetyl isoflavone glucosides	Malonyl isoflavone glucosides
		mainly obtained after fermentation	native / formed upon moist heating	formed upon dry heating	native
R_1 = OH	R_2 = H	Genistein	Genistin	Acetyl genistin	Malonyl genistin
R_1 = H	R_2 = H	Daidzein	Daidzin	Acetyl daidzin	Malonyl daidzin
R_1 = H	R_2 = OCH_3	Glycitein	Glycitin	Acetyl glycitin	Malonyl glycitin

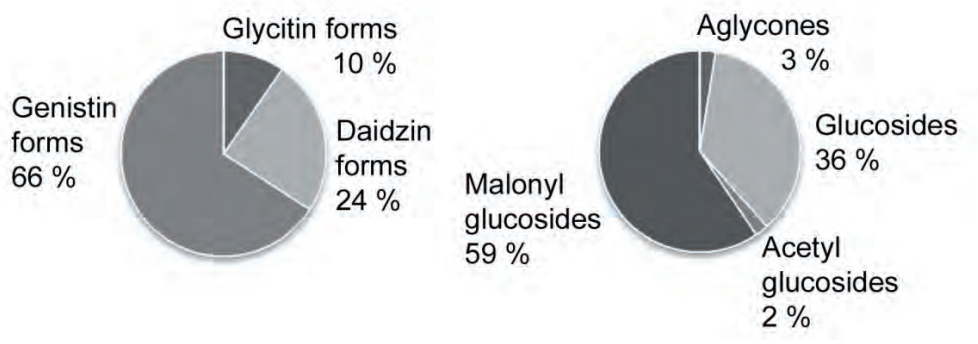


Figure 1. Composition of isoflavone forms in unprocessed soybeans (adapted from ***(37)***).

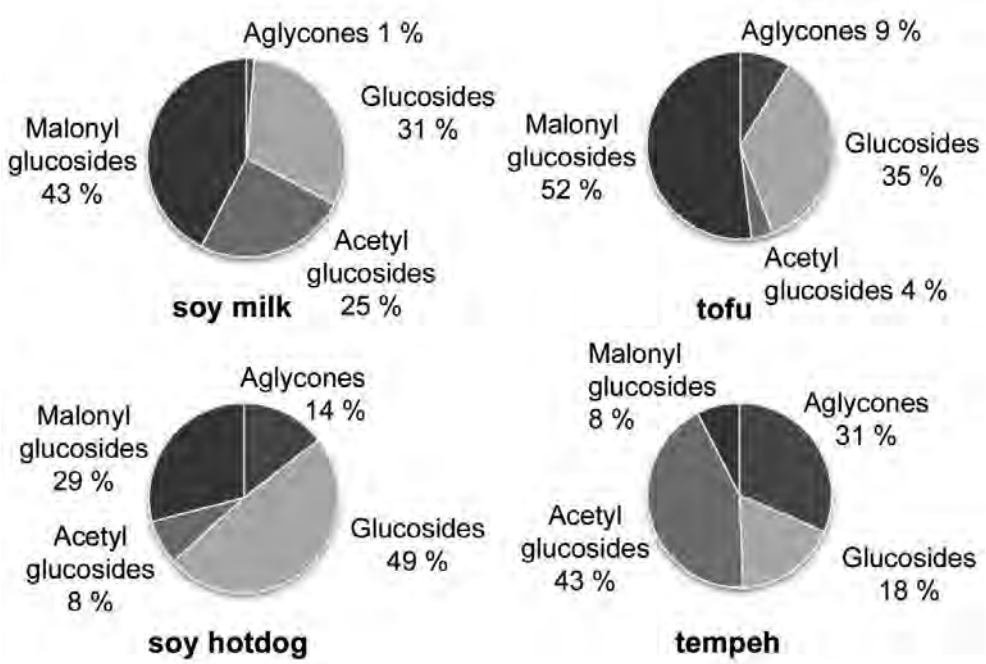


Figure 2. Composition of isoflavone forms in soy products (adapted from ***(46)***).

Tea

Another example of a food product rich in flavonoids, and known for its bitter taste, is tea. In contrast to many other flavonoid-rich food products, flavonoids in green tea occur mainly in their aglycone forms. Green tea (unfermented) contains higher concentrations of catechins than oolong tea (semi-fermented) or black tea (fermented), and is also reported as the most bitter tasting tea *(1)*. In total (on a dry weight basis), green tea consists of 30-42 % (*w/w*) phenolic compounds *(47)*. Amongst them, the largest group consists of catechins (**Figure 3**), whereas smaller amounts of phenolic acids (*e.g.* 5-galloylquinic acid and 5-caffeoylquinic acid) and flavonol glycosides (*e.g.* quercetin 3-*O*-rutinoside and kaempferol 3-*O*-glucoside) are present. The compositions of phenolic compounds, flavanols in particular, in green tea is illustrated in **Figure 4**. Besides phenolic compounds, tea contains also the bitter alkaloids theobromine and caffeine (3-4 % *w/w*). During fermentation into oolong and black tea, a large number of different structures are formed upon oxidation of flavanols, resulting in thearubigins, theaflavins, and others. The exact composition of the high molecular weight compounds, thearubigins in particular, and their molecular structures are largely unknown *(47, 48)*.

The flavanols in tea are commoly referred to as “catechins”. They consist mainly of the stereoisomers catechin (C) and epicatechin (EC), gallocatechin (GC) and epigallocatechin (EGC), and the galloylated epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (see **Figure 3**). With respect to their taste properties, they are known as bitter and astringent, the latter being less pronounced *(12)*. Epicatechin is reported as more bitter than catechin *(49)*, and the galloylated catechins ECG and EGCG are perceived as most bitter *(11)*. The oxidation products theaflavins and thearubigins are to a large extent not commercially available, and their taste properties are not well-established.

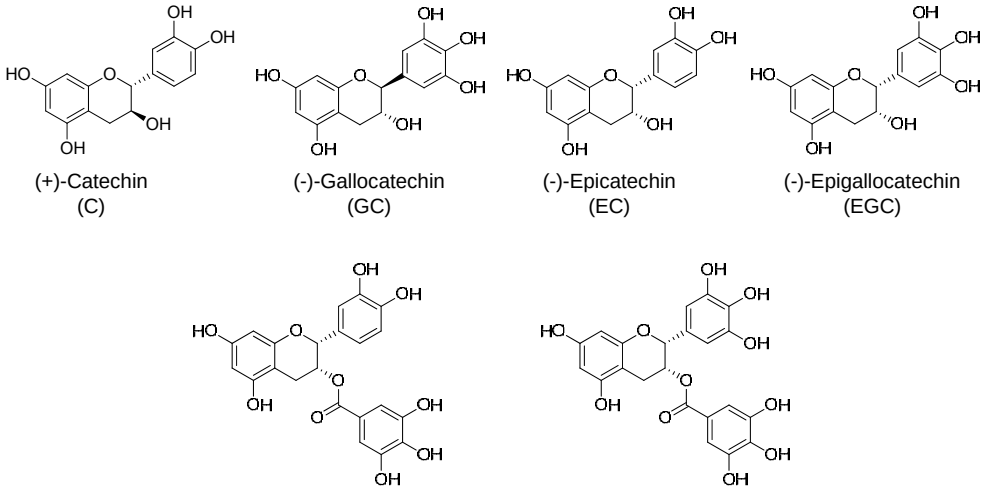


Figure 3. Structures of most abundant catechins in green tea (from ***(50)***).

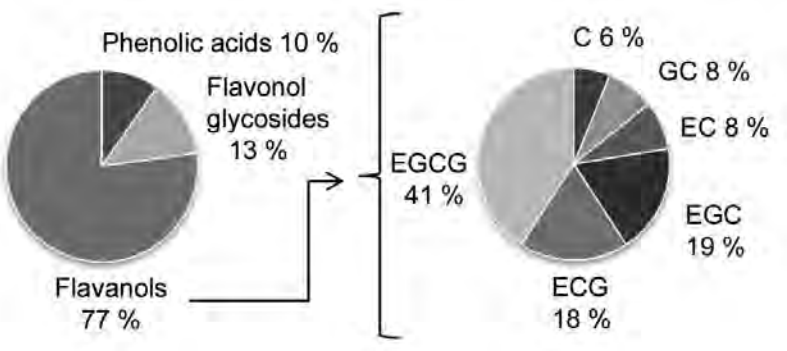


Figure 4. Example of composition of phenolic compounds in green tea (adapted from ***(47, 50)***).

TASTE RECEPTORS

Taste perception

The perception of food is to a large extent determined by its taste, but actually it results from a combination of all sensory senses: smell, taste, vision (*e.g.* color, appearance), touch (*e.g.* texture, temperature, pain, astringency), and even sound (*e.g.* breaking of textures). Interactions which may happen between senses are called "cross-modal interactions", finally leading to the overall sensory perception *(51)*. In this thesis, the focus lays exclusively on taste.

There are five basic tastes: sweet, salty, sour, bitter, and umami. Debate is still ongoing regarding possible fat receptors. The function of taste is probably the evaluation of the nutritional content of food (sweet: carbohydrates, salt: minerals, umami: proteins), but it also acts as warning system against spoilage and toxicity (sour and bitter). Furthermore, it has an important role in creating enjoyment during food consumption *(52)*. Taste is perceived via taste receptors activated by non-volatile compounds. These taste receptors are located on the surface of taste receptor cells, embedded in taste buds. A taste bud can contain taste receptor cells for all five taste qualities. Taste buds are located mainly in the papillae on the tongue, but also in other tissues of the mouth and throat. Sweet, bitter and umami receptors belong to the group of so-called G-protein coupled receptors (GPCRs), also known as seven-transmembrane domain receptors, whereas salt and sour receptors are ion channels *(52)*.

Bitter taste receptors

In 2000, two research groups discovered bitter taste receptors in human and mouse *(53-55)*. They were first called T2Rs *(53)*, TRBs *(55)*, or TAS2Rs *(56)*. Recently, the Gene Nomenclature Committee of the Human Genome Organization (HUGO) adapted the human TAS2R nomenclature *(57)*. Human bitter taste receptor genes comprise of ~ 25 full length members *(58)*. Surprisingly, bitter taste receptors have not only been identified in oral tissues, but also in *e.g.* the respiratory system and the gastrointestinal tract. Their functions are not fully understood yet. Instead of contribution to taste perception, they might play a role in digestion and metabolism (gastrointestinal tract) and act as a warning system for inhalation of harmful substances (respiratory system) *(59)*.

The signal transduction mechanisms of bitter receptors, belonging to the GPCR family, have been characterized as follows. Upon activation of a bitter receptor by a bitter compound, heterotrimeric G-proteins, consisting of $G\alpha_{gustducin}$, $G\beta_3$ (or $G\beta_1$), and $G\gamma_{13}$, couple to the receptor *(60, 61)*. Subsequently, the G-proteins dissociate into $G\alpha_{gustducin}$ and the $G\beta\gamma$-subunit. Two pathways ($\alpha_{gustducin}$-PDE- pathway and $\beta\gamma$-PLC-β_2-IP_3/DAG pathway) are described (**Figure 5**), which lead to the elevation of intracellular calcium levels, the

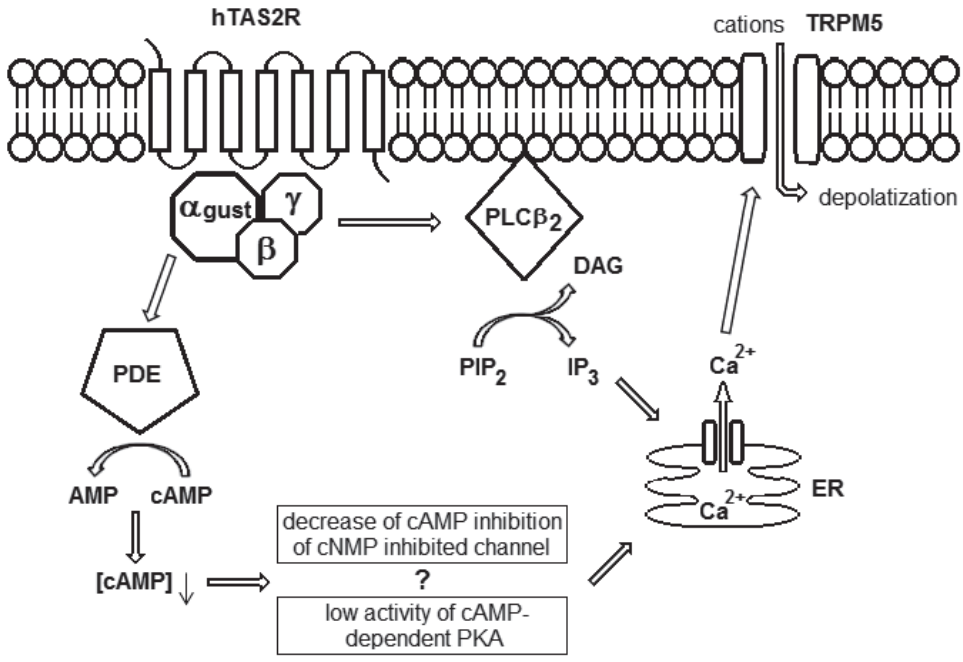


Figure 5. Proposed signal transduction mechanism in bitter receptor cells (adapted from ***(62, 65)***).

depolarization of the taste cell, and finally neurotransmitter release *(62)*. In detail, the Gβγ-subunit activates phospholipase $C\beta_2$ ($PLC\text{-}\beta_2$) *(63)*, which cleaves phosphoinositol biphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 provokes the release of Ca^{2+} from the endoplasmatic reticulum (ER) into the cytoplasm *(64)*. The other pathway via $\alpha_{gustducin}$-PDE is not entirely clear yet. $G\alpha_{gustducin}$ stimulates phosphodiesterase (PDE) to reduce the cyclic adenosine monophosphate (cAMP) levels. The decreased cAMP concentration may disinhibit cyclic nucleotide-inhibited (cNMP) channels and result in elevation of intracellular Ca^{2+} *(62)*. Another suggested mechanism is that the decreased cAMP concentration leads to low activity of cAMP-dependent protein kinase (PKA). High PKA levels might lead to chronic inhibition of Ca^{2+} release. The role of $\alpha_{gustducin}$ might thus be to maintain cAMP levels low to ensure adequate Ca^{2+} signaling *(65)*. Ca^{2+} triggers the opening of the TRPM5 channel, influx of cations, resulting in taste cell depolarization, and subsequently neurotransmitter release, that activates the taste nerves, finally giving a signal to the brain *(62, 66)*.

Cell-based bitter receptor assays

Native taste receptor cells from the human oral tissue are difficult to culture longer than a few days, and it is challenging to obtain large amounts of human material. Therefore,

heterologous expression systems in easy maintainable cells are used to study response of bitter receptors towards bitter tastants. To this end, almost exclusively human embryonic kidney (HEK)293 cells are used. As the native taste receptor specific G-proteins are lacking in HEK293 cells, an easy-measurable readout system was created by co-expression of one of the hTAS2Rs and $G\alpha_{15}$ *(54)*. This Gα subunit has been shown to couple to a wide range of receptors and can activate a Ca^{2+} signaling pathway *(54, 67)*. Intracellular Ca^{2+} release is easily detectable by calcium-sensitive dyes in combination with fluorescence measurements. $G\alpha_{15}$ was used in the early hTAS2R studies *(54, 56)*, but later on, the sensitivity of the bitter receptor assay has been increased by the use of $G\alpha_{16}$, containing the last 44 amino acids of the native $G\alpha_{gustducin}$ *(68)*. An alternative way to measure bitter receptor activation has been reported for bitter receptors expressed in *Spodoptera frugiperda* SF9 insect membranes, measuring guanosine 5'-*O*-(gamma-thio)-triphosphate (GTPγS)-binding to α-gustducin (or its homolog, transducin) *(54, 69)*. Despite the advantage of a higher resemblance to the transduction mechanism in native cells, the latter approach has the disadvantage of being a low throughput method. Hence the majority of bitter receptor studies have been conducted in the HEK293 $G\alpha_{16gust44}$ system.

For cell-based bitter receptor assays in HEK293 cells, stably expressing $G\alpha_{16gust44}$, transient *(70)* as well as stable *(71)* expression of the hTAS2R gene has been reported. For this, HEK293 T cells and HEK293 T-REx Flp-In cells are used, respectively. With transient (temporary) transfection the receptor DNA is temporarily expressed. As most of the DNA is not integrated into the cell genome, it is degraded within a few days. Therefore, transient transfection is merely useful for rapid analysis or when only a few experiments are performed. On the other hand, stable cell lines, in which the receptor gene is stably integrated into the cell genome, are more practical for frequent analyses. They have the advantage that transfection only needs to be performed once and the expression level of the gene is constant over time. An accompanying advantage of the Flp-In system is that the integration side of the integrated gene is always the same, due to targeted recombination. In **Figure 6**, a simplified scheme of a bitter receptor assay in stable cell lines is shown. The DNA of the respective hTAS2R is integrated in the cell nucleus (in **Figure 6** indicated in blue) and the receptor expression is induced by the addition of doxycycline. After incubation with calcium sensitive fluorescent dye, the bitter molecules are added. Upon activation of the bitter receptor, the heterotrimeric G-proteins couple to the receptor. Subsequently, the G-proteins dissociate into $G\alpha_{16}$ and the Gβγ-subunit, which starts the signaling cascade resulting in intracellular calcium release. Due to the presence of a calcium-sensitive fluorescent dye, receptor activation can be recorded as fluorescent signal in a fluorescence plate reader. In order to establish dose-response behavior of a bitter receptor agonist, the activation of the respective hTAS2R is measured at different concentrations of the bitter compound. The maximum signal of activation for each concentration is subsequently plotted in a sigmoidal dose-response curve, from which

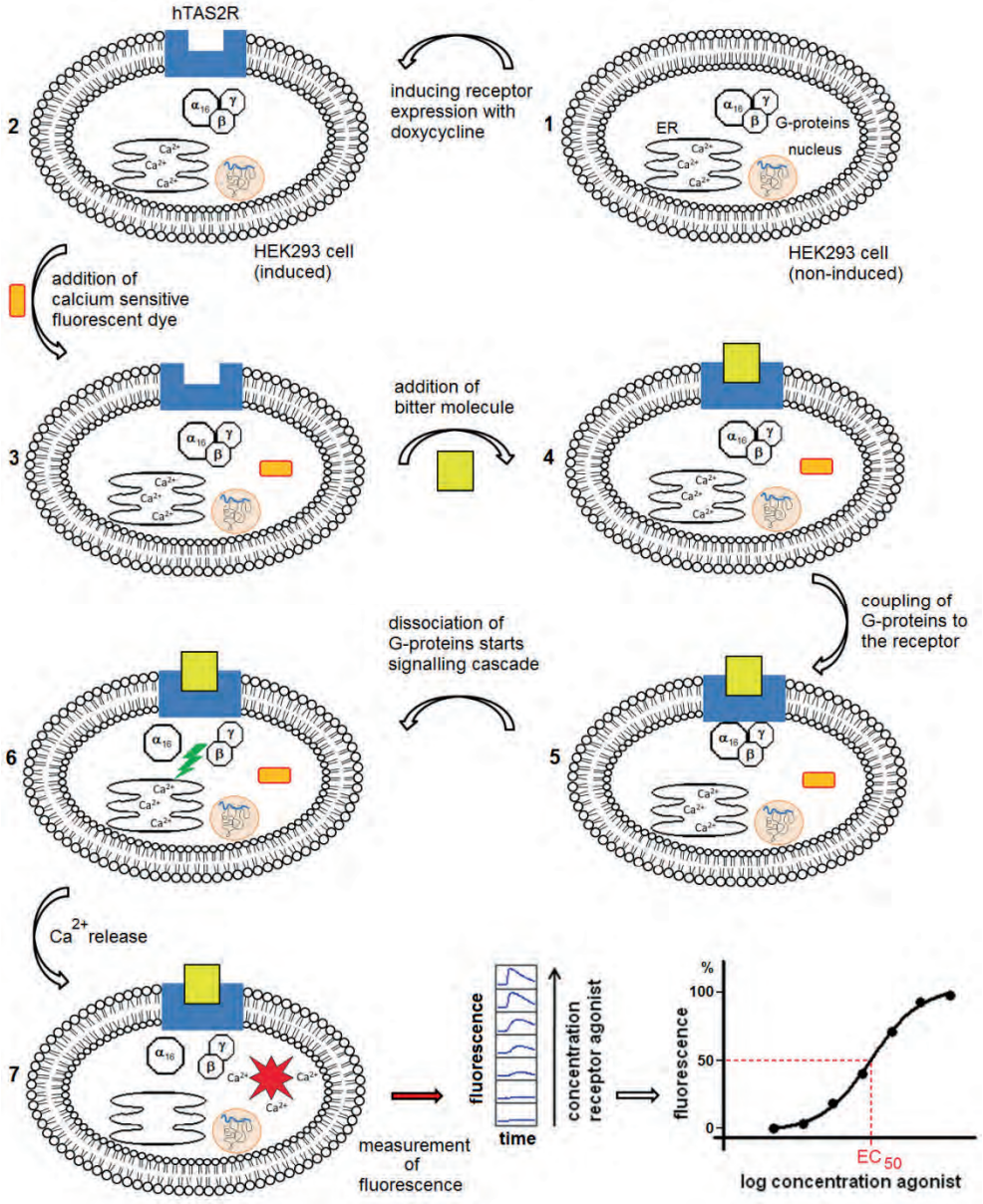


Figure 6. Simplified bitter taste receptor cell-assay, stably expressing the hTAS2R gene (partially adapted from ***(72)***).

receptor activation threshold, half maximal activation concentration (EC_{50}) and maximal receptor activation by the respective agonist can be obtained.

Characteristics of bitter taste receptors

Bitter receptors are the most diverse amongst the family of taste receptors. They can recognize structurally very diverse bitter compounds (*e.g.* salts, peptides, lactones, phenolic compounds, terpenes, alkaloids). Native bitter taste receptor cells can express multiple hTAS2Rs *(73)*, and it has been shown, that they are able to form oligomers in HEK cells *(74)*. However, no functional consequences seemed to result from this ability, and using single hTAS2Rs in HEK cells in order to identify agonists seems to be appropriate.

Bitter receptors are seven-transmembrane receptors, as they contain seven α-helices passing through the cell membrane. Besides the seven transmembrane (TM) regions, they comprise of three extracellular loops (EL) and three intracellular loops (IL). Amongst the 25 hTAS2Rs, the lengths of the loops, extracellular N-termini, and intracellular C-termini are quite variable *(75)*. **Figure 7** shows a snake plot of a generic bitter taste receptor. Most literature suggests the presence of a single ligand binding site. This binding pocket is most probably located within the transmembrane-region *(76-81)*. Predicted or experimentally confirmed amino acid residues involved in agonist interaction are accumulated in TM III, VI, and VII *(76)*. However, a possible influence of EL1 and EL2 in agonist selectivity has

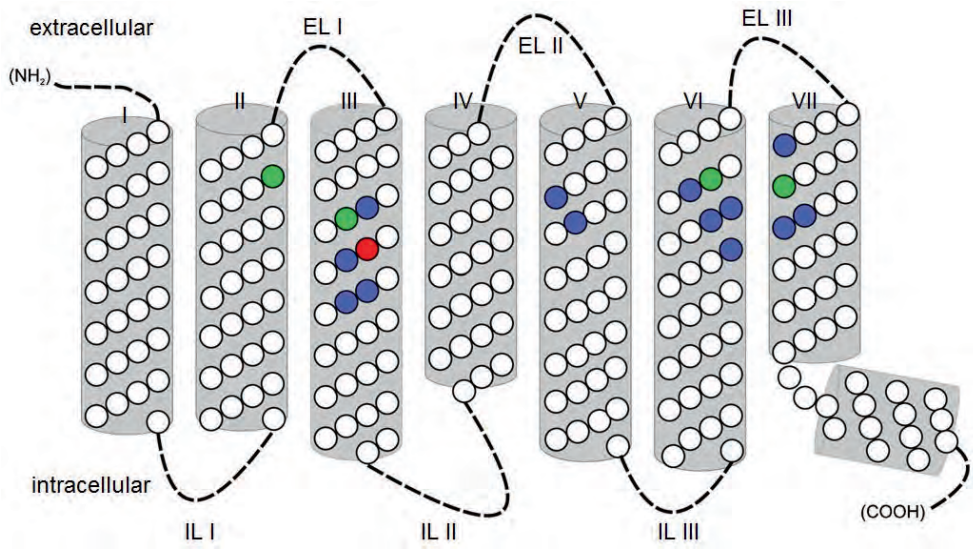


Figure 7. Snake plot of a generic hTAS2R. Amino acid positions involved in agonist interaction in the receptors hTAS2R1 ***(81, 82)***, -16 ***(77, 83)***, -30 ***(69)***, -38 ***(79)***, and -46 ***(78)*** are indicated by colors (red, predicted in four receptors, green, two hits, blue, one hit.) (adapted from ***(76)***).

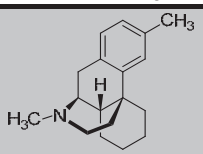
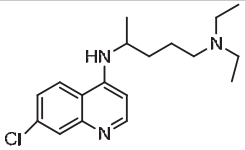
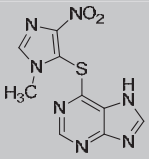
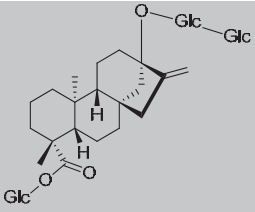
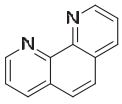
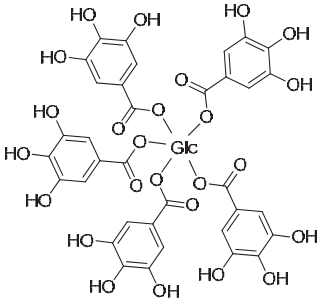
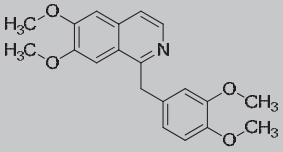
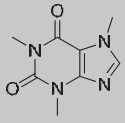
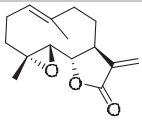
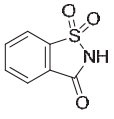
been reported as well *(69, 80)*. So far, information on amino acid residues involved in ligand binding is limited to hTAS2R1, -16, -30, -31, -38, -43, and -46.

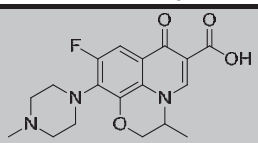
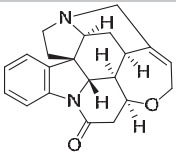
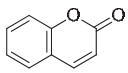
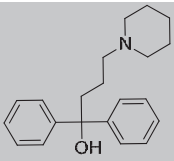
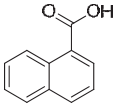
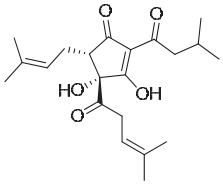
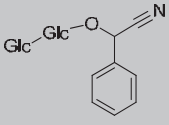
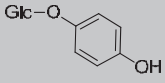
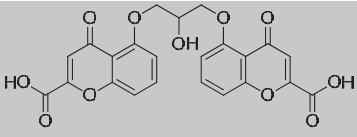
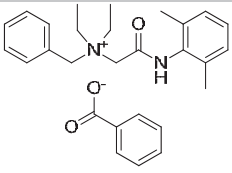
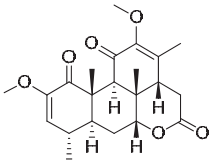
A *state-of-the-art* overview of hTASRs and examples of their agonists, divided into dietary and non-dietary origin, is given in **Table 3**. Some bitter receptors are broadly tuned, meaning that they are activated by several classes of compounds (hTAS2R1, -4, -10, -14, -39, and -46), whereas others have, so far, a very limited agonist spectrum of one or two agonists only (hTAS2R3, -13, -41, -49, and -50). Two bitter taste receptors, hTAS2R16 and hTAS2R38, seem to respond quite specifically to almost exclusively one kind of chemical structure (mainly β-glucopyranosides for hTAS2R16 and mainly sulfur-containing compounds for hTAS2R38) *(56, 84, 85)*. For some bitter receptors (hTAS2R3, -9, -13, -20, and -41), only non-dietary agonists have been identified so far. For four hTAS2Rs, no agonists have been identified yet. Such receptors are called "orphan receptors". Also, not all compounds known as bitter have been assigned to hTAS2Rs yet, but the progress in the last years is enormous. In **Figure 8** this progress is illustrated, showing that *e.g.* only half of all human bitter receptors were deorphanized in the beginning of this Ph.D. research (May 2009), whereas at the time of writing (August 2013), only four receptors were not assigned to bitter compounds yet. Especially the number of compounds identified as agonists has risen enormously.

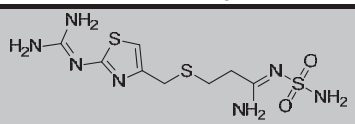
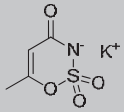
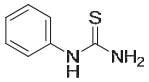
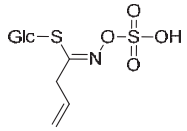
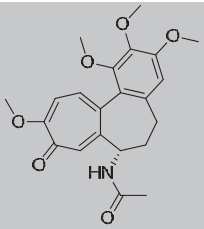
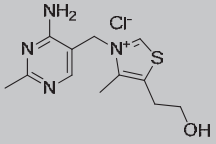
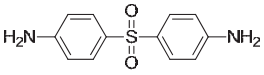
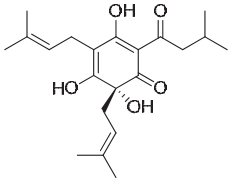
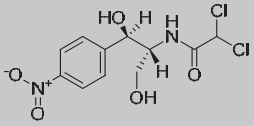
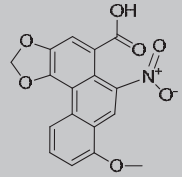
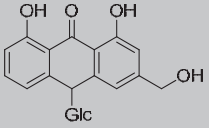
The compounds known as most intensely bitter are the synthetic compound denatonium *(86)* and the natural compound amarogentin *(87)*. Not only obviously bitter compounds are hTAS2R agonists. The sweet compounds acesulfame K, saccharin, and steviol glycosides exhibit bitter after taste. This observation has been explained by the identification of bitter receptors activated by these sweeteners *(88, 89)*.

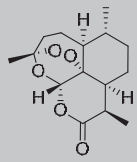
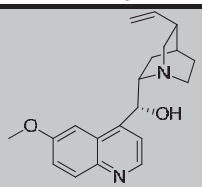
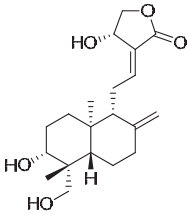
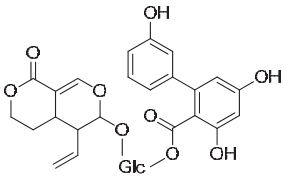
Furthermore, in bitter receptors, a considerable number of single-nucleotide polymorphisms (SNPs) is known. The most prominent example is hTAS2R38, in which SNPs lead to "taster" and "non-taster" phenotypes. "Tasters" perceive 6-*n*-propylthiouracil (PROP) and phenylthiocarbamide (PTC) as bitter, whereas "non-tasters" do not. Likewise, at receptor level, the hTAS2R38-PAV (taster) variant is activated by both substances, whereas the hTAS2R38-AVI (non-taster) variant is not *(84)*. This example shows also that *in vitro* measurements of bitter receptors expressed in HEK cells can resemble *in vivo* situations. Remarkably, the ratio of "tasters" versus "non-tasters" based on hTAS2R38 polymorphisms in the human population is approximately 50 % / 50 %, though with regional differences *(90)*. In contrast, for some other receptors, *e.g.* hTAS2R14, SNP ratios of 99 % / 1% in the human population are reported *(75)*, and functional SNPs are unknown *(91)*. Besides the SNP variants, also the density of taste buds seems to play a role in bitterness sensitivity *(92)*.

Table 3. Overview of bitter taste receptors with examples of their respective agonists. Recently changed former receptor names and references are given in parentheses.

Bitter receptor	Example of agonist non-dietary	Example of agonist dietary
hTAS2R1	 Dextromethorphan ***(85)***	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser ***(93)***
hTAS2R3	 Chloroquine ***(85)***	unknown
hTAS2R4	 Azathioprine ***(85)***	 Stevioside ***(89)***
hTAS2R5	 1,10-Phenantroline ***(85)***	 β-1,2,3,4,6-Penta-O-galloyl-D-glucopyranose ***(94)***
hTAS2R7	 Papaverine ***(95)***	 Caffeine ***(85)***
hTAS2R8	 Parthenolide ***(85)***	 Saccharin ***(95)***

Bitter receptor	Example of agonist non-dietary	Example of agonist dietary
hTAS2R9	 Ofloxacin ***(96)***	unknown
hTAS2R10	 Strychnine ***(56)***	 Coumarin ***(85)***
hTAS2R13	 Diphenidol ***(85)***	unknown
hTAS2R14	 1-Naphthoic acid ***(70)***	 cis-Isohumulone ***(97)***
hTAS2R16	 Amygdalin ***(56)***	 Arbutin ***(56)***
hTAS2R19 (hTAS2R48)	unknown	unknown
hTAS2R20 (hTAS2R49)	 Cromolyn ***(85)***	unknown
hTAS2R30 (hTAS2R47)	 Denatonium benzoate ***(69)***	 Quassin ***(85)***

Bitter receptor	Example of agonist non-dietary	Example of agonist dietary
hTAS2R31 (hTAS2R44)	 Famotidine ***(85)***	 Acesulfame K ***(88)***
hTAS2R38	 Phenylthiocarbamide (PTC) ***(84)***	 Sinigrin ***(85)***
hTAS2R39	 Colchicine ***(85)***	 Thiamine ***(85)***
hTAS2R40	 Dapsone ***(85)***	 Cohumulone ***(97)***
hTAS2R41	 Chloramphenicol ***(98)***	unknown
hTAS2R42	unknown	unknown
hTAS2R43	 Aristolochic acid ***(88)***	 Aloin ***(99)***
hTAS2R45	unknown	unknown

Bitter receptor	Example of agonist non-dietary	Example of agonist dietary
hTAS2R46	 Artemisinin ***(100)***	 Quinine ***(85)***
hTAS2R50	 Andrographolide ***(101)***	 Amarogentin ***(101)***
hTAS2R60	unknown	unknown

Structure-activity relationships of bitter compounds that activate hTAS2Rs

The correlation between the chemical structure of a compound and its biological activity is generally described as structure-activity relationship. The analysis of structure-activity relationships leads to the identification of chemical groups responsible for the activity of the compound. As bitter compounds can be very variable in structure, it is difficult to identify general molecular characteristics responsible for bitterness. It was reported that bitter compounds require one polar group and one hydrophobic group, and that the threshold of bitter compounds depended on the apolar moiety, but were also related to steric factors and charge distribution within the molecule *(87)*. However, it has been shown that small structural variations can lead to large changes in activity, *e.g.* the amino acid L-phenylalanin tastes bitter whereas D-phenylalanin tastes sweet, and isosakuranetin-7-*O*-rutinoside tastes neutral, whereas isosakuranetin-7-*O*-neohesperidoside tastes bitter *(3)*. At the receptor level, few studies have investigated structure-activity relationships. It has been shown that hTAS2R16 was activated by various β-glucopyranosides (hydrophobic residue attached to glucose by a β-glycosidic bond), but not by the corresponding α-glucopyranosides or β-galactopyranosides. The potency of receptor activation was influenced by the hydrophobicity of the residue *(56)*. Furthermore, steviol glycosides were identified as agonists of hTAS2R4 and hTAS2R14. They showed higher sensitivity towards hTAS2R4, and it was investigated that the length of the sugar chain, pyranose substitution, and the C16 double bond determined the taste of steviol glycosides *(89)*. For most bitter receptors agonists, structure-activity relationships have not been investigated yet.

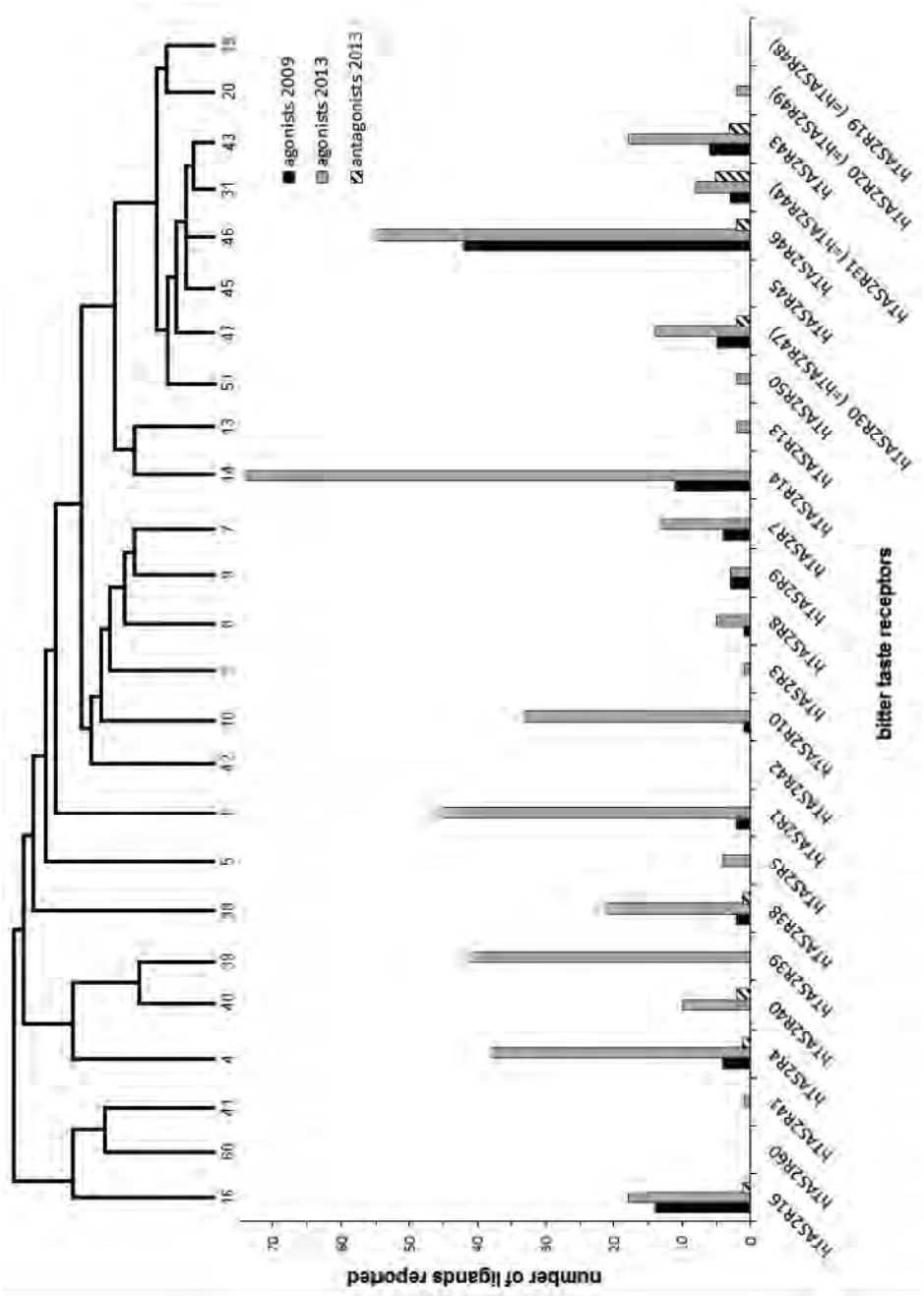


Figure 8. Number of ligands reported per hTAS2R ***(54, 56, 69-71, 80, 83-85, 88, 89, 93-110)*** in May 2009 and August 2013. The dendrogram (top) illustrates the sequence relationship within the hTAS2R family (adapted from ***(76)***).

REDUCTION OF BITTER TASTE

Strategies for masking or reduction of bitter taste

For the majority of bitter tasting food products, efforts have been made to either reduce the level of bitter tasting compounds or to mask the bitter taste. In vegetables, for example, selective breeding has been applied for a long time, leading to *e.g.* Brussels sprouts varieties, which have low bitterness *(1)*. Besides traditionally bitter food products, there are also products in which bitterness is a newly introduced problem. These are either functional foods, fortified with health promoting, but bitter, ingredients, or they are low-sugar / low-fat / low-salt products, developed due to health concerns in the context of overweight, diabetes, and cardiovascular disease. The reduced amount of sugar, fat, and / or salt can lead to the perception of previously masked, undesired taste properties (sour, bitter, astringent) *(72)*. Designing healthier products while maintaining consumer acceptability has, therefore, become an important challenge.

Table 4. Overview of bitterness reduction methods (partially adapted from ***(72)***).

Bitterness reducing method	Description	Examples
removal / reduction of bitter compounds	selective breeding	reduction of sinigrin in ***Brassica*** vegetables ***(111)***
	physical removal (filters, resins, solvents, precipitation)	fining of wine with gelatins ***(112)***
	conversion (enzymes, microorganisms, cooking)	enzymatic deglycosylation of naringin ***(113)***
addition of tastants / flavors	masking effect (salts, sugars, acids, strong or co-congruent flavors)	reduced bitter perception of pharmaceuticals by sodium, glutamate and AMP ***(114)***
encapsulation of bitter compounds	physical barrier surrounding the molecule	encapsulation of poly-phenols with maltodextrins by spray-drying ***(115)***
complexation of bitter compounds	molecular complexation	debittering of tea extract polyphenols by addition of casein ***(116)***
	molecular inclusion	elimination of bitter taste of milk casein hydrolysates by β-cyclodextrins ***(117)***
blocking of bitter receptors	full antagonism	(4-(2,2,3-trimethylcyclo-pentyl)butanoic acid blocks activation of hTAS2R31 ***(109)***
	partial agonism	3β-hydroxypeleolide reduces activation of hTAS2R46 ***(103)***

An overview of bitterness reduction strategies is given in **Table 4**. Besides application in foods, many strategies are used in the pharmaceutical industry. The choice of methods often depends on whether the compounds causing bitterness are naturally present in the

food product or are added. The taste of health promoting ingredients should be masked, because removal would also remove their potential health benefits. Methods such as encapsulation of compounds by spray drying are not applicable when the bitter compounds are already present in the food raw material. Often, a combination of methods has to be used to eliminate bitter taste.

Complexation of bitter compounds

A molecular complex is formed by loose association involving two or more molecules. The bonding between the components of a complex is of a reversible nature and weaker than a covalent bond *(118)*.

One form of complexation applied in pharmaceutical and food industry is molecular inclusion by cyclodextrins *(117)*. It is used in order to reduce unpleasant tastes or smells, or to enhance the water solubility of hydrophobic compounds. Cyclodextrins represent a family of cyclic oligosaccharides, consisting of glucose units bound through α-(1,4) linkages. α-, β-, and γ-cyclodextrins are composed of 6, 7, and 8 units, respectively *(119)*. They form a cylinder-shaped structure (host), comprising of a hydrophilic outer surface and a hydrophobic cavity. This cavity can include a (hydrophobic) guest molecule, mostly by a host:guest ratio of 1:1, providing that the guest molecule fits geometrically into the cavity *(117)*. In food applications, cyclodextrins are most often used as β-form. They have been shown to mask bitter taste of various dietary compounds, *e.g.* protein hydrolysates, vitamins and phenolic compounds *(117, 119)*.

Another form of complexation makes use of the interactions between proteins and phenolic compounds. Complex formation between proteins and phenolic compounds is mainly driven by hydrophobic interactions and hydrogen bonding. Hydrophobic interactions occur primarily between apolar amino acid residues (mainly proline) and the aromatic rings of phenolic compounds. Hydrogen bonds are formed between the amine or carbonyl groups of amino acid residues and the hydroxyl groups of phenolic compounds *(120)*. The formation and stability of a protein-phenolic complex is largely determined by the accessibility of proline residues as well as the structural properties of phenolic compounds. Proteins having open structures favor binding to phenolics, in contrast to proteins having globular structures. With respect to phenolic compounds, conformation, flexibility, galloylation and degree of polymerization have been shown to be important drivers of protein-phenolic interactions *(121)*.

Reduction of bitter taste by receptor blockers

One of the newest approaches to reduce bitter taste is the use of so-called bitter receptor blockers. These are antagonists of the bitter taste receptor, which inhibit receptor activation by the bitter compound. A simplified mechanism of receptor blocking is shown in **Figure**

9. When the bitter receptor blocker occupies the binding pocket, the bitter receptor is not activated and the signaling cascade resulting in intracellular calcium release is not started. To our knowledge, it is not known whether the G-protein binds to the receptor in presence of a blocker in the binding pocket. In order to establish the dose-response behavior of a bitter receptor blocker, the activation of the respective hTAS2R is measured at constant agonist and various antagonist concentrations. Based on the fluorescent signals, a dose-response curve of receptor inhibition is plotted, from which a half maximal inhibitory concentration (IC_{50}) can be calculated.

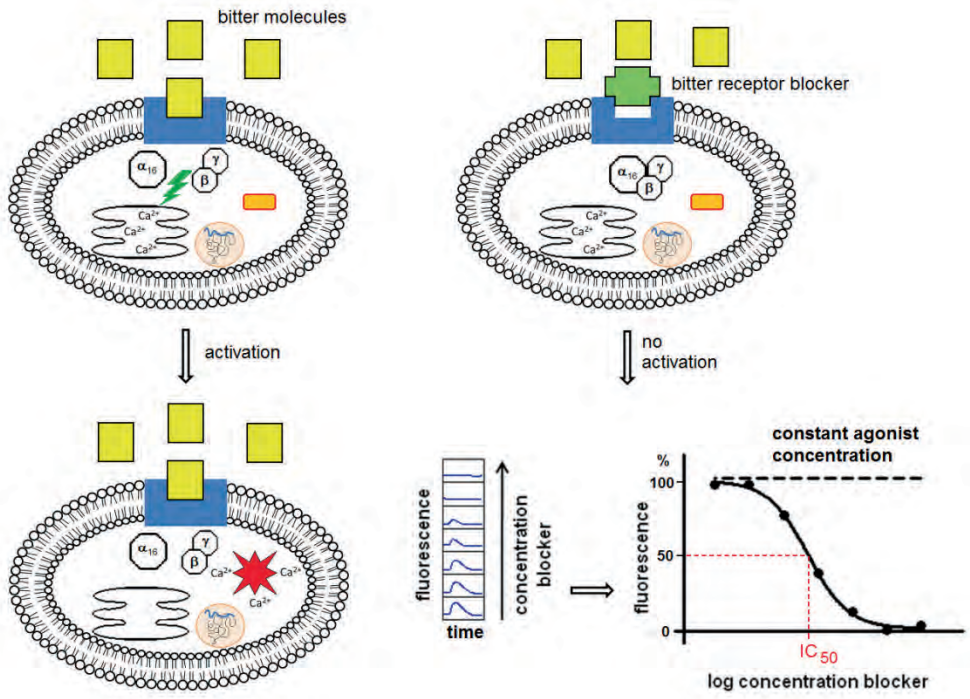


Figure 9. Simplified mechanism of bitter taste receptor activation and blocking.

The first hTAS2R antagonist was published in 2010 *(109)*. The small molecule (4-(2,2,3-trimethylcyclopentyl)butanoic acid (also denoted as GIV 3727) was reported as inhibitor of hTAS2R31, and was also able to inhibit three other taste receptors in a dose-dependent way (hTAS2R4, hTAS2R40, and hTAS2R43). Additionally to blocking of the receptor response, this compound effectively reduced the bitter aftertaste of artificial sweeteners acesulfame K and saccharin in sensory tests, without changing the sweet taste perception. In 2011, inhibitory activity of two sesquiterpene lactones was discovered,

namely of 3β-hydroxydihydrocostunolide (3HDC) for hTAS2R30, hTAS2R40, and hTAS2R46, and of 3β-hydroxypelenolide (3HP) for hTAS2R30, hTAS2R31, hTAS2R43, and hTAS2R46 *(103)*. The latter compound was shown to activate hTAS2R46 weakly, thus the reduction of receptor activation was caused by partial agonism. Although a partial agonist activates the receptor as well, it competes with the full agonist for receptor binding and its action results in a much lower receptor activation compared to the full agonist. Furthermore, it was shown *(103)* that a compound can act as agonist towards one taste receptor, but as antagonist towards another taste receptor, as the two compounds 3HDC and 3HP, besides their inhibitory properties, showed agonistic properties towards other bitter receptors.

At present, few compounds have been published to act as antagonists on bitter taste receptors. This is shown in the ligand overview in **Figure 8**. It should be noted that only dose-dependent receptor inhibition has been included in the figure, as dose-independent inhibition (*e.g.* reported by *(122)*) might have been caused by mechanisms other than receptor antagonism.

Mechanisms of receptor antagonism

There are two basic molecular mechanisms of receptor antagonism. When the antagonist blocks the access of the agonist to the receptor through steric hindrance, this is referred to as orthosteric antagonism. When the antagonist binds to another binding site on the receptor than the agonist, a change of affinity of the receptor to the agonist is induced through a change in conformation of the receptor. This is referred to as allosteric antagonism *(123)*. In experimental pharmacology, cell lines expressing the receptor of interest are pre-incubated with the antagonist and then challenged with increasing agonist concentrations *(124)*. In the orthosteric mechanism, agonist and antagonist compete for the same binding site, and the relative affinity and concentrations of agonist and antagonist determine which molecule occupies the binding site. **Figure 10** illustrates that it depends on the kinetics of the system whether this results in surmountable antagonism (dextral displacement of the dose-response curve without depression of maximal amplitude) (**A**), in an intermediate state between surmountable and insurmoutable antagonism (dextral displacement and moderate depression of the maximal response) (**B**), or in insurmountable antagonism (depression of maximal amplitude) (**C**). The first case is observed when agonist and antagonist are in equilibrium with each other, meaning that there is sufficient time for the antagonist to dissociate from the receptors, and the agonist to bind to unbound receptors *(125)*. The second case is called “hemi-equilibrium”, whereby agonist, antagonist and receptors have partially come to equilibrium with each other *(123)*. The last phenomenon is observed when the dissociation rate of the antagonist from the receptors is slower than a competing process with the agonist *(126)*. It should be noted that more than one molecular mechanism can produce the same pattern of dose-response curves, *e.g.* insurmountable antagonism (**C**) can

be caused by orthosteric inhibition with insufficient equilibration time, by allosteric inhibition, or by irreversible inhibition (covalently bound antagonist) *(125, 126)*. Allosteric antagonists produce saturable effects, *i.e.* after a maximum antagonistic effect is obtained, increases in antagonist concentration have no further effect. Furthermore, their effect can be agonist specific, which is not possible for orthosteric antagonists *(125)*.

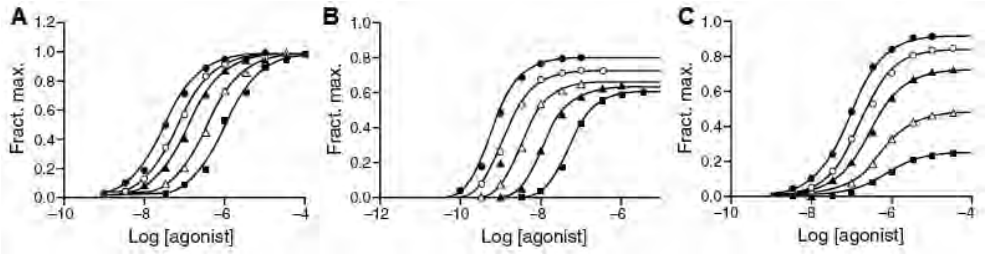


Figure 10. Mechanisms of orthosteric antagonism ***(125)***. Surmountable (competitive) (**A**), surmountable → insurmountable (hemi-equilibria) (**B**), insurmountable (non-competitive) (**C**). ● no antagonist added; from ○ until ■: increasing antagonist concentrations.

OUTLINE OF THIS THESIS

Many (iso)flavonoids have been associated with potential beneficial health effects. Therefore, consumption of (iso)flavonoid-rich food products, and enrichment of foods with (iso)flavonoids is becoming increasingly popular. This trend might be compromised by undesirable taste properties of these compounds. Several (iso)flavonoids have been reported to be bitter, albeit contradictory findings have been made in sensory tests. Other (iso)flavonoids have unknown taste properties, as they have never been incorporated in high concentrations in food products. Therefore, objective measures are necessary to identify which (iso)flavonoids activate bitter receptors and thus have the potential to contribute to bitterness of a food product, and which are the molecular signatures involved. Furthermore, little has been reported about reducing bitterness of (iso)flavonoids at the molecular level. Nonetheless, enhanced application of (iso)flavonoids might necessitate targeted measures against bitter taste.

This thesis can be divided into two parts. The first part of this thesis addresses the identification of bitter taste receptors for bitter dietary compounds. It was hypothesized that compounds causing bitter taste in soy products can be assigned to bitter taste receptors and that there are signatures that underlie receptor activation. Out of all 25 human bitter taste receptors, two receptors, hTAS2R14 and hTAS2R39, were identified to be activated by the bitter soy compound genistein **(Chapter 2**). Structure-activity relationships were established for the activation of hTAS2R14 and hTAS2R39 by structurally similar

isoflavonoids. Moreover, a substitution pattern of isoflavonoids most favorable for activation of these two bitter receptors was determined. Next, it was hypothesized that bitter flavonoids hold a molecular signature involved in bitter receptor activation, which enables prediction of intrinsic bitterness. Therefore, in **Chapter 3**, a large group of structurally similar flavonoids was measured on the same two receptors. The aim was to investigate chemical characteristics for (iso)flavonoids to activate hTAS2R14 and hTAS2R39. This investigation was underpinned by 2D-fingerprint and 3D-pharmacophore modeling, and led to the identification of the molecular features necessary for (iso)flavonoids to activate hTAS2R14 and hTAS2R39, and the differences therein.

The second part of this thesis addresses debittering strategies. Based on known bitter masking effects of flavanones in sensory studies, it was assumed that flavanones have the ability to block (iso)flavonoid bitter receptors on the molecular level. **Chapter 4** describes the identification and characterization of flavanones as antagonists for hTAS2R39. Their ability to reduce hTAS2R39 activation by ECG, their pharmacological properties, as well as their ability to block hTAS2R14 were investigated. In **Chapter 5** it was hypothesized that bitter taste reduction for dietary flavonoids by complexation with food proteins can be predicted by use of *in vitro* assays. The potential of β-casein, β-lactoglobulin and different gelatins to reduce hTAS2R39 activation by complexation of bitter tea EGCG was evaluated in *in vitro* binding studies, and transferred to the *in vivo* situation, in which a trained sensory panel determined bitterness. Finally, **Chapter 6** discusses the findings presented in this thesis, addresses prospects and limitations of the bitter receptor cell assay, presents additional results on testing (iso)flavonoids for possible antagonistic properties, and compares taste evaluation by sensory tests, receptor assays and modeling. Furthermore it evaluates strategies for bitter taste reduction, and applies the findings to soy products and tea.

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Chapter 2

2

Soy isoflavones and other isoflavonoids activate the human bitter taste receptors hTAS2R14 and hTAS2R39

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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 µM on hTAS2R14 and hTAS2R39, and EC_{50} values of 29 and 49 µM, 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 towards 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, acetyl genistin, and malonyl genistin. 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.

INTRODUCTION

Soybeans (***Glycine max*** L. Merrill) are consumed by Asian populations on a regular basis. Growing evidence of 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 for 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 malonyl glucosides, acetyl glucosides, and unsubstituted glucosides ***(3)***. The quantities of isoflavones in soybeans depend on cultivar and year of cultivation ***(4)***. Commonly, the malonyl glucosides and unsubstituted glucosides dominate in soybeans, whereas acetyl glucosides and aglycones are present in very small amounts ***(3)***. Processing can lead to the conversion of malonyl glucosides into acetyl glucosides (decarboxylation due to dry heating) and unsubstituted glucosides (mainly due to moist heating) ***(5, 6)***. Fermentation strongly increases the amount of aglycones due to action of β-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, e.g. 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[1], -42, -45, -48[2], and -60. Despite of 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, amongst 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.

[1] hTAS2R41 has been deorphanized in 2013, see Chapter 1
[2] The name of hTAS2R48 has been changed into hTAS2R19, see Chapter 1

MATERIALS AND METHODS

Materials

Isoflavonoids were purchased from Indofine Chemical Company (Hillsborough, NJ, USA), 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, Steinheim, Germany) at a 100 mM stock concentration. Trypan blue solution (0.4 %) was purchased from Sigma-Aldrich (Steinheim, Germany).

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, USA) stably expressing the chimeric G-protein α-subunit G 16-gust44 (cloned into pcDNA4 (Invitrogen)) ***(20)*** and one of the 25 human bitter receptor genes (cloned into pcDNA5/FRT (Invitrogen)), were used. In order 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 ***(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 ***μ***g/mL), geneticin (400 ***μ***g/mL) and hygromycin (100 ***μ***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 Corporation, Sunnyvale, CA, USA). 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 ***μ***L/well and cultured for 24 h. Transcription of the receptors was induced by adding 0.25 μg/mL doxycycline (Sigma-Aldrich). Cells were induced for 24 h and then loaded with the calcium-sensitive fluorescent dye Fluo-4-AM (2.5 ***μ***M, Molecular Probes, Eugene, OR, USA), 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 µM for genistein and genistin. For acetyl genistin and malonyl genistin, this was 100 µM due to limited availability of the compounds. Both hTAS2R14 and hTAS2R39 were screened for activation by the other isoflavonoids at 500 µM. In case of activation they were measured at different concentrations up to 1 mM in order to establish dose-response curves. For hTAS2R16, the applied concentrations were 100 µM acetyl genistin and malonyl genistin, 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 were 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, non-induced 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

In order to investigate the effect of the isoflavonoids on the viability of the cells, dye exclusion tests were conducted after 2 min 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, USA). 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 Corporation) 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 in order to

normalize to background fluorescence ***(19)***. Besides the response of induced cells, also the response of non-induced cells was measured as negative control for every compound at every concentration on the same plate. In cases that a non-specific 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 non-induced cells was taken at the reading point at which the signal of the induced cells was maximal. Threshold values of the agonists towards receptor activation were determined as first concentration showing significant difference to the baseline and to the response of non-induced cells. Differences were considered to be significant at $\boldsymbol{P}<0.05$, using a t-test (two sided, non-paired) (SAS 9.2, Sas Institute Inc., Cary, NC, USA). Between four and twelve concentrations were used to fit non-linear regression curves using Graph Pad Prism (version 4 for Windows, Graph Pad Software, San Diego, CA, USA). 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 non-induced cells, if applicable). For compounds that evoked non-specific 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, acetyl genistin and malonyl genistin, were tested using HEK293 cell lines, each cell line expressing one of the 25 different human bitter receptors. This was done in order 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, acetyl genistin, and malonyl genistin (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 (e.g. ***(13, 15, 21)***). hTAS2R39 was just recently deorphanized and it was stated that this receptor belonged to a group of bitter receptors that has a

penchant for natural compounds ***(13)***. Its agonist spectrum was smaller than that of the broadly tuned receptor hTAS2R14.

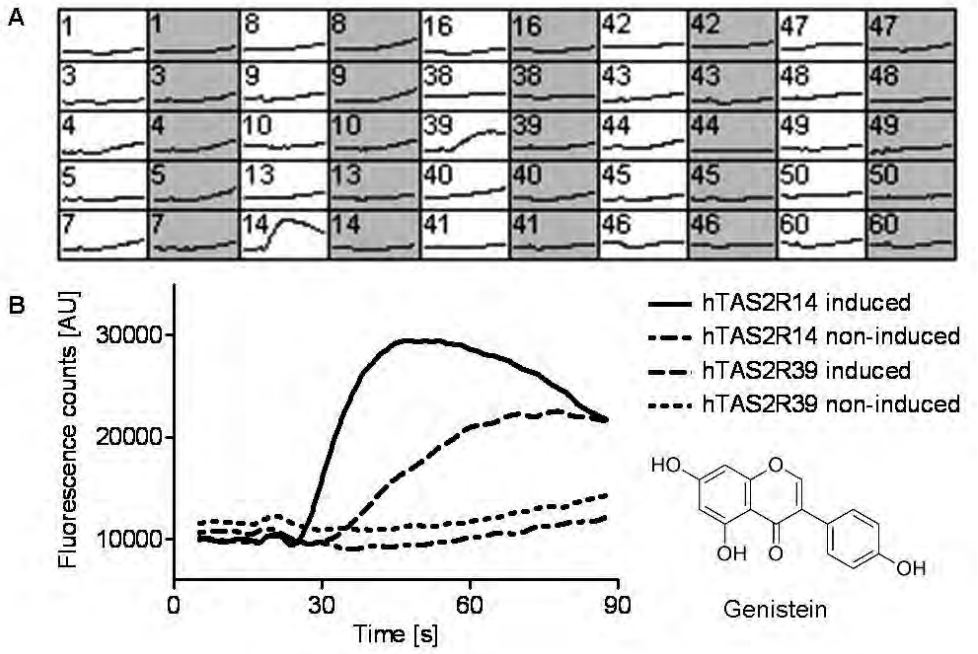

Figure 1. Bitter receptor activation by genistein. (**A**) Screening of HEK293 cells, each stably expressing one of the 25 hTAS2Rs[3], towards activation by genistein (400 µM). The signals of induced cells are depicted with white background. Non-induced cells were used as negative controls (grey background). (**B**) Calcium traces of hTAS2R14 and hTAS2R39 and their respective negative controls.

Dose-response behavior of genistein and its glucoside forms on hTAS2R14 and hTAS2R39

To compare the activation of hTAS2R14 and hTAS2R39 by genistein accurately, responses were recorded in the concentration range of 0.5-500 µM of genistein. Genistein displayed threshold values of 4 and 8 µM on hTAS2R14 and hTAS2R39, respectively (dose–response curves shown in **Figure 2**). The EC_{50} values after correction for non-specific signals were calculated to be 28.9 µM ± 8.2 µM for hTAS2R14 and 49.4 µM ± 8.9 µM for hTAS2R39. Additionally, the three glucoside forms of genistein were investigated. None of them clearly activated hTAS2R14, whereas acetyl genistin activated hTAS2R39 from 125 µM onwards, and genistin and malonyl genistin activated hTAS2R39 at 500 µM. EC_{50} values of the glucoside forms could not be determined because no maximum was reached in the concentration-response curves.

[3] The names of hTAS2R44, -47, -48, and -49 have meanwhile been changed into hTAS2R31, -30, -19, and -20, respectively, see Chapter 1

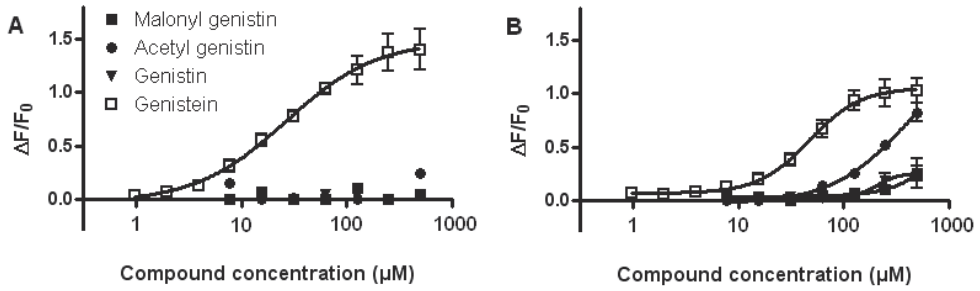

Figure 2. Normalized concentration-response curves of (**A**) hTAS2R14 and (**B**) hTAS2R39 containing HEK293 cells stimulated with malonyl genistin, acetyl genistin, genistin, and genistein.

Structure–activity relationship of selected isoflavonoids on hTAS2R14 and hTAS2R39

In order to understand the molecular signature involved in bitter receptor activation of genistein, 15 additional compounds were selected besides the four genistein forms, based on the structure of the soy isoflavone genistein: 13 isoflavones with different substitution pattern of the skeleton, one isoflavan and one 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 the 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.

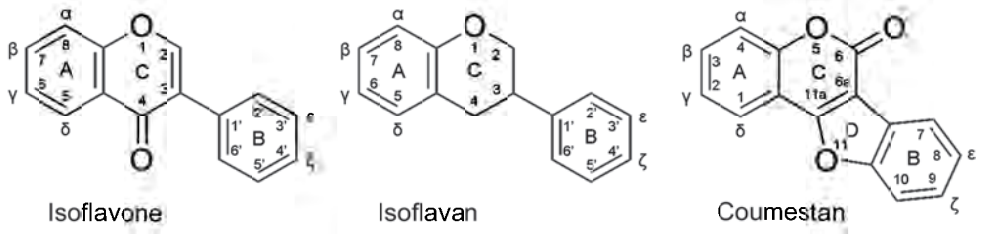

Figure 3. Chemical structure of the isoflavone, isoflavan, and coumestan skeleton. A-D denote the different rings of the isoflavonoids, 1-6' and 1-11a the commonly used carbon positions (inside), and alpha-zeta the positions used in this study (outside). The carbon positions can be substituted with -OH,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

These structurally similar isoflavonoids were screened for their ability to activate hTAS2R14 and hTAS2R39. When activation occurred at 500 µM, different concentrations were measured to map their dose-response behavior. Thresholds values and, when appropriate, EC_{50} values are given in **Table 1** for hTAS2R14 and **Table 2** for hTAS2R39. In total, 13 compounds activated hTAS2R14 and 15 compounds were active on hTAS2R39.

Based on the threshold values for each of the two receptors, the compounds were classified into four groups, I ≤32 µM (low), 32 µM < II ≤ 125 µM (medium), and 125 µM < III ≤ 500 µM (high), and IV no activation up to 500 µM (not active, (n.a.)). hTAS2R14 was activated by four compounds at low threshold, by two compounds at medium threshold, and seven compounds had a high threshold. Six compounds were considered inactive on hTAS2R14. For hTAS2R39, less compounds belonged to the low (2x) and the medium threshold group (2x), but more compounds belonged to the high threshold group (11x). 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 non-specific 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 in order 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 **Supporting information, Figure S2**). Furthermore, all compounds were tested for autofluorescence. None of the isoflavonoids in this study showed fluorescent behaviour under the conditions used in the assay.

Effect of glucosylation

Aglycones showed similar effects on hTAS2R14 and hTAS2R39, whereas glucosides showed different effects. Thirteen out of the fourteen aglycones activated hTAS2R14, while there were eleven 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 out of five isoflavone glucosides, viz. glycitin, genistin, acetylgenistin, and malonyl genistin (**Figure 2**). This finding suggested that glucosylation inhibited activation of hTAS2R14.

As β-glucopyranosides are reported to activate hTAS2R16 ***(27)***, the five glucosides daidzin, glycitin, genistin, acetyl genistin, and malonyl genistin were additionally tested on hTAS2R16. None of them activated this receptor. A very recent publication reported the inhibition of hTAS2R16 by probenecide ***(28)***, a compound commonly used in G-protein coupled receptor assays to prevent the efflux of calcium-sensitive dyes from the cells ***(29)***. Therefore, experiments on hTAS2R16 were repeated without probenecide in the buffer. Also without probenecide, no activation of hTAS2R16 by soy isoflavone glucosides occurred.

Table 1. Summary of threshold and EC_{50} values of various isoflavonoids on hTAS2R14.

Compound name (code)	Subgroup	Positions α	β	γ	δ	ε	ζ	EC_{50} [μM]	Threshold [μM]	group
Genistein (**Gein**)[a,b]	Isoflavone	H	OH	H	OH	H	OH	28.9	4	
(+/-)-Equol (**10**)[c]	Isoflavan	H	OH	H	H	H	OH	47.2	8	I
Prunetin (**6**)	Isoflavone	H	OCH_3	H	OH	H	OH	n.d.	16	
7-Hydroxy-isoflavone (**3**)	Isoflavone	H	OH	H	H	H	H	n.d.	32	
Biochanin A (**1**)[a]	Isoflavone	H	OH	H	OH	H	OCH_3	n.d.	63	
7,8,4'-Trihydroxy-isoflavone (**8**)	Isoflavone	OH	OH	H	H	H	OH	124	63	II
Isoflavone (**5**)	Isoflavone	H	H	H	H	H	H	n.d.	250	
6,7,4'-Trihydroxy-isoflavone (**7**)	Isoflavone	H	OH	OH	H	H	OH	378	250	
7,3',4'-Trihydroxy-isoflavone (**9**)	Isoflavone	H	OH	H	H	OH	OH	264	250	
Coumestrol (**11**)[a]	Coumestan	H	OH	H	H	H	OH	358	250	III
Formononetin (**2**)[a]	Isoflavone	H	OH	H	H	H	OCH_3	n.d.	500	
Daidzein (**Dein**)[a,b]	Isoflavone	H	OH	H	H	H	OH	n.d.	500	
Glycitein (**Glyein**)[a]	Isoflavone	H	OH	OCH_3	H	H	OH	n.d.	500	
7-Hydroxy-6-methoxy-isoflavone (**4**)	Isoflavone	H	OH	OCH_3	H	H	H	n.d.	n.sp.	
Acetyl genistin (**AGe**)[a]	Isoflavone glucoside	H	***O***-Glc-6"-***O***-Acetyl	H	OH	H	OH	n.d.	n.a.	
Daidzin (**D**)[a,b]	Isoflavone glucoside	H	***O***-Glc	H	H	H	OH	n.d.	n.a.	IV
Genistin (**Ge**)[a,b]	Isoflavone glucoside	H	***O***-Glc	H	OH	H	OH	n.d.	n.a.	
Glycitin (**Gly**) [a]	Isoflavone glucoside	H	***O***-Glc	OCH_3	H	H	OH	n.d.	n.a.	
Malonyl genistin (**MGe**)[a,b]	Isoflavone glucoside	H	***O***-Glc-6"-***O***-Malonyl	H	OH	H	OH	n.d.	n.a.	

Not specific (n.sp.) means that activity could not be determined due to non-specific signals in the negative control. Four groups of thresholds for activation of hTAS2R14 correspond to I (low) (≤ 32 μM), II (medium) (>32 μM - 125 μM), III (high) (>125 μM - 500 μM) and IV not active up to 500 μM (n.a.).
n.d., not determined, [a] occurring in soybean (***Glycine max*** L. Merrill) ***(3, 25)***, [b] compound frequently reported as bitter ***(7-11)***, [c] S-(-)equol is an intestinal metabolite of daidzein ***(26)***.

Table 2. Summary of threshold and EC_{50} values of various isoflavonoids on hTAS2R39.

Compound name (code)	Subgroup	Positions α	β	γ	δ	ε	ζ	EC_{50} [μM]	Threshold [μM]	group
Genistein (**Gein**)[a,b]	Isoflavone	H	OH	H	OH	H	OH	49.4	8	I
(+/-)-Equol (**10**)[c]	Isoflavan	H	OH	H	H	H	OH	55.8	32	
7,8,4'-Trihydroxy-isoflavone (**8**)	Isoflavone	OH	OH	H	H	H	OH	184	63	II
Acetyl genistin (**AGe**)[a]	Isoflavone glucoside	H	***O***-Glc-6"-***O***-Acetyl	H	OH	H	OH	n.d.	125	
7-Hydroxy-isoflavone (**3**)	Isoflavone	H	OH	H	H	H	H	315	250	III
6,7,4'-Trihydroxy-isoflavone (**7**)	Isoflavone	H	OH	OH	H	H	OH	n.d.	250	
7,3',4'-Trihydroxy-isoflavone (**9**)	Isoflavone	H	OH	H	H	OH	OH	n.d.	250	
Coumestrol (**11**)[a]	Coumestan	H	OH	H	H	H	OH	n.d.	250	
Biochanin A (**1**)[a]	Isoflavone	H	OH	H	OH	H	OCH_3	n.d.	500	
Formononetin (**2**)[a]	Isoflavone	H	OH	H	H	H	OCH_3	n.d.	500	
Daidzein (**Dein**)[a,b]	Isoflavone	H	OH	H	H	H	OH	n.d.	500	
Genistin (**Ge**)[a,b]	Isoflavone glucoside	H	***O***-Glc	H	OH	H	OH	n.d.	500	
Glycitein (**Glyein**)[a]	Isoflavone	H	OH	OCH_3	H	H	OH	n.d.	500	
Glycitin (**Gly**)[a]	Isoflavone glucoside	H	***O***-Glc	OCH_3	H	H	OH	n.d.	500	
Malonyl genistin (**MGe**)[a,b]	Isoflavone glucoside	H	***O***-Glc-6"-***O***-Malonyl	H	OH	H	OH	n.d.	500	
Prunetin (**6**)	Isoflavone	H	OCH_3	H	OH	H	OH	n.d.	n.sp.	IV
7-Hydroxy-6-methoxy-isoflavone (**4**)	Isoflavone	H	OH	OCH_3	H	H	H	n.d.	n.sp.	
Isoflavone (**5**)	Isoflavone	H	H	H	H	H	H	n.d.	n.a.	
Daidzin (**D**)[a,b]	Isoflavone glucoside	H	***O***-Glc	H	H	H	OH	n.d.	n.a.	

Not specific (n.sp.) means that activity could not be determined due to non-specific signals in the negative control. Four groups of thresholds for activation of hTAS2R39 correspond to I (low) (≤ 32 μM), II (medium) (>32 μM - 125 μM), III (high) (>125 μM - 500 μM) and IV not active up to 500 μM (n.a.).
n.d., not determined, [a] occurring in soybean (***Glycine max*** L. Merrill) ***(3, 25)***, [b] compound frequently reported as bitter ***(7-11)***, [c] S-(-)equol is an intestinal metabolite of daidzein ***(26)***.

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 β and ζ) 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.

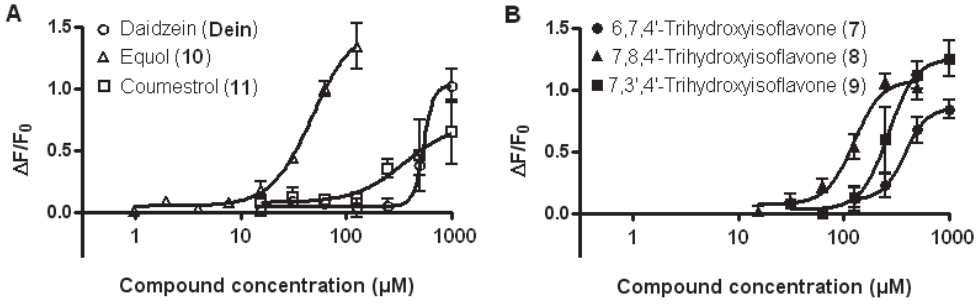

Figure 4. Concentration-response curves of hTAS2R14 for (**A**) the isoflavone daidzein (**Dein**), the isoflavan equol (**10**), and the coumestan coumestrol (**11**), and (**B**) the three isoflavones 6,7,4'-trihydroxyisoflavone (**7**), 7,8,4'-trihydroxyisoflavone (**8**), and 7,3',4'-trihydroxyisoflavone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

Substitutions of A- and B-rings

In **Figure 4B**, the activation of hTAS2R14 by three isoflavones is compared. These three isoflavones (**7**,**8**,**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 α and β (compound **8**) led to higher efficacy and potency than two hydroxyl groups on positions β and γ (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 comparing (**Glyein**) to (**4**), (**1**) to (**2**), or (**Gein**) to (**Dein**). For the activation of hTAS2R39, substitution of the A-ring is important, as the unsubstituted compound (**5**) was inactive. 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 exception of prunetin (**6**) on hTAS2R14). Hydroxylation of β and ζ 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 % ***(30)***. 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**, **2**, **3**, **7**, **8**, **9**, **10**, **11**). Until now, only four compounds were described that stimulated both hTAS2R14 and hTAS2R39, which are azathioprine, chlorpheniramine, diphenidol and quinine ***(13)*** (see **Supporting information**). These four compounds and the newly identified isoflavonoid-agonists do not share structural characteristics regarding 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, 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 ***(31)***. 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.

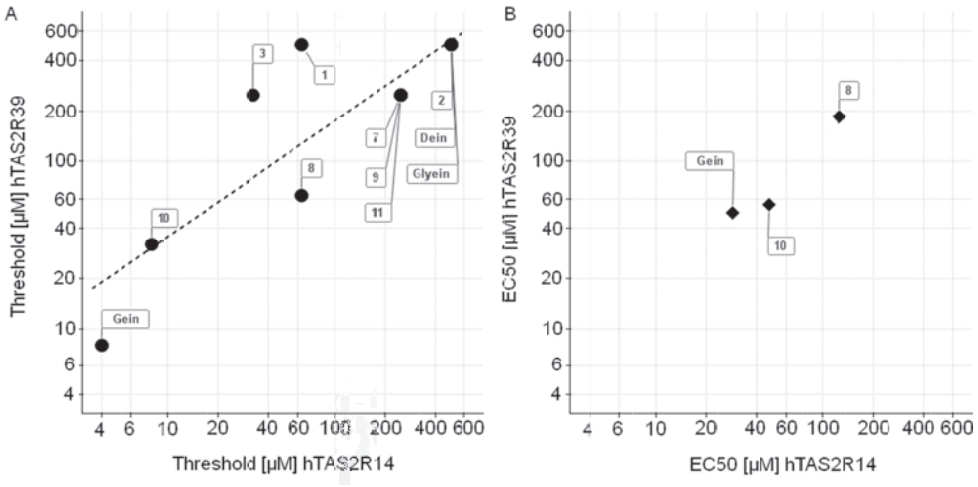

Figure 5. Correlation of (**A**) threshold values and (**B**) EC_{50} values of hTAS2R14 and hTAS2R39. The dashed line in (**A**) represents the linear regression line (R^2=0.75).

New dietary agonists of receptor hTAS2R39

So far, 17 agonists had been described for hTAS2R39 ***(13, 18, 32)*** (see **Supporting information**). Of these agonists, four are flavonoids and therewith structurally similar to isoflavonoids. The other 13 agonists belong to different chemical classes, and they are structurally very different form 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, viz. **Gein**, **Dein**, **Glyein**, **Ge**, **Gly**, **AGe**, **MGe**, **1**, **2**, **3**, **7**, **8**, **9**, **10**, and **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 ***(27)***, that β-glucopyranosides with a hydrophobic group attached to C1 of the pyranose activated the human bitter receptor hTAS2R16. The β-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 β-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.

Relevance for sensory perception

Okubo et al. ***(9)*** described the threshold values for bitterness and astringency together to be in nM to µM range (10^{-6} M to 10^{-7} M for genistein, 5x 10^{-7} M to 5x 10^{-8} M for daidzein). Kudou et al. ***(10)*** determined threshold values for undesirable taste and determined 10^{-5} M for genistein and 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 (4x 10^{-3} M for genistein, 2.9x 10^{-3} M for daidzein) are in the mM range, whereas the bitter receptor threshold values determined in our study are in µM 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 which 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 which occur with a frequency of 99 % (GPCR Natural Variance database http://nava.liacs.nl ***(33)***). 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 ***(34)***, adaptational states and hormone levels can cause variable taste responses ***(35)***. With the bitter receptor activation assay, we have now measured the intrinsic bitterness of a number of (dietary) isoflavonoids, in 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 ***(36)*** varied between 0.4 and 14.3 µg/g in soy milk and 2.4 and 60.4 µg/g in tofu. This is approximately 2 to 225 µM, which is exceeding 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 amount of the other three forms increases ***(37-39)***. 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 µg/g (soy milk ***(40)*** and

fried tofu ***(41)***, respectively), which is approximately 0 to 250 µM. Therewith, 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 µg/g (soy milk ***(40)***) and 562 µg/g (tofu, ***(41)***), which is approximately 50 µM to 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 as neutraceutical, is more estrogenic than its precursor ***(26)***. Also coumestrol, which is formed from daidzein by combined malting by a food-grade fungus, is more estrogenic than its precursor ***(42)***. 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 formulating food products enriched in these compounds.

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SUPPORTING INFORMATION

Table S1. Agonists of hTAS2R14 and hTAS2R39.

Compounds	Agonist		Detected by
	hTAS2R14	hTAS2R39	
1,8-Naphthalaldehydic acid	+		Behrens et al. 2004
1-Naphthoic acid	+		Behrens et al. 2004
1-Nitronaphthalene	+		Behrens et al. 2004
Picrotin	+		Behrens et al. 2004
Picrotoxinin	+		Behrens et al. 2004
	+	-	Meyerhof et al. 2010
Piperonylic acid	+		Behrens et al. 2004
Sodium benzoate	+		Behrens et al. 2004
	+	-	Meyerhof et al. 2010
(-)-α-Thujone	+		Behrens et al. 2004
	+	-	Meyerhof et al. 2010
Aristolochic acid	+		Sainz et al. 2007
	+		Pronin et al. 2007
	+	-	Meyerhof et al. 2010
Herbolide D	+		Brockhoff et al. 2007
Casein hydrolysate (peptides)	+		Maehashi et al. 2008
trans-Isocohumulone	+		Intelmann et al. 2009
trans-Isohumulone	+		Intelmann et al. 2009
trans-Isoadhumulone	+		Intelmann et al. 2009
cis-Isocohumulone	+		Intelmann et al. 2009
cis-Isohumulone	+		Intelmann et al. 2009
cis-Isoadhumulone	+		Intelmann et al. 2009
Lupulone	+		Intelmann et al. 2009
Adlupulone	+		Intelmann et al. 2009
8-Prenylnaringenin	+		Intelmann et al. 2009
Humulone isomers	+	-	Meyerhof et al. 2010
Hoodia gordonii glycoside H.g.-12	+	-	Le Nevé et al. 2010
Hoodia gordonii aglycone	+	-	Le Nevé et al. 2010
Absinthin	+	-	Meyerhof et al. 2010
Arborescin	+	-	Meyerhof et al. 2010
Arglabin	+	-	Meyerhof et al. 2010
Artemorin	+	-	Meyerhof et al. 2010
Campher	+	-	Meyerhof et al. 2010
Caffeine	+	-	Meyerhof et al. 2010
Cascarillin	+	-	Meyerhof et al. 2010
Cumarin	+	-	Meyerhof et al. 2010
Cucurbitacin B	+	-	Meyerhof et al. 2010
Falcarindiol	+	-	Meyerhof et al. 2010
Noscapine	+	-	Meyerhof et al. 2010
Papaverine	+	-	Meyerhof et al. 2010
Parthenolide	+	-	Meyerhof et al. 2010
Quassin	+	-	Meyerhof et al. 2010
Benzamide	+	-	Meyerhof et al. 2010
Benzoin	+	-	Meyerhof et al. 2010

Compounds	Agonist		Detected by
	hTAS2R14	hTAS2R39	
Carisoprodol	+	-	Meyerhof et al. 2010
Chlorhexidine	+	-	Meyerhof et al. 2010
Diphenhydramine	+	-	Meyerhof et al. 2010
Diphenylthiourea	+	-	Meyerhof et al. 2010
Divinylsulfoxid	+	-	Meyerhof et al. 2010
Flufenamic acid	+	-	Meyerhof et al. 2010
Haloperidol	+	-	Meyerhof et al. 2010
4-Hydroxyanisol	+	-	Meyerhof et al. 2010
Quinine	+	+	Meyerhof et al. 2010
Azathioprine	+	+	Meyerhof et al. 2010
Chlorpheniramine	+	+	Meyerhof et al. 2010
Diphenidol	+	+	Meyerhof et al. 2010
Amarogentin	-	+	Meyerhof et al. 2010
Chloramphenicol	-	+	Meyerhof et al. 2010
Colchicine	-	+	Meyerhof et al. 2010
Thiamine	-	+	Meyerhof et al. 2010
Acetaminophen	-	+	Meyerhof et al. 2010
Chloroquine	-	+	Meyerhof et al. 2010
Denatonium benzoate	-	+	Meyerhof et al. 2010
(-)-Epicatechin		+	Narukawa et al. 2011
(-)-Epicatechin gallate		+	Narukawa et al. 2011
(-)-Epigallocatechin		+	Narukawa et al. 2011
(-)-Epigallocatechin gallate		+	Narukawa et al. 2011
Peptide: Phe-Phe-Pro-Arg		+	Ueno et al. 2011
Peptide: Pro-Arg		+	Ueno et al. 2011
Isoflavone	+	-	this study
Prunetin	+	-	this study
Biochanin A	+	+	this study
Coumestrol	+	+	this study
Daidzein	+	+	this study
(+/-)-Equol	+	+	this study
Formononetin	+	+	this study
Genistein	+	+	this study
Glycitein	+	+	this study
7-Hydroxyisoflavone	+	+	this study
6,7,4'-Trihydroxyisoflavone	+	+	this study
7,3',4'-Trihydroxyisoflavone	+	+	this study
7,8,4'- Trihydroxyisoflavone	+	+	this study
Acetyl genistin	-	+	this study
Genistin	-	+	this study
Glycitin	-	+	this study
Malonyl genistin	-	+	this study

+ activation, - no activation

previously described agonists of hTAS2R14 and hTAS2R39

new agonists of hTAS2R14 and hTAS2R39

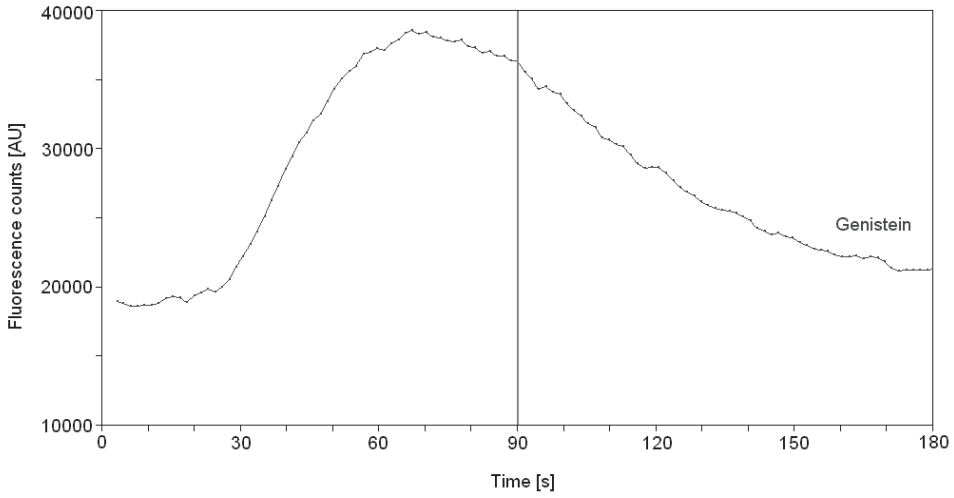

Figure S1. Activation of hTAS2R39 by genistein (measuring time: 180 s). The grey line indicates the end of normally performed measuring after 90 s.

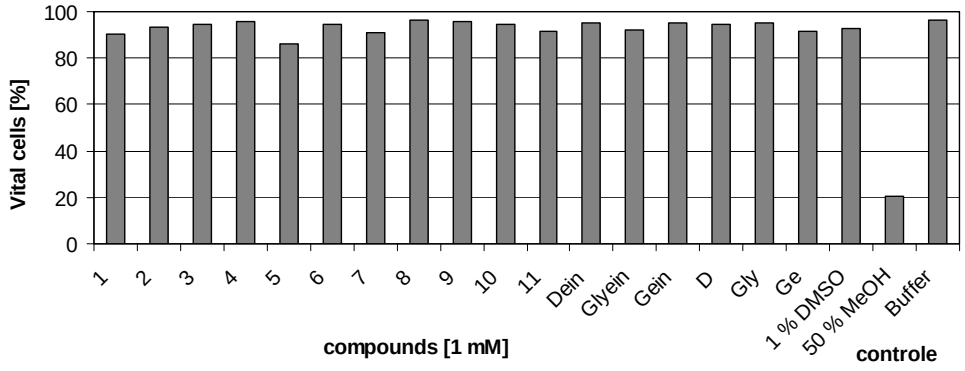

Figure S2. Cell viability after 2 min incubation with isoflavonoids, Tyrode's buffer (negative control for toxicity), and methanol (positive control for toxicity).

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Chapter 3

Bitter taste receptor activation by flavonoids and isoflavonoids: Modeled structural requirements for activation of hTAS2R14 and hTAS2R39

Based on: Wibke S.U. Roland, Leo van Buren, Harry Gruppen, Marianne Driesse, Robin J. Gouka, Gerrit Smit, Jean-Paul Vincken. Bitter taste receptor activation by flavonoids and isoflavonoids: Modeled structural requirements for activation of hTAS2R14 and hTAS2R39. *Journal of Agricultural and Food Chemistry* **2013** *61* (44), 10454-10466.

ABSTRACT

Many flavonoids and isoflavonoids have undesirable bitter taste, which hampers their use as food bioactives. The aim of this study was to investigate the effect of a large set of structurally similar (iso)flavonoids on the activation of bitter receptors hTAS2R14 and hTAS2R39, and to predict their structural requirements to activate these receptors. In total, 68 compounds activated hTAS2R14 and 70 compounds activated hTAS2R39, amongst which 58 ligands were overlapping. Their activation threshold values varied over a range of three log units between 0.12 and 500 μM. Ligand-based 2D-fingerprint and 3D-pharmacophore models were created to detect structure activity relationships. The 2D-models demonstrated excellent predictive power in identifying bitter (iso)flavonoids and discrimination from inactive ones. The structural characteristics for an (iso)flavonoid to activate hTAS2R14 (or hTAS2R39), were determined by 3D-pharmacophore models to be composed of two (or three) hydrogen bond donor sites, one hydrogen bond acceptor site, and two aromatic ring structures, of which one had to be hydrophobic. The additional hydrogen bond donor feature for hTAS2R39 ligands indicated the possible presence of another complementary acceptor site in the binding pocket, compared to hTAS2R14. Hydrophobic interaction of the aromatic feature with the binding site might be of higher importance in hTAS2R14 than in hTAS2R39. Together, this might explain why OH-rich compounds showed different behavior on the two bitter receptors. The combination of *in vitro* data and different *in silico* methods created a good insight in activation of hTAS2R14 and hTAS2R39 by (iso)flavonoids and provided a powerful tool in prediction of their potential bitterness. By understanding the “bitter motif”, introduction of bitter taste in functional foods enriched in (iso)flavonoid bioactives might be avoided.

INTRODUCTION

Phenolic compounds, such as flavonoids and isoflavonoids, are in the focus of health research. Isoflavonoids are mainly occurring in legumes, such as soybeans, and have been associated with prevention of some cancers, cardiovascular disease, menopausal complaints and osteoporosis *(1)*. Flavonoids occur in many different plants and are widely present in our diet. Amongst others, they might play a role in the reduced incidence of cancer and cardiovascular diseases *(2, 3)*. Unfortunately, many (iso)flavonoids have a negative impact on sensory perception as they can taste bitter *(4)*. The bitter taste of soybean isoflavones has been described in several sensory studies *(5, 6)*. Recently, we showed that several soybean isoflavones activated the human bitter taste receptors hTAS2R14 and hTAS2R39 and partially elucidated the isoflavonoid substitution pattern favorable for activation of both receptors *(7)*.

Bitter taste receptor hTAS2R14 is known to be activated by a large number of compounds originating from different chemical classes *(7-13)*. For hTAS2R39 on the other hand, a rather moderate number of agonists has been reported *(7, 9, 14-17)* and this receptor seemed to be less broadly tuned than hTAS2R14 *(9)*. Recently, hTAS2R39 has been reported to be activated by tea catechins *(14)*, which belong to the group of flavonoids.

There are also numerous flavonoids, which do not traditionally occur in our diet, and for which the taste is often not known. It is hypothesized that the bitter taste characteristics can be predicted, once the molecular signatures of (iso)flavonoids for activation of hTAS2R14 and hTAS2R39 are known. Originating in pharmaceutical science, the concept of molecular modeling gains importance in food science *(18)*, and might be employed in facilitating such predictions. A 2D-fingerprint model is based on a binary representation of a molecule in which each bit indicates the presence or absence of a molecular fragment *(19)*. A 3D-pharmacophore model operates with a set of features together with their relative spatial orientation, which relates to a set of chemical features in a molecule. These features are recognized by amino acid residues in the receptor binding site with complementary functions, ultimately explaining that molecule's biological activity *(20, 21)*. Recently, Ley et al. *(22)* described a pharmacophore model, which on docking in a hTAS2R10 structural model allowed identification of a masking agent for the bitter taste of caffeine. Furthermore, ligand-based pharmacophore modeling has been applied to understand structure-activity relationships of odors *(23)*, and to explain interactions between flavor compounds and β-lactoglobulin *(24)*.

The objective of the present study was to study the behavior of flavonoids towards activation of hTAS2R14 and hTAS2R39. Their activation would indicate that the compounds tested have a bitter taste. To this end, a large subset of flavonoids, or flavonoid analogues, was tested, and the threshold and EC_{50} values of the active compounds were determined. A second objective was to investigate the chemical space of flavonoids, and isoflavonoids, in relation to the activation of bitter receptors hTAS2R14 and hTAS2R39.

By linking the receptor activation with the compound's molecular structure, 2D-fingerprint models and ligand-based 3D-pharmacophore models for each of the two bitter receptors were established. These models map the structural requirements for ligands with an isoflavonoid, flavonoid, or similar structure for these two receptors. Altogether, they distinguish active from inactive (iso)flavonoids and enable predicting bitterness of similar compounds with unknown taste properties.

MATERIALS AND METHODS

Receptor assays

Materials

Compounds tested were purchased from Indofine Chemical Company (Hillsborough, NJ, USA), Extrasynthese (Genay, France), Sigma-Aldrich (Steinheim, Germany), Brunschwig (Amsterdam, The Netherlands), Bioconnect (Huissen, The Netherlands), or WAKO (Neuss, Germany). The majority of compounds were ≥99% (18 compounds), ≥98% (44 compounds) or ≥97% (15 compounds) pure. Compounds **38** and **71** were ≥96% pure, **22**, **39**, **43**, **45**, **50**, **51**, **59**, **70**, **78**, and **94** were ≥95% pure, **33**, **54**, **56**, **74**, **79**, **80**, and **88** were ≥90% pure, and **37** was ≥85% pure. Each compound was dissolved in DMSO (Sigma-Aldrich, Steinheim, Germany) to a 100 mM stock concentration. Trypan blue solution (0.4 % w/v) was purchased from Sigma-Aldrich (Steinheim, Germany).

Tyrode's buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM Hepes, pH 7.4) with 0.5 mM probenecid (Sigma-Aldrich) was used for dilution of compound-DMSO stock solutions and for calcium imaging assays. All compounds were tested for autofluorescence and toxic effects on the cells used at a concentration of 1 mM as described before *(7)*, without observing abnormalities.

In vitro assessment of hTAS2R14 and hTAS2R39 activation by intracellular calcium release

Activation of bitter taste receptors expressed in HEK293 cells leads to release of intracellular Ca^{2+} *(25)*. This was measured using the fluorescent calcium dye Fluo-4-AM (2.5 μM, Molecular Probes, Eugene, OR, USA) in a FlexStation II 384 or FlexStation III (Molecular Devices Corporation, Sunnyvale, CA, USA) for 90 s (excitation 485 nm / emission 520 nm). The expression of hTAS2R14 and hTAS2R39 in HEK293 cells, the maintenance of the cells, and the measuring procedure were performed as reported earlier *(7)*.

In total, a set of 97 compounds are described in this study, of which 19 isoflavonoids were reported in our earlier publication *(7)*, and 78 other compounds were tested

additionally. 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 hTAS2R14 and hTAS2R39 for activation was performed at 500 µM concentrations of test compounds. In case of activation, test compounds were measured at concentration ranges up to 1 mM in order to establish dose-response curves. Non-induced cells, not expressing the bitter receptors, were always measured in parallel to verify specificity of receptor activation. As positive controls for receptor activation, in each plate duplicate measurements with epicatechin gallate *(14)* for hTAS2R39 or with naphthoic acid *(8)* (or genistein) for hTAS2R14 were performed. All experiments were conducted in duplicate on two or more different days. Some compounds were not completely soluble at high concentrations and, therefore, their real potency might be underestimated.

Calcium assay data processing and statistical analysis

Data processing and statistical analysis were done as reported previously *(7)*. SoftMax Pro 5.4 software (Molecular Devices Corporation) was used to plot the fluorescence signals. The fluorescence value ($\Delta F/F_0$), representing receptor activity, was 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 signal of the baseline in order to normalize background fluorescence *(26)*. Besides the response of induced cells, also the response of non-induced cells (not expressing the bitter receptor) was measured as negative control for every compound at every concentration on the same plate. In cases that a non-specific 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 non-induced cells was taken at the reading point at which the signal of the induced cells was maximal. Threshold values of the agonists towards receptor activation were determined as first concentration showing significant difference to the baseline and to the response of non-induced cells. Differences were considered to be significant at $P<0.05$, using a t-test (two sided, non-paired) (SAS 9.2, Sas Institute Inc., Cary, NC, USA). Dose-response curves were established as non-linear regression curves using Graph Pad Prism (version 4 for Windows, Graph Pad Software, San Diego, CA, USA). The error bars reflect the standard error of the mean (SEM). EC_{50} values were calculated for compounds that reached a maximum in the dose-response curve (after subtraction of the response of non-induced cells, if applicable). For compounds that evoked high non-specific signals at higher concentrations, only the data for the appropriate concentrations were shown in the figures.

Modeling

2D-fingerprint modeling

2D-modeling was performed using Pipeline Pilot v8.0, (Accelrys, San Diego, CA, USA). The chemical structures of the 97 compounds tested were derived from the SMILES (simplified molecular-input line-entry system) notations (**supplementary material S1**). Results at the screening concentration of 500 μM were used. Compounds showing ambiguous results (22 compounds for hTAS2R14; 21 compounds for hTAS2R39) were excluded. This resulted in 75 and 76 compounds suitable for modeling of receptor activation of hTAS2R14 and hTAS2R39, respectively. The majority of these compounds was used for model building (training set), containing both active and inactive compounds, whereas ~10 % of the structurally most diverse compounds were excluded from model building for validation purposes (test set).

The optimal fingerprint model was obtained by building numerous models based on different fingerprint types. This optimization was done with the training set, using the Bayesian interference method and leave-one-out settings *(19, 27)*. Three-fold cross-validations were performed to evaluate the models, in which ranking of the best models was primarily based on the Receiver Operating Characteristic (ROC) scores (see **supplementary material S2-S4**). The highest ranking models were further validated by the test sets, resulting in the final model selection. ECFP-10 and ECFP-8 fingerprint models were selected for hTAS2R14 and hTAS2R39, respectively. Their ROC scores were close to 1, which indicated excellent accuracy. Extended-Connectivity Fingerprints (ECFP) use charge and hybridization of the atom (extended (E)) and connectivity (C) and return a list of fragments present in the molecule *(19)*, of which in this case the maximum diameter of a fragment was 10 and 8 bond lengths, respectively.

For identification of key molecular features for bitter receptor activation, 20 “good” (“G”) and 20 “bad” (“B”) fingerprint fragments were calculated for each model. Each of them has a Bayesian score. If this score is positive, the likelihood that the fragment is a member of the active subset increases, and *vice versa*. A selection of four illustrative fragments is shown in the results section; the full list is specified in the **supplementary material (S5-S9).**

3D-pharmacophore modeling

3D-pharmacophore modeling was performed using Discovery Studio v3.1 (Accelrys). In contrast to 2D-fingerprint modeling, a training set for 3D-pharmacophore modeling is based on only a few highly active compounds. The selection for pharmacophore training ligands on bitter receptor activation was based on activation thresholds, as EC_{50} values were not available for all (iso)flavonoids. Compounds were categorized by their threshold values into highly active (threshold ≤32 μM), moderately active (threshold > 32 – 500 μM)

and inactive compounds (not active up to 500 µM) (**Table 2**). 24 compounds for hTAS2R14 and 20 compounds for hTAS2R39 showed ambiguous results or high non-specific signals and were excluded from modeling. Besides low threshold, the training ligands had to be diverse in backbone structure and substitution pattern. Seven highly active compounds were chosen as training ligands for each receptor (**Table 1**). One of the training ligands selected for building the hTAS2R39 pharmacophore was an anthocyanidin and required special attention. Due to the pH dependence of the anthocyanidin structure (shifting equilibria between flavylium cation structure, quinoid tautomers, hemiketal, and chalcone) *(28, 29)*, multiple forms were included for modeling.

Training compounds were subjected to internal strain energy minimization and conformational analysis (maximum number of conformers = 200; generation type: best quality; energy range = 10 kcal / mol above the calculated global minimum). Using the hiphop and hiphop refine algorithms of Discovery Studio, the chemical features optimized for exploring the spatial pharmacophore map of this group of compounds were “hydrogen bond acceptor”, “hydrogen bond donor”, “hydrophobic aromatic”, “hydrophobic aliphatic”, and “ring aromatic”. The “hydrophobic aromatic” feature describes an aromatic ring which is hydrophobic, the spatial orientation of which is irrelevant. The “ring aromatic” feature describes an aromatic ring which can be hydrophobic or hydrophilic, depending on its substitutions. Furthermore, the plane of this aromatic ring has a fixed orientation towards the receptor binding site. The pharmacophore models were trained with qualitative data and thus the features are not weighed and the relevance of each feature was considered as equal. The quality of mapping is described by fit values. A feature that maps exactly on the respective atom has a fit value of 1, and a feature that does not map has a fit value of 0. The fit values and relative energies of all compounds are summarized in the **supplementary material S1**. Mapping is done by rotation and translation of the molecule to optimize the superimposition of the molecule on the features, meanwhile additionally the torsion angles are altered whereby the maximal relative energy should not exceed 10 kcal / mol, compared to its optimal energetic state of 0 kcal / mol.

The pharmacophore protocol created 10 hypotheses for each run. These hypotheses were analyzed to find the optimal model. Hypotheses were scored based on the confusion matrix table, ROC plots, rank score, direct hit/partial hit and maximal fit value (additional information given in **supplementary material S10-S14**). The optimization involved variation of features and/or locations to optimize sensitivity and specificity of the structure based activity prediction.

Pharmacophore building for ligands of hTAS2R14 resulted in a 5-feature pharmacophore, whereas for ligands of hTAS2R39, it resulted in a 6-feature pharmacophore. In the case of the model built for hTAS2R14, a ligand had to map all five features in order to be predicted as active, whereas in the case of the model built for hTAS2R39, ligands were allowed to map five out of six features, without disqualifying

Table 1. Chemical structures (related to structure depicted in **Figure 1**) of compounds tested and their behavior on hTAS2R14 and hTAS2R39 concerning activity, threshold, and EC_{50} values. Activity was determined at 500 µM screening concentration (+ active, - inactive, +/- results ambiguous).

Compound	No	Activity hTAS2R		Threshold hTAS2R		EC_{50} hTAS2R	
		14	39	14	39	14	39
Flavones							
Acacetin	1	-	+/-	n.a.	n.sp.	n.d.	n.d.
Apigenin	2	+	+	8	1	n.d.	n.d.
Chrysin	3	+	+	63	16	n.d.	n.d.
Chrysoeriol	4	+	+/-	500	n.a.	n.d.	n.d.
5,2'-Dihydroxyflavone	5	-	+	n.a.	500	n.d.	n.d.
5,3'-Dihydroxyflavone	6	+/-	+/-	n.a.	n.a.	n.d.	n.d.
5,4'-Dihydroxyflavone	7	+	+	250	500	n.d.	n.d.
6,4'-Dihydroxyflavone	8	+	+	125	500	n.d.	n.d.
7,4'-Dihydroxyflavone	9	+	+	16	125	n.d.	n.d.
5,7-Dimethoxyflavone[d]	10	+	+	16	32	n.d.	n.d.
6,7-Dimethoxyflavone	11	+	-	125	n.a.	n.d.	n.d.
Flavone	12	+	+	8	8	20.5	45.9
Gardenin A	13	-	+/-	n.a.	n.a.	n.d.	n.d.
Genkwanin	14	-	+	n.a.	500	n.d.	n.d.
5-Hydroxyflavone	15	+/-	+	n.a.	500	n.d.	n.d.
4'-Hydroxyflavone	16	+/-	+	n.a.	500	n.d.	n.d.
5-Hydroxy-3'-methoxyflavone	17	-	-	n.a.	n.a.	n.d.	n.d.
4'-Hydroxy-6-methoxyflavone	18	+	-	125	n.a.	n.d.	n.d.
4'-Hydroxy-7-methoxyflavone	19	+	+	250	250	n.d.	n.d.
Luteolin	20	+	+	2	0.5	6.0	7.3
6-Methoxyflavone	21	+/-	-	n.a.	n.a.	n.d.	n.d.
6-Methoxyluteolin[a]	22	+	+	4	8	11.0	22.9
Scutellarein[a,b]	23	+	+	8	8	35.0	40.3
Tricetin	24	+/-	+	n.a.	250	n.d.	n.d.
5,7,2'-Trihydroxyflavone	25	+	+	8	4	21.1	35.3
5,3',4'-Trihydroxyflavone	26	+/-	+/-	n.a.	n.sp.	n.d.	n.d.
7,3',4'-Trihydroxyflavone	27	+	+	16	16	67.3	141
5,7,4'-Trimethoxyflavone	28	+	+/-	250	n.a.	n.d.	n.d.
Flavonols							
Datiscetin[a]	29	+	+	2	16	10.0	41.6
Fisetin	30	+/-	+	n.sp.	1	n.d.	n.d.
Flavonol	31	+/-	+/-	n.a.	n.a.	n.d.	n.d.
Gossypetin[c]	32	-	+	n.a.	250	n.d.	388
Herbacetin	33	+	+	125	125	n.d.	n.d.
Isorhamnetin	34	+	+	125	0.12	n.d.	n.d.
Kaempferol	35	+	+	8	0.5	n.d.	n.d.
6-Methoxyflavonol	36	-	+/-	n.a.	n.sp.	n.d.	n.d.
Morin	37	+	+	8	2	n.d.	n.d.
Myricetin[c]	38	+	+	250	1	n.d.	n.d.
Quercetagetin	39	+/-	+	250	2	n.d.	n.d.
Quercetin	40	+/-	+/-	n.sp.	n.sp.	n.d.	n.d.

Backbones A: flavone (R_3=H) / flavonol (R_3=OH), B: flavanone (R_3=H) / flavanonol (R_3=OH), C: flavan (R_3=H) / flavanol (R_3=OH), D: chalcone, E: dihydrochalcone, F: anthocyanidin (R_3=OH) / deoxyanthocyanidin (R_3=H), G: isoflavone, H: isoflavan, I: coumestan, J: stilbene, K: aurone, L: xanthone. Side groups a: ***6"-O***-acetyl glucose, b: ***6"-O***-malonyl glucose, c: glucose, d: 4-((2S)-3-(hydroxylmethyl)-1,4-dioxan-2-yl)-2-methoxyphenol, e: gallic acid. (based on **Figure 1**)

Back-bone	R_1	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
A		H	OH	H	OH	H	H	H	OCH_3	H
A		H	OH	H	OH	H	H	H	OH	H
A		H	OH	H	OH	H	H	H	H	H
A		H	OH	H	OH	H	H	OCH_3	OH	H
A		H	OH	H	H	H	OH	H	H	H
A		H	OH	H	H	H	H	OH	H	H
A		H	OH	H	H	H	H	H	OH	H
A		H	H	OH	H	H	H	H	OH	H
A		H	H	H	OH	H	H	H	OH	H
A		H	OCH_3	H	OCH_3	H	H	H	H	H
A		H	H	OCH_3	OCH_3	H	H	H	H	H
A		H	H	H	H	H	H	H	H	H
A		H	OH	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	OCH_3
A		H	OH	H	OCH_3	H	H	H	OH	H
A		H	OH	H	H	H	H	H	H	H
A		H	H	H	H	H	H	H	OH	H
A		H	OH	H	H	H	H	OCH_3	H	H
A		H	H	OCH_3	H	H	H	H	OH	H
A		H	H	H	OCH_3	H	H	H	OH	H
A		H	OH	H	OH	H	H	OH	OH	H
A		H	H	OCH_3	H	H	H	H	H	H
A		H	OH	OCH_3	OH	H	H	OH	OH	H
A		H	OH	OH	OH	H	H	H	OH	H
A		H	OH	H	OH	H	H	OH	OH	OH
A		H	OH	H	OH	H	OH	H	H	H
A		H	OH	H	H	H	H	OH	OH	H
A		H	H	H	OH	H	H	OH	OH	H
A		H	OCH_3	H	OCH_3	H	H	H	OCH_3	H
A		OH	OH	H	OH	H	OH	H	H	H
A		OH	H	H	OH	H	H	OH	OH	H
A		OH	H	H	H	H	H	H	H	H
A		OH	OH	H	OH	OH	H	OH	OH	H
A		OH	OH	H	OH	OH	H	H	OH	H
A		OH	OH	H	OH	H	H	OCH_3	OH	H
A		OH	OH	H	OH	H	H	H	OH	H
A		OH	H	OCH_3	H	H	H	H	H	H
A		OH	OH	H	OH	H	OH	H	OH	H
A		OH	OH	H	OH	H	H	OH	OH	OH
A		OH	OH	OH	OH	H	H	OH	OH	H
A		OH	OH	H	OH	H	H	OH	OH	H

Compound	No	Activity hTAS2R		Threshold hTAS2R		EC_{50} hTAS2R	
		14	39	14	39	14	39
3,6,3',4'-Tetrahydroxyflavone	41	+/-	+	8	2	n.d.	n.d.
3,7,4'-Trihydroxyflavone	42	+	+	1	0.5	n.d.	n.d.
Flavanones							
Eriodictyol[a]	43	+	+	32	16	61.4	62.0
Flavanone	44	+	+/-	32	n.a.	n.d.	n.d.
Hesperitin	45	+/-	+/-	16	8	n.d.	n.d.
Homoeriodictyol[b]	46	+	+	32	32	63.9	84.9
4'-Hydroxyflavanone[c]	47	-	+/-	n.a.	n.a.	n.d.	n.d.
Liquiritigenin	48	+	+	32	16	59.2	64.5
6-Methoxyflavanone	49	+/-	-	n.a.	n.a.	n.d.	n.d.
Naringenin	50	+	+	16	8	36.2	32.9
Pinocembrin	51	+	+	8	4	39.1	48.9
Flavanonols							
Fustin	52	+	+	500	250	n.d.	n.d.
Silibinin[c]	53	+	+	8	8	56.1	99.2
(+)-Taxifolin	54	+	+	63	125	n.d.	n.d.
Flavanols							
(+)-Catechin[c]	55	+	+	500	250	n.d.	n.d.
(-)-Epicatechin (EC)	56	+/-	+	500	250	n.d.	n.d.
(-)-Epicatechin gallate (ECG)	57	+	+	125	32	n.d.	151
(-)-Epigallocatechin[d] (EGC)	58	-	+	n.a.	500	n.d.	n.d.
(-)-Epigallocatechin gallate[b] (EGCG)	59	+	+	250	32	n.d.	161
Chalcones[e]							
Butein	60	+	+	16	125	n.d.	n.d.
Chalcone	61	+	+/-	32	n.a.	n.d.	n.d.
3,2'-Dihydroxychalcone	62	+/-	+	8	8	24.5	53.6
4,2'-Dihydroxychalcone	63	+/-	+/-	n.a.	n.a.	n.d.	n.d.
2',4'-Dihydroxychalcone	64	+/-	+/-	n.sp.	n.sp.	n.d.	n.d.
Eriodictyolchalcone[b]	65	+	+	32	16	40.7	55.5
4'-Hydroxychalcone	66	+/-	+/-	16	n.sp.	n.d.	n.d.
Isoliquiritigenin	67	+/-	+/-	16	16	n.d.	n.d.
2,2',4'-Trihydroxychalcone	68	+	+	8	2	n.d.	n.d.
4,2',5'-Trihydroxychalcone[d]	69	+	+	125	2	n.d.	n.d.
Dihydrochalcones[e]							
Phloretin[a]	70	+	+	16	8	30.2	41.3
Anthocyanidins							
Cyanidin chloride[b]	71	+	+	250	32	n.d.	187
Pelargoninidin chloride	72	+	+	63	32	n.d.	n.d.
Deoxyanthocyanidins							
Apigeninidin chloride[d]	73	-	-	n.a.	n.a.	n.d.	n.d.

Back-bone	R_1	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
A		OH	H	OH	H	H	H	OH	OH	H
A		OH	H	H	OH	H	H	H	OH	H
B		H	OH	H	OH			OH	OH	
B		H	H	H	H			H	H	
B		H	OH	H	OH			OH	OCH_3	
B		H	OH	H	OH			OCH_3	OH	
B		H	H	H	H			H	OH	
B		H	H	H	OH			H	OH	
B		H	H	OCH_3	H			H	H	
B		H	OH	H	OH			H	OH	
B		H	OH	H	OH			H	H	
B		OH	H	H	OH			OH	OH	
B, d		OH	OH	H	OH			**d**	**d**	
B		OH	OH	H	OH			OH	OH	
C		OH	OH		OH			OH	OH	H
C		OH	OH		OH			OH	OH	H
C, e		**e**	OH		OH			OH	OH	H
C		OH	OH		OH			OH	OH	OH
C, e		**e**	OH		OH			OH	OH	OH
D										
D	H		OH		OH	H	H	OH	OH	
D	H		H		H	H	H	H	H	
D	H		OH		H	H	H	OH	H	
D	H		OH		H	H	H	H	OH	
D	H		OH		OH	H	H	H	H	
D	OH		OH		OH	H	H	OH	OH	
D	H		H		OH	H	H	H	H	
D	H		OH		OH	H	H	H	OH	
D	H		OH		OH	H	OH	H	H	
D	H		OH		H	OH	H	H	OH	
E	OH		OH		OH	H	H	H	OH	
F		OH	OH		OH			OH	OH	
F		OH	OH		OH			H	OH	
F		H	OH		OH			H	OH	

Compound	No	Activity hTAS2R		Threshold hTAS2R		EC_{50} hTAS2R	
		14	39	14	39	14	39
Isoflavones							
Acetyl genistin[b,c,d,f]	74	-	+	n.a.	125	n.d.	n.d.
Biochanin A[f]	75	+	+	63	500	n.d.	n.d.
Daidzein[f]	76	+	+	500	500	n.d.	n.d.
Daidzin[f]	77	+/-	+/-	n.a.	n.a.	n.d.	n.d.
7,4'-Dimethoxy-5-hydroxyisoflavone[d]	78	-	-	n.a.	n.a.	n.d.	n.d.
7,4'-Dimethoxy-isoflavone	79	+	-	500	n.a.	n.d.	n.d.
Formomonetin[c,f]	80	+	+	500	500	n.d.	n.d.
Genistein[a,b,f]	81	+	+	4	8	28.9	49.4
Genistin[f]	82	-	+	n.a.	500	n.d.	n.d.
Glycitein[f]	83	+	+	500	500	n.d.	n.d.
Glycitin[f]	84	-	+	n.a.	500	n.d.	n.d.
7-Hydroxyisoflavone[f]	85	+	+	32	250	n.d.	315
7-Hydroxy-6-methoxyisoflavone[f]	86	+/-	+/-	n.sp.	n.sp.	n.d.	n.d.
Isoflavone[d,f]	87	+	-	250	n.a.	n.d.	n.d.
Malonyl genistin[f]	88	-	+	n.a.	500	n.d.	n.d.
Prunetin[f]	89	+	+/-	16	n.sp.	n.d.	n.d.
6,7,4'-Trihydroxyisoflavone[f]	90	+	+	250	250	n.d.	n.d.
7,8,4'-Trihydroxyisoflavone[f]	91	+	+	63	63	124	184
7,3',4'-Trihydroxyisoflavone[f]	92	+	+	250	250	n.d.	n.d.
Isoflavans							
Equol[a,f]	93	+/-	+/-	8	32	47.2	55.8
Coumestans[e]							
Coumestrol[f]	94	+	+	250	250	n.d.	n.d.
Stilbenes[e]							
Resveratrol	95	+	+	16	63	30.3	109
Aurones[e]							
Sulfuretin[b]	96	+	+	16	16	21.5	48.0
Xanthones							
Xanthone[d]	97	+	+	250	500	n.d.	n.d.

[a] training set used for pharmacophore building of hTAS2R14.
[b] training set used for pharmacophore building of hTAS2R39.
[c] test set used for fingerprint model of hTAS2R14.
[d] test set used for fingerprint model of hTAS2R39.
[e] to facilitate comparison of position of substitution with the majority of the flavonoids, the residue numbering for chalcones, coumestans, stilbenes, and aurones, the usually applied residue numbering was adapted.
[f] published previously ***(7)***.

Back-bone	R_1	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
G, a			OH	H	**a**	H		H	OH	
G			OH	H	OH	H		OH	OCH_3	
G			H	H	OH	H		H	OH	
G, c			H	H	**c**	H		H	OH	
G			OH	H	OCH_3	H		H	OCH_3	
G			H	H	OCH_3	H		H	OCH_3	
G			H	H	OH	H		H	OCH_3	
G			OH	H	OH	H		H	OH	
G, c			OH	H	**c**	H		H	OH	
G			H	OCH_3	OH	H		H	OH	
G, c			H	OCH_3	**c**	H		H	OH	
G			H	H	OH	H		H	H	
G			H	OCH_3	OH	H		H	H	
G			H	H	H	H		H	H	
G, b			OH	H	**b**	H		H	OH	
G			OH	H	OCH_3	H		H	OH	
G			H	OH	OH	H		H	OH	
G			H	H	OH	OH		H	OH	
G			H	H	OH	H		OH	OH	
H					OH				OH	
I					OH				OH	
J			OH		OH				OH	
K					OH			OH	OH	
L										

n.a., not active up to 500 µM.
n.d., not determined.
n.sp., not specific, means that activity could not be determined due to non-specific signals in the non-induced cells.

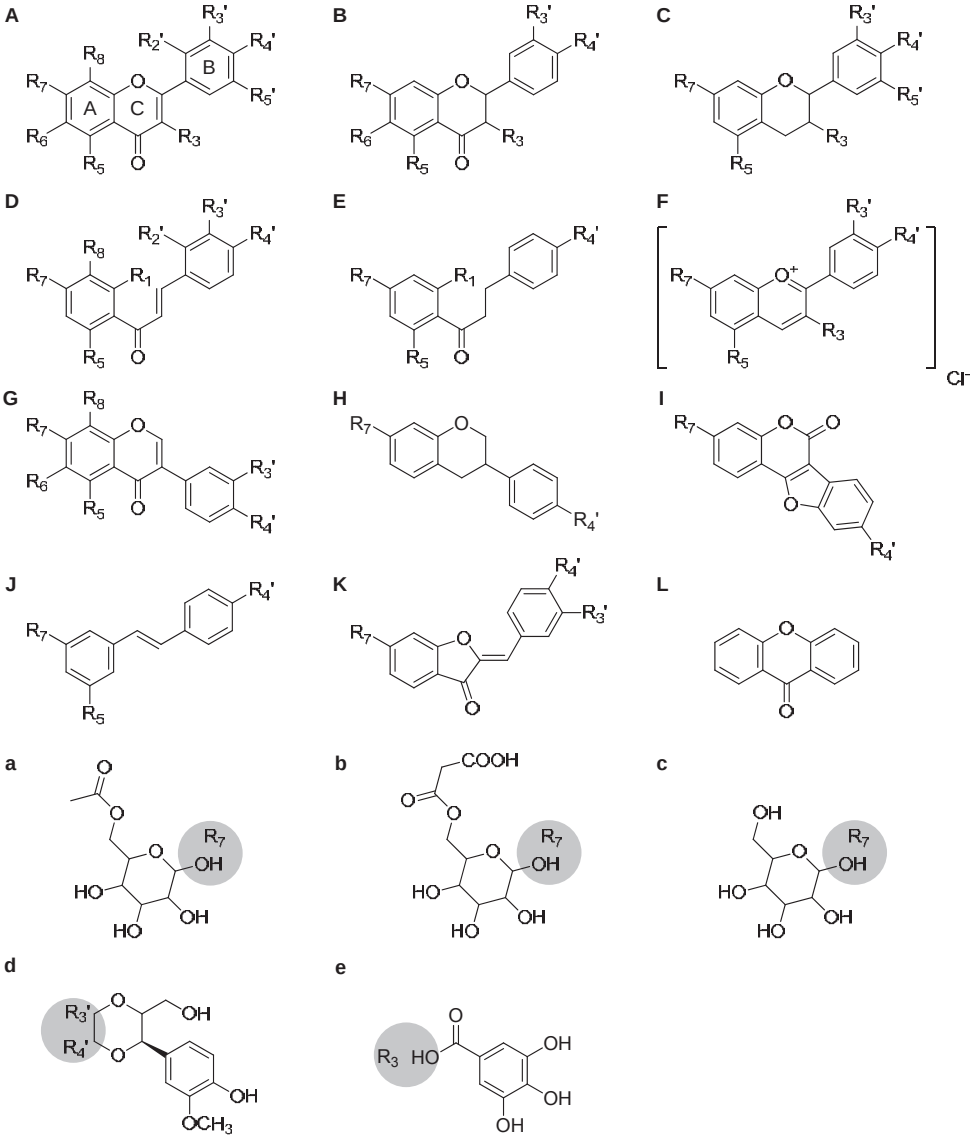

Figure 1. Backbone structures (A-L) and larger substituents (a-e).

them as active. Quality of mapping and correctness of predictions were analyzed with a heat map (**supplementary material S15-S16**).

RESULTS

Compound selection and qualitative screening for hTAS2R activation

Ninety seven compounds were screened for activation of bitter receptors hTAS2R14 and hTAS2R39, based on variation in backbone structure and their substitution pattern (**Table 1 and Figure 1**). One example is shown in **Figure 2A**. 6,7-Dimethoxyflavone (**11**) clearly activated hTAS2R14, whereas it did not activate hTAS2R39. All test compounds were categorized into "+", "-", and "+/-", depending on their extent of receptor activation. The latter designation was given when results were ambiguous even after three repetitions in duplicate. *In vitro* results identified that 60 compounds activated hTAS2R14 and 67 compounds activated hTAS2R39 at the screening concentration of 500 μM. In contrast, 15 compounds did not activate hTAS2R14, whereas 9 compounds did not activate hTAS2R39. The remaining compounds could not be unambiguously classified at the screening concentration (**Table 1**).

Dose-response behavior of selected (iso)flavonoids on hTAS2R14 and hTAS2R39

All (iso)flavonoids (or similar) that activated one or both of the two bitter receptors at the screening concentration were measured at different concentrations in order to establish dose-response curves, the threshold and EC_{50} values of which are summarized in **Table 1**. Their threshold values varied over a range of three log units between 0.12 and 500 μM.

Although many compounds activated both bitter receptors, the threshold concentrations for each individual receptor sometimes varied. For example, resveratrol (**95**) (**Figure 2B**) had a threshold value with hTAS2R14 of 16 μM and an EC_{50} value of 30.3 μM, whereas with hTAS2R39 this was 63 μM and 109 μM, respectively. An even larger difference in bitter receptor activation was observed for epigallocatechin gallate (**59**) (**Figure 2C**), with a threshold of 32 μM and an EC_{50} of 161 μM for hTAS2R39, and a threshold of 250 μM for hTAS2R14. The EC_{50} value for hTAS2R14 could not be calculated. Other compounds showed almost identical behavior towards both bitter receptors. For example, scutellarein (**23**; **Figure 2D**) activated both receptors from 8 μM onwards with EC_{50} values of 35.0 and 40.3 μM for hTAS2R14 and hTAS2R39, respectively.

The influence of small structural changes on bitter receptor activation

Small structural changes had different effects on receptor activation. A few examples for hTAS2R39 are shown in **Figure 3**. Phloretin (**70**) and naringenin (**50**) have the same A- and B-ring substitutions, but differ in an open and closed C-ring, respectively (**Figure 3A**). Nevertheless, both compounds have the same threshold of 8 μM, and differ only slightly in EC_{50} values (41.3 μM for (**70**) and 32.9 μM for (**50**)). In this case, the change of C-ring structure had a small effect. In contrast, eriodictyolchalcone (**65**) and luteolin (**20**), differing

similarly in C-ring configuration, showed different behavior in receptor activation (**Figure 3B**). The thresholds were 16 µM for (**65**) and 0.5 µM for (**20**), whereas the EC_{50} values were 55.5 µM for (**65**) and 7.3 µM (**20**).

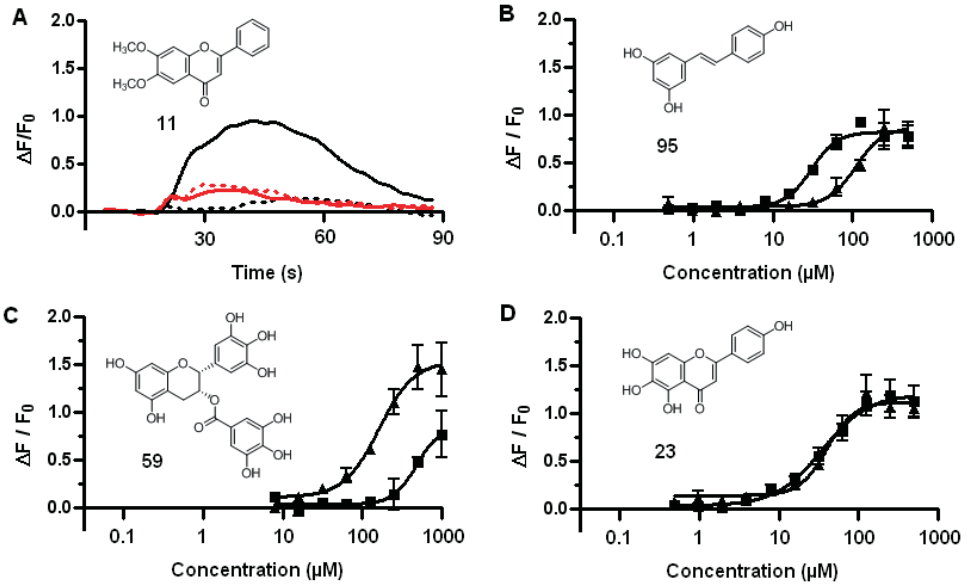

Figure 2. Effect of 500 µM 6,7-dimethoxyflavone (**11**) on hTAS2R14 (—) and hTAS2R39 (—), and on the corresponding non-induced cells, not-expressing the bitter receptors (- - -) and (- - -) (**A**); dose-response curves of resveratrol (**95**) (**B**), epigallocatechin gallate (**59**) (**C**), and scutellarein (**23**) (**D**) on both bitter receptors hTAS2R14 (■) and hTAS2R39 (▲).

In some cases, similar effects were observed for variation in B-ring structure. For example, the structural difference between eriodictyol (**43**) and homoeriodictyol (**46**) did not affect receptor activation (**Figure 3C**). For other compounds, a change in B-ring substituents had an effect, *e.g.* comparing luteolin (**20**) (two OH-groups on the B-ring, highly active) to tricetin (**24**) (three OH-groups on the B-ring, threshold 250 µM, curve not shown). **Figure 3D** compares three flavones that vary in A-ring substitution: 3',4',7-trihydroxyflavone (**27**), 6-methoxyluteolin (**22**), and luteolin (**20**) with effects on threshold (16 µM, 8 µM, and 0.5 µM, respectively), and EC_{50} values (141 µM, 22.9 µM, and 7.3 µM, respectively).

There was no obvious universal relation between structural denominator and activity. Considering the large number of compounds tested, modeling was employed to detect structure/activity relationships.

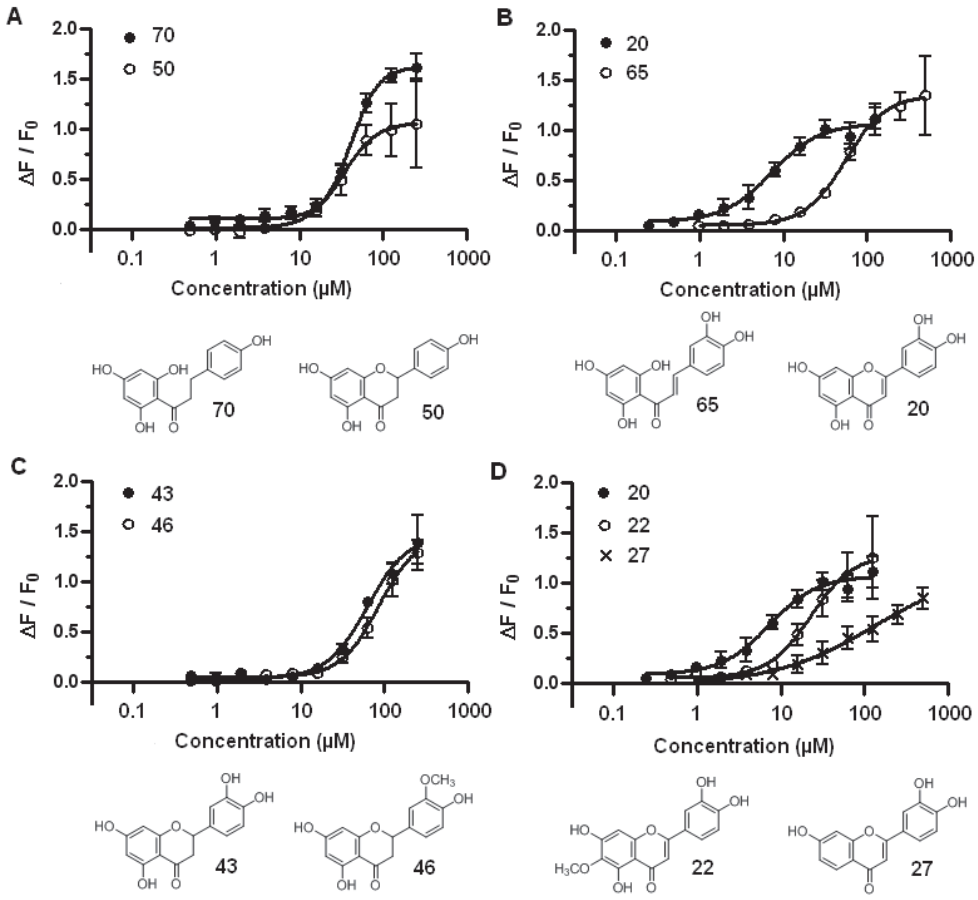

Figure 3. Comparison of dose-response curves on hTAS2R39 of phloretin (**70**) and naringenin (**50**) (**A**), luteolin (**20**) and eriodictyolchalcone (**65**) (**B**), eriodictyol (**43**) and homoeriodictyol (**46**) (**C**), and 3',4',7-trihydroxyflavone (**27**), 6-methoxyluteolin (**22**), and luteolin (**20**) (**D**).

2D-fingerprint modeling

The best separation between active and inactive compounds was achieved by the ECFP-10 and ECFP-8 fingerprint models for hTAS2R14 and hTAS2R39, respectively. **Figure 4** displays the predictions for the complete set of training and test molecules used for modeling. The ECFP-10 model for hTAS2R14 agonists predicted 93% of all compounds correctly. Two compounds were falsely predicted as active and three compounds were falsely predicted as inactive. The ECFP-8 model for hTAS2R39 agonists predicted 96% of all compounds correctly. One compound was falsely predicted as active and two compounds were falsely predicted as inactive.

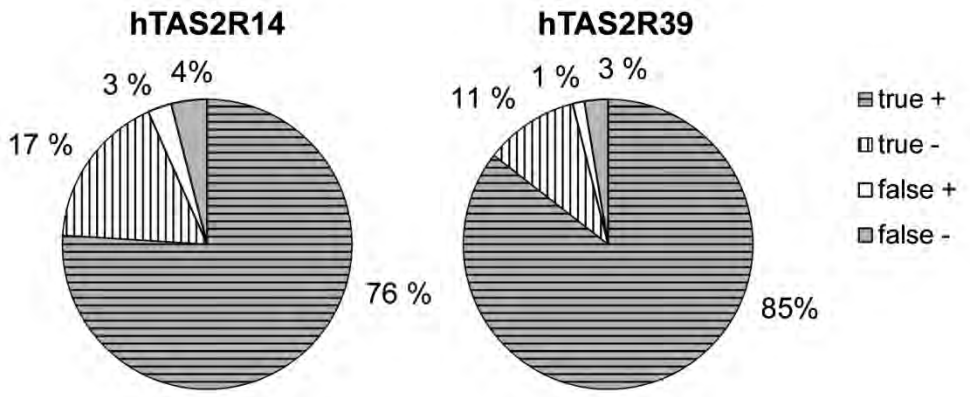

Figure 4. Predictions of 2D-fingerprint models with true positives, true negatives, false positives, and false negatives (in percentage) for hTAS2R14 (n=75) and hTAS2R39 (n=76) ligands.

In order to identify key molecular features involved in bitter receptor activation, "good" and "bad" fingerprint fragments were established (see **supplementary material S5-S9**), of which a selection of four illustrative fragments is shown here for the hTAS2R14 model (**Figure 5**). Based on the fragments it was observed that the flavanones and isoflavones were likely to trigger hTAS2R14. Flavones could activate this receptor as well, but there were fragments within some flavones assigned to the "bad" fragments, implying that some flavones caused less or no activation. Methoxylation and glycosylation seemed to impair activation of this receptor. Receptor hTAS2R39 had a high probability of activation by flavanones and flavonols and showed a similar behavior towards flavones as compared to hTAS2R14. Methoxylation fragments were not beneficial for activation by isoflavonoids. Glycosylation was not an obvious bad feature for this receptor. The fingerprint fragments helped in understanding which molecular features were obviously favorable and unfavorable for receptor activation were, but it was not possible to develop a most common substructure.

In conclusion, the 2D-models possessed excellent predictive value for identification of bitter (iso)flavonoids activating hTAS2R14 or hTAS2R39, but did not give sufficient insight into understanding of the general molecular signature involved in bitter receptor activation of (iso)flavonoids. Therefore, 3D modeling was used as a next step in understanding which chemical characteristics influence bitter receptor interaction.

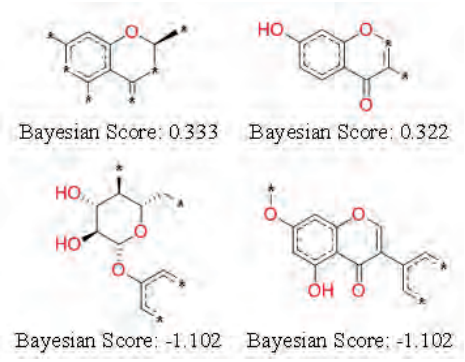

Figure 5. Selected "good" (positive Bayesian score) and "bad" (negative Bayesian score) fingerprint fragments for hTAS2R14.

3D-pharmacophore modeling

The structural requirements for (iso)flavonoids to activate hTAS2R14 were best described by a 5-feature pharmacophore (**Figure 6A**), of which all five features had to be mapped. It comprised two hydrogen donor features, one hydrogen acceptor feature, one "hydrophobic aromatic" feature, and one "ring aromatic" feature. The ROC-plot area under the curve value was of 0.751 (for confusion matrices and ROC plots, see **supplementary material S11-S14**). This model was able to correctly predict the activation or absence of activation of two thirds of the ligands and performed better for highly active than for moderately active compounds (the division into threshold groups is shown in **Table 2)**. Of the highly active compounds, 81% was predicted correctly, but for the moderately active compounds this was 52%. Two thirds of the inactive compounds were predicted correctly. The reason behind and remedy for incorrect predictions will be discussed in another section.

The best results for modeling the activation of hTAS2R39 by (iso)flavonoids were achieved with a 6-feature pharmacophore (shown in **Figure 6B)**, which allowed the ligands to map five out of six features. It comprised three hydrogen donor features, one hydrogen acceptor feature, one "hydrophobic aromatic" feature, and one "ring aromatic" feature. The ROC-plot area under the curve value was 0.873, while returning 9 false positives and 3 false negatives. This model was able to map 84% of the compounds correctly. Divided into threshold groups, 94% of the highly active compounds, 75% of the moderately active compounds, and 79% of the inactive compounds were predicted correctly.

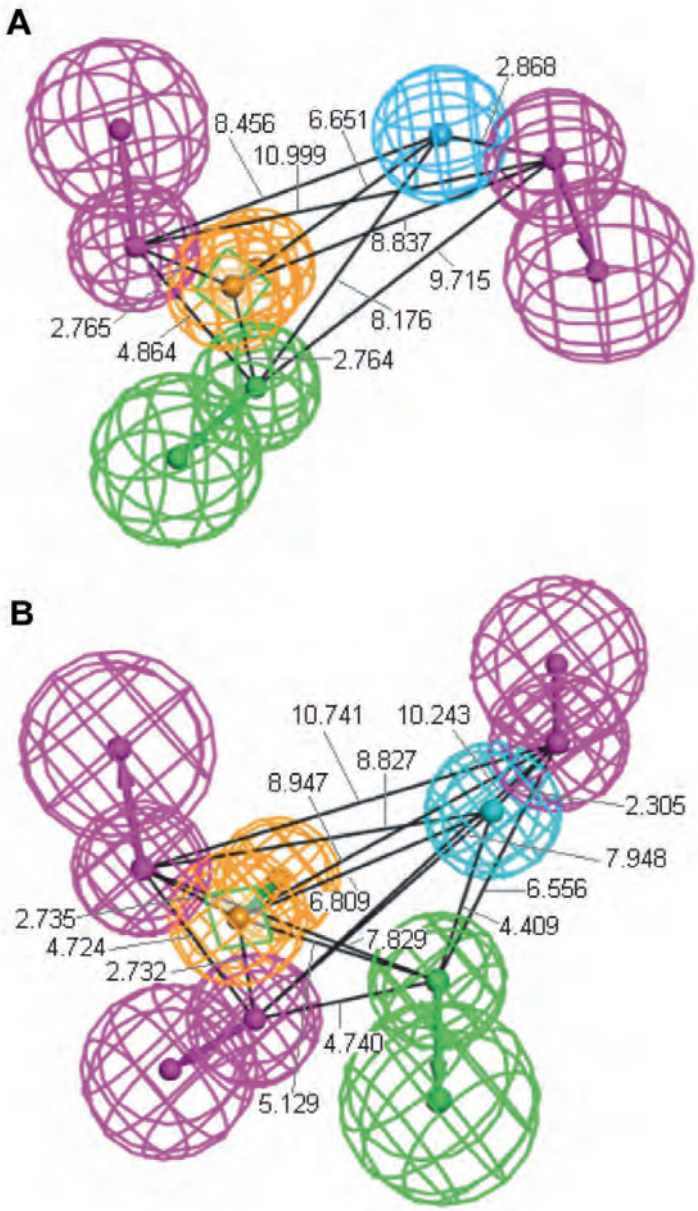

Figure 6. Pharmacophores for hTAS2R14 (**A**) and hTAS2R39 (**B**). The colors of the spheres represent the following features: pink - hydrogen donors, green - hydrogen acceptors, blue - hydrophobic aromatic, and yellow - ring aromatic. The green rectangle represents a plain within a ring structure. The small spheres indicate the presence of the feature on the ligand and the large spheres indicate the possible position of the amino acids on the receptor, interacting with this feature. The direction of interaction is shown with an arrow. The numbers represent the distance between the features in Ångström.

Visualization of mapped molecules

In **Figure 7A**, the pharmacophore for hTAS2R14 ligands is shown including the mapped molecule luteolin (**20**). The features of the pharmacophore almost precisely mapped the respective atoms of the flavonoid. This was reflected in an excellent fit value of 4.9, very close to the maximal attainable fit value of 5. The molecule was mapped with a relative energy of 0.14 kcal / mol, meaning that it required little energy to fit the conformation of the molecule into the pharmacophore. Compound **78** (7,4'-dimethoxy-5-hydroxyisoflavone) was unable to activate hTAS2R14, which is illustrated by suboptimal mapping (**Figure 7B**). One hydrogen bond donor feature did not map at all, which is indicated by a darker shade of pink in this feature than when the feature mapped. The structure of 7,4'-dimethoxy-5-hydroxyisoflavone (**78**) was applied with the relative energy 6.4 kcal / mol. So even with rotation, translation, and torsion, and in spite of a reasonable fit value of 2.1, the molecule did not map, in accordance with the compound being inactive. Often, high fit values indicate good mapping, but low fit values do not necessarily mean mismapping of molecules. As long as all features are met somehow, a bad fit value can still lead to activation. This is for example the case for 7,8,4'-trihydroxyisoflavone (**91**), which has a fit value of 0.8, but still maps the features and activates the receptor.

In **Figure 7C**, kaempferol (**35**) is mapped on the pharmacophore for hTAS2R39. The points in the middle of the sphere and the position of the respective atoms were close to each other. The mapping of the ligand was good, which is reflected in a fit value of 5.0 (out of 6 maximum attainable). **Figure 7D** shows an inactive flavonoid on hTAS2R39, namely 4'-hydroxy-6-methoxyflavone (**18**). As shown by the darker shade of pink, two of the three hydrogen donor features did not map at all.

To illustrate the influence of the absence of a C-ring, the mapped molecules for a chalcone (butein (**60**)) and a stilbene (resveratrol (**95**)) are depicted in **Figures 7E** and **7F**, respectively. As a result of the flexibility of butein (**60**), it mapped most of the features of the hTAS2R39 very well, except for the shifted hydrogen bond acceptor feature, resulting in a good fit value of 4.8. Its relative energy used for modeling was very low (0.08 kcal / mol).

Although the aromatic rings of resveratrol (**95**) are in closer proximity of each other than in flavonoids, it mapped the hTAS2R14 pharmacophore with all five features (fit value 3.6, relative energy 0.06 kcal / mol), indicating that the pharmacophore model might also be applicable to compounds that are structurally similar to flavonoids. Thus, a small variation in distance might have minor influence on binding. This is not self-evident, as the length of a molecule can be crucial for receptor activation, which can be observed for estrogen receptor agonists *(30)*.

Furthermore, the C-ring seems to be of less influence for bitter receptor activation than the A-and the B-rings. This is coherent with the observations in our previous study on

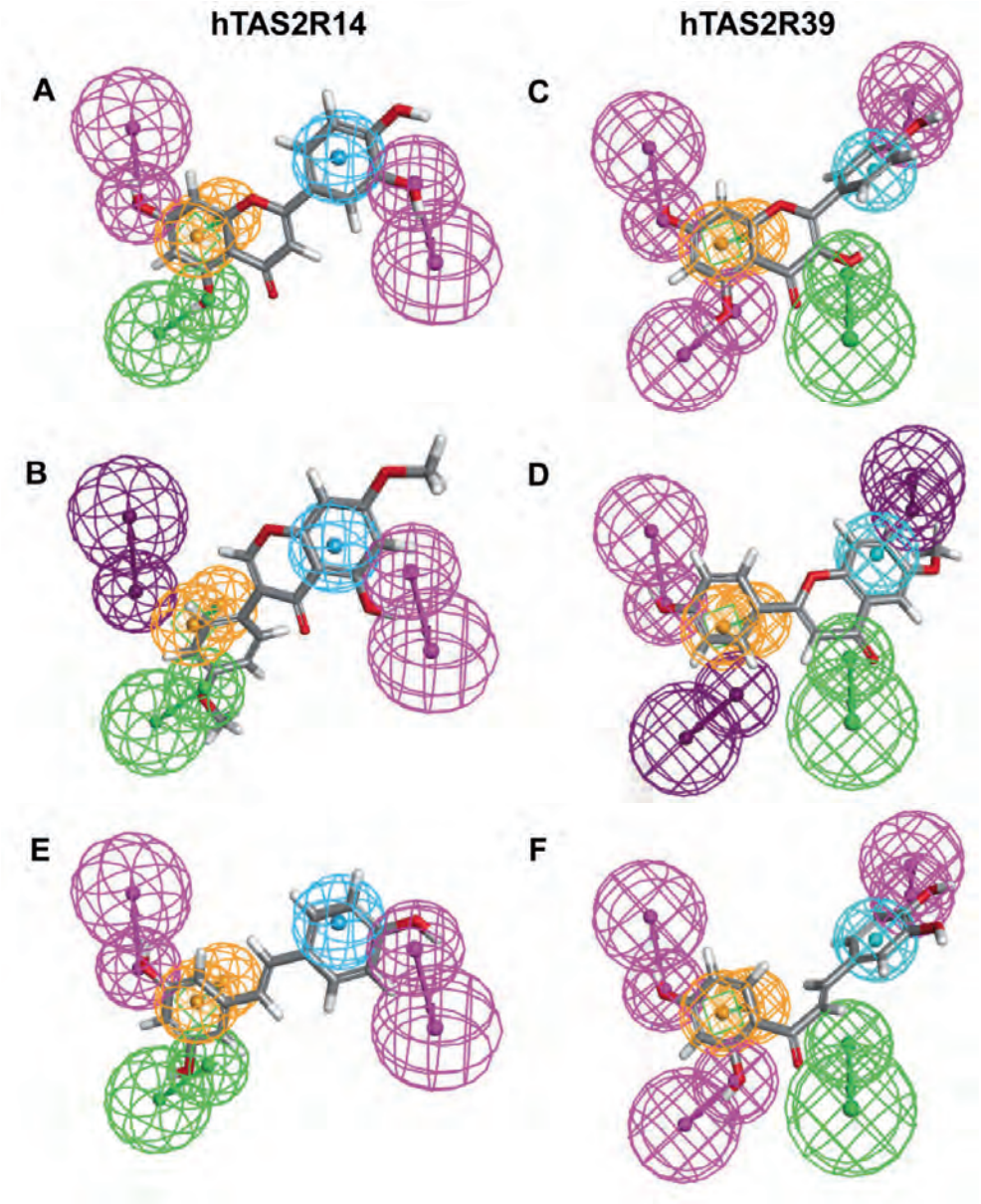

Figure 7. Examples of hTAS2R14 ligand pharmacophores including luteolin (**20**) (**A**), 7,4'-dimethoxy-5-hydroxy-isoflavone (**78**) (**B**), and resveratrol (**95**) (**E**). Examples of hTAS2R39 ligand pharmacophores including kaempferol (**35**) (**C**), 4'-hydroxy-6-methoxy-flavone (**18**) (**D**), and butein (**60**) (**F**).

isoflavonoids *(7)*, showing that the hydroxylation positions on A-and B-rings were more crucial for activation than the exact structure in the center (e.g. C-ring) of the molecule.

DISCUSSION

(Iso)flavonoid agonists of hTAS2R14 and hTAS2R39 and their effects on bitter taste receptors and taste perception

Amongst aglycones, catechins are the most important group of flavonoids occurring in our diet. We identified catechins as agonists of two bitter receptors, hTAS2R14 and hTAS2R39. Previously, only activation of hTAS2R39 was reported *(14)*. On hTAS2R39, we identified thresholds of ECG, EGCG, EC, and EGC, to be 32, 32, 250, and 500 µM, respectively. Previously reported thresholds were between 10 and 30 µM for all four catechins *(14)*. Thus, our thresholds for galloylated catechins were in the same range as the previously reported thresholds, but we observed by a factor of ten higher thresholds for the non-galloylated catechins. The same trend applied to the observed EC_{50} values on hTAS2R39. We obtained EC_{50} values of 150.6 µM and 161.2 µM for ECG and EGCG, respectively, and values for EC and EGC could not be calculated within the suitable concentration range (EC_{50} values reported by Narukawa *(14)* for ECG, EGCG, EGC, EC: 88.2, 181.6, 395.5, 417.7 µM, respectively). In another recent publication *(16)*, EC was identified as an agonist for three bitter receptors: hTAS2R39 (threshold 1 mM, EC_{50} 3.8 mM), hTAS2R4 (threshold 2 mM, EC_{50} 30.2 mM), and hTAS2R5 (threshold 1 mM, EC_{50} 3.2 mM). We additionally identified hTAS2R14 (threshold 500 µM; no EC_{50} determined). Thus, differences are reported between the various bitter receptor assays, which might be caused by differences in experimental conditions. It should also be noted that *in-vitro* threshold values reported in the elevated µM or in the mM range are very high, and, *e.g.* in the case of EC in green tea, are unlikely to contribute to the bitter taste of the product.

Amongst the many new agonists of hTAS2R14 and hTAS2R39 reported in this study, some compounds were known as bitter before (*e.g.* taxifolin, resveratrol), some compounds had unknown taste properties (e.g. synthetic flavonoids), while three compounds were previously reported as bitter taste maskers (homoeriodictyol, eriodictyol and phloretin *(22, 31)*). We identified homoeriodictyol and eriodictyol as agonists of hTAS2R14 (thresholds 32 µM for both compounds) and hTAS2R39 (thresholds 32 µM and 16 µM, respectively). These two compounds were reported to mask bitterness of caffeine in sensory tests without exhibiting strong taste characteristics at 100 ppm *(31)*. The molecular mechanism of masking by homoeriodictyol and eriodictyol requires further clarification. For phloretin, it was reported that its masking activity (50 mg / L (= 182 µM)) for caffeine in sensory tests competed with its bitterness observed at elevated concentrations (100 mg / L (= 365 µM)) *(22)*. It was suggested that this might be an overlapping effect of antagonistic and agonistic

activity, as described for sesquiterpene lactones *(32)*. We identified phloretin as agonists of hTAS2R14 and hTAS2R39 (thresholds 16 µM and 8 µM, respectively). Thus, the bitterness of phloretin could be caused by activation of hTAS2R14 and hTAS2R39, and possibly other receptors. Remarkably, the bitterness threshold of phloretin *in-vivo* was ~20-40 fold higher than *in-vitro*. It has been observed before *(10)* that bitter compounds (hop acids) can display higher sensory than receptor thresholds. In the case of hop acids, this has been ascribed to their interaction with the oral mucosa. A bitter receptor assay might thus overestimate bitterness, as interaction with mucosa or other *in-vivo* conditions is not accounted for. Nevertheless, overestimation is not necessarily the case, as similar *in-vivo* and *in-vitro* thresholds have been reported for β-D-glucopyranosides *(33)* and tea catechins *(14)*.

Evaluation of the 2D and 3D models

Even though they only operated with planar compound structures, the 2D-fingerprint models were very effective in predicting active compounds, and discriminating them from inactive compounds, leading to low numbers of false predictions. This might be due to the relatively planar nature of many (iso)flavonoids. The 2D-fingerprint models can be used as a quick *in silico* screening tool in compound library screening in order to identify (iso)flavonoid (or similar) compounds that might taste bitter. Due to lower complexity, they are more suitable than the 3D-pharmacophore models for screening large compound databases. On the contrary, 2D-fingerprint models created only a partial understanding of the molecular features involved in bitter receptor activation. The 3D-pharmacophore models provided a broader insight into (iso)flavonoid bitter receptor interaction. The structural characteristics for an (iso)flavonoid to activate hTAS2R14 (or hTAS2R39), were determined to be composed of two (or three) hydrogen bond donor sites, one hydrogen bond acceptor site, one hydrophobic ring structure and one aromatic ring structure.

The receptor activation threshold values lie within a range of three orders of magnitude, which is generally considered as a relatively small range for quantitative structure activity relationship (QSAR) modeling. In the attempt of establishing a QSAR model, a clear correlation between features and threshold could not be determined. But even without a quantitative prediction, the pharmacophore models are a powerful tool in prediction of potential bitterness.

Explanation of false predictions by 3D-pharmacophore models

Several compounds were falsely predicted as positive or negative by the 3D-pharmacophore models (see **Table 2**).

Table 2. Classification of ligands into threshold groups highly active (≤32 µM), moderately active (>32 µM - 500 µM), and inactive (not active up to 500 µM). For 3D-pharmacophore modeling, the number of ligands per threshold group are given for hTAS2R14 and hTAS2R39, and subdivided by their model prediction into true / false positives / negatives. Several compounds measured were not included into modeling.

Activity	hTAS2R14					hTAS2R39				
	Total	True		False		Total	True		False	
		+	-	+	-		+	-	+	-
High	27	22	-	-	5	35	33	-	-	2
Moderate	23	12	-	-	11	28	21	-	-	7
Inactive	23	-	15	8	-	14	-	11	3	-

The highly active false negatives, *i.e.* the actives from the cell assay which did not map to the pharmacophores, had a lack of substituents on the B-ring of the (iso)flavonoid in common. In most cases, as shown in **Figure 8A** for pinocembrin (**51**), the donor feature next to the hydrophobic aromatic feature in the pharmacophore for hTAS2R14 ligands could not be mapped by these molecules. Consequently, the model classified these molecules as inactive. The pharmacophore model for hTAS2R39 ligands was able to recognize more of these unsubstituted ligands, probably due to the mapping setting which allowed the ligands to miss one feature. The question arises whether the donor feature next to the hydrophobic aromatic group might be irrelevant or of less importance for binding. Another explanation could be that a second mode of binding to the receptor for ligands without substitution on the B-ring occurs. Thus, the current pharmacophore models are limited to (iso)flavonoids that contain two (for hTAS2R14) or at least one (for hTAS2R39) substituted aromatic ring(s). Nevertheless, naturally occurring (iso)flavonoids are mostly substituted (exceptions are *e.g.* pinocembrin and chrysin), and therefore applicable to the model.

False positives, *i.e.* molecules which mapped to the pharmacophore(s), even though they did not activate the receptor(s), were obtained as well. Only three compounds were falsely predicted as positive for hTAS2R39, and eight compounds for hTAS2R14. Out of these eight, five were isoflavones glucosylated on position 7 of the A-ring (example given for **74** in **Figure 8B**), which might cause steric hindrance for optimal receptor binding. In order to prevent these false positive predictions, excluded volumes might be added to the pharmacophore model for receptor hTAS2R14, as shown in **Figure 8C**. However, this option should only be used for isoflavones, as structures with other backbones have not been tested. Furthermore, since only the glucoside substitutions on position 7 typical for isoflavones were tested, no statement can be made about other glycosylation positions frequently occurring in flavonoids, such as position 3. Thus, the resulting model for hTAS2R14 ligands is limited to aglycones, while the model for hTAS2R39 ligands can be used for certain glycosides as well. An additional reason for false positive prediction might be a solubility issue. Some compounds (for example **1** and **14**) had limited solubility at high

concentrations, thus their real potential to act as bitter compound might have been underestimated.

In conclusion, the prediction of the generated pharmacophore models for activation of receptors hTAS2R14 and hTAS2R39 by (iso)flavonoids was successful, and most false positive and false negative predictions could be explained, leading to an understanding of 88% of hTAS2R14 ligands, and 94% of hTAS2R39 ligands within this study. The combination of *in vitro* and *in silico* data created a good insight in activation of hTAS2R14 and hTAS2R39 by (iso)flavonoids.

Comparison of ligand-based pharmacophore models for hTAS2R14 and hTAS2R39

The difference between the two pharmacophore models indicated that despite the largely overlapping ligands of both receptors, there was a difference in the molecular structure and substitution pattern of ligands recognized by receptor hTAS2R14 and hTAS2R39. The additional donor feature of the pharmacophore model for hTAS2R39 ligands indicated the possible presence of another complementary acceptor site in the binding pocket of hTAS2R39 compared to hTAS2R14. This might explain why the OH-rich compounds showed different behavior on the two bitter receptors, as can be observed for compounds **24**, **32**, and **58**, which activate only hTAS2R39, but not hTAS2R14, and for compounds **38**, **39**, **57**, and **59,** which have a much higher activity on hTAS2R39 than on hTAS2R14. Another explanation might be that the hydrophobic aromatic feature, and therewith hydrophobic interaction with the binding site, is of higher importance in hTAS2R14 than in hTAS2R39, as compounds with three OH groups on the B-ring (**24**, **38, 58**, and **59)** have a decreased hydrophobicity on this aromatic ring and no or decreased activity on hTAS2R14.

Both pharmacophore models have two donor features which seem to be equally relevant for discrimination between the active and inactive ligands. The aromatic features and the acceptor feature did not seem to have a strong discriminator function, but they might play a role in ligand alignment.

Tuning breadth of bitter taste receptors

To date, hTAS2R14 has been regarded as a more broadly tuned receptor, compared to hTAS2R39 *(9)*. This idea was based on the activation of hTAS2R14 by structurally very diverse compounds, and the fact that fewer agonists for hTAS2R39 were known. During recent years, numerous new agonists have been reported for hTAS2R39 *(7, 14, 15, 17)*, including the large number of flavonoids from the present study, which exceeds the number of hTAS2R39 agonists known before. Hence, hTAS2R39 reveals to be activated by many more agonists than initially thought. Also the number of hTAS2R14 agonists keeps increasing (*(7, 13)* and this study).

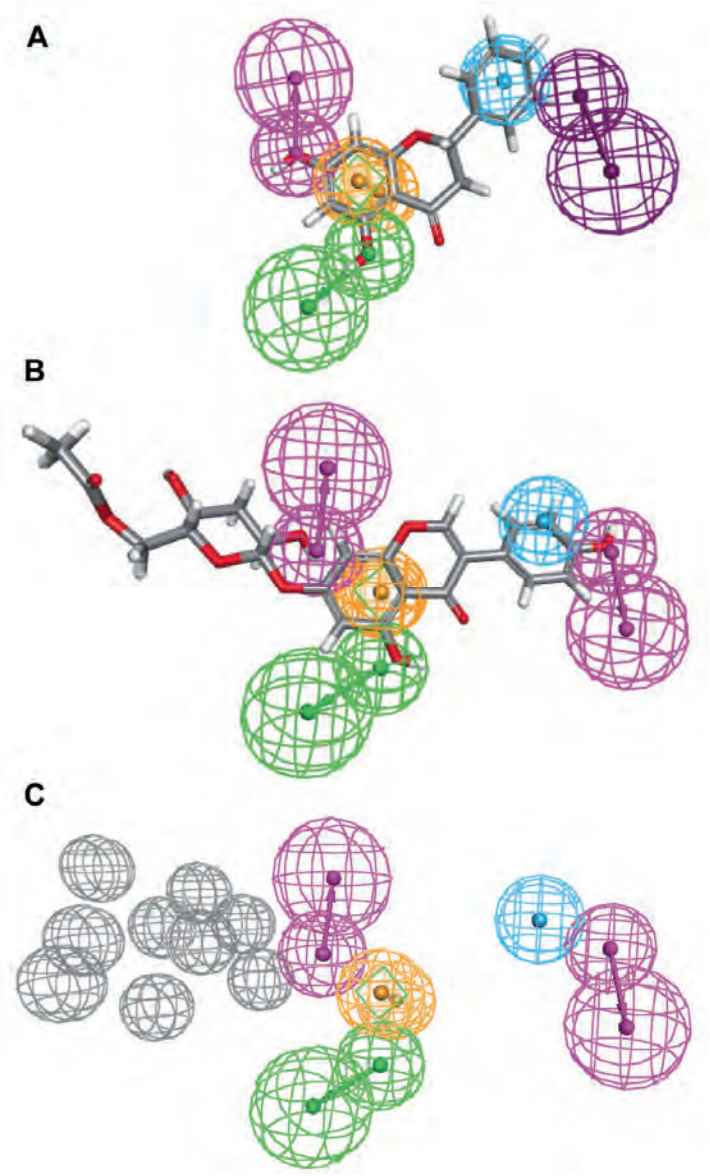

Figure 8. The falsely negative predicted compound pinocembrin (**51**) on the hTAS2R14 ligand pharmacophore (**A**); the falsely positive predicted compound acetyl genistin (**74**) on the hTAS2R14 ligand pharmacophore (**B**); excluded volumes on the hTAS2R14 ligand pharmacophore (**C**).

With time, the number of hTAS2R agonists will grow further, and it is questionable whether tuning breadth should simply be related to the number of bitter receptor agonists. Our present results show that the substitution pattern of (iso)flavonoids was of higher importance for receptor activation than their backbone structure (***e.g.*** whether the agonist is a flavone or a flavanone). Our pharmacophore modeling revealed, which signatures underlay binding to hTAS2R14 and -39. Therefore, it might be better to describe tuning breadth of bitter receptors in terms of the number of molecular signatures recognized by the receptor. In this way, a collection of molecules with similar signature will only count as one with respect to tuning breadth, and tuning breadth is less likely to be overestimated.

In conclusion, this study identified many flavonoids as intrinsically bitter, and elucidated the structural requirements for bitterness of (iso)flavonoids. Understanding their "bitter motif" might prevent the introduction of bitter taste in the design of functional foods enriched in (iso)flavonoid bioactives.

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SUPPORTING INFORMATION

Table S1. Fit values and relative energies of tested compounds with number codes and SMILES mapped to the pharmacophore models for hTAS2R14 and hTAS2R39.

No.	Fit Value		Relative Energy		SMILES
	14	39	14	39	
1	3,98	3,93	0,92	9,86	O=C2C1=C(O)C=C(O)C=C1OC(C3=CC=C(OC)C=C3)=C2
2	3,14	3,93	0,04	0,07	O=C2C1=C(O)C=C(O)C=C1OC(C3=CC=C(O)C=C3)=C2
3	3,98	3,35	0,00	0,98	O=C2C1=C(O)C=C(O)C=C1OC(C3=CC=CC=C3)=C2
4	3,93	4,38	6,34	2,64	O=C2C1=C(O)C=C(O)C=C1OC(C3=CC=C(O)C(OC)=C3)=C2
5	2,83	3,19	3,49	0,00	O=C2C1=C(O)C=CC=C1OC(C3=CC=CC=C3O)=C2
6	3,97	2,57	0,05	2,04	O=C2C1=C(O)C=CC=C1OC(C3=CC=CC(O)=C3)=C2
7	2,13	3,25	0,09	2,18	O=C2C1=C(O)C=CC=C1OC(C3=CC=C(O)C=C3)=C2
8	1,77	3,02	0,00	0,00	O=C2C1=CC(O)=CC=C1OC(C3=CC=C(O)C=C3)=C2
9	2,93	3,72	0,03	0,08	OC2=CC1=C(C=C2)C(C=C(C3=CC=C(O)C=C3)O1)=O
10	2,90	2,64	0,00	0,86	O=C1C=C(C3=CC=CC=C3)OC2=C1C(OC)=CC(OC)=C2
11	2,90	2,51	0,00	1,42	O=C1C=C(C3=CC=CC=C3)OC2=C1C=C(OC)C(OC)=C2
12	2,09	2,44	0,00	0,00	O=C2C1=CC=CC=C1OC(C3=CC=CC=C3)=C2
13	1,69	2,74	8,16	0,00	COC1=CC(=CC(=C1OC)OC)C2=CC(=O)C3=C(O2)C(=C(C(=C3O)OC)OC)OC
14	2,54	3,29	9,05	1,61	O=C2C1=C(O)C=C(OC)C=C1OC(C3=CC=C(O)C=C3)=C2
15	3,00	2,47	0,00	0,00	O=C2C1=C(O)C=CC=C1OC(C3=CC=CC=C3)=C2
16	2,27	3,20	0,03	0,04	O=C2C1=CC=CC=C1OC(C3=CC=C(O)C=C3)=C2
17	2,61	2,94	6,30	6,27	O=C2C1=C(O)C=CC=C1OC(C3=CC=CC(OC)=C3)=C2
18	2,25	3,48	0,00	8,81	O=C1C=C(C3=CC=C(O)C=C3)OC2=C1C=C(OC)C=C2
19	3,57	3,47	8,52	7,34	O=C1C=C(C3=CC=C(O)C=C3)OC2=C1C=CC(OC)=C2
20	4,95	4,19	0,14	1,13	O=C2C1=C(O)C=C(O)C=C1OC(C3=CC=C(O)C(O)=C3)=C2
21	2,12	2,64	0,00	0,87	O=C1C=C(C3=CC=CC=C3)OC2=C1C=C(OC)C=C2
22	5,00	4,65	2,58	9,12	O=C1C=C(C3=CC=C(O)C(O)=C3)OC2=C1C(O)=C(OC)C(O)=C2
23	3,09	3,96	0,00	0,73	OC1=C(O)C(O)=C(C(C=C(C3=CC=C(O)C=C3)O2)=O)C2=C1
24	3,98	3,34	0,00	1,12	O=C2C1=C(O)C=C(O)C=C1OC(C3=CC(O)=C(O)C(O)=C3)=C2
25	3,82	0,30	3,49	3,25	O=C2C1=C(O)C=C(O)C=C1OC(C3=CC=CC=C3O)=C2
26	1,72	0,16	0,04	2,13	O=C2C1=C(O)C=CC=C1OC(C3=CC=C(O)C(O)=C3)=C2
27	3,73	2,95	0,03	0,03	O=C1C=C(C3=CC=C(O)C(O)=C3)OC2=C1C=CC(O)=C2
28	2,94	2,69	8,02	7,06	O=C1C=C(C3=CC=C(OC)C=C3)OC2=C1C(OC)=CC(OC)=C2
29	2,90	0,37	2,41	2,41	C1=CC=C(C(=C1)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O

No.	Fit Value		Relative Energy		SMILES
	14	39	14	39	
30	3,52	3,56	0,01	0,01	C1=CC(=C(C=C1C2=C(C(=O)C3=C(O2)C=C(C=C3)O)O)O)O
31	2,19	2,69	2,44	2,45	O=C2C1=CC=CC=C1OC(C3=CC=CC=C3)=C2O
32	4,63	4,93	0,00	3,41	OC1=CC(O)=C(O)C2=C1C(C(O)=C(C3=CC=C(O)C(O)=C3)O2)=O
33	2,92	4,85	0,01	0,07	C1=CC(=CC=C1C2=C(C(=O)C3=C(O2)C(=C(C=C3O)O)O)O)O
34	3,82	5,42	9,31	7,00	COC1=C(C=CC(=C1)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O
35	2,96	4,97	0,00	3,23	O=C2C1=C(O)C=C(O)C=C1OC(C3=CC=C(O)C=C3)=C2O
36	2,20	2,65	0,86	0,03	O=C1C(O)=C(C3=CC=CC=C3)OC2=C1C=C(OC)C=C2
37	2,90	4,95	0,89	0,89	C1=CC(=C(C=C1O)O)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O
38	3,99	4,07	1,03	3,32	C1=C(C=C(C(=C1O)O)O)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O
39	4,97	4,92	3,25	3,33	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C(=C(C=C3O2)O)O)O)O)O)O
40	4,97	4,93	9,66	3,32	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O
41	2,56	3,08	0,00	0,01	O=C1C(O)=C(C3=CC=C(O)C(O)=C3)OC2=C1C=C(O)C=C2
42	2,89	4,34	0,00	3,24	OC2=CC1=C(C=C2)C(C(O)=C(C3=CC=C(O)C=C3)O1)=O
43	4,90	4,30	2,00	0,09	O=C1CC(C3=CC=C(O)C(O)=C3)OC2=C1C(O)=CC(O)=C2
44	2,19	2,69	1,52	1,52	O=C2C1=CC=CC=C1OC(C3=CC=CC=C3)C2
45	4,89	4,59	9,83	8,18	COC1=C(C=C(C=C1)[C@@H]2CC(=O)C3=C(C=C(C=C3O2)O)O)O
46	3,70	4,39	6,03	5,88	COC1=C(C=CC(=C1)[C@@H]2CC(=O)C3=C(C=C(C=C3O2)O)O)O
47	2,02	3,09	1,47	0,99	O=C1CC(C3=CC=C(O)C=C3)OC2=C1C=CC=C2
48	2,95	3,68	1,87	1,59	OC2=CC1=C(C=C2)C(CC(C3=CC=C(O)C=C3)O1)=O
49	2,12	2,64	1,48	1,48	O=C1CC(C3=CC=CC=C3)OC2=C1C=C(OC)C=C2
50	3,09	4,22	1,89	0,00	O=C3C1=C(O)C=C(O)C=C1OC(C3)C2=CC=C(O)C=C2
51	3,99	3,79	1,46	2,71	C1[C@H](OC2=CC(=CC(=C2C1=O)O)O)C3=CC=CC=C3
52	2,23	2,55	1,71	3,04	C1=CC(=C(C=C1C2C(C(=O)C3=C(O2)C=C(C=C3)O)O)O)O
53	4,24	3,70	5,66	8,78	COC1=C(C=CC(=C1)[C@@H]2[C@H](OC3=C(O2)C=C(C=C3)[C@@H]4[C@H](C(=O)C5=C(C=C(C=C5O4)O)O)O)CO)O
54	2,48	5,51	0,02	0,11	O=C1[C@H](O)C(C3=CC=C(O)C(O)=C3)OC2=C1C(O)=CC(O)=C2
55	4,77	5,57	2,57	2,64	OC1=CC(O)=CC2=C1C[C@H](O)C(C3=CC=C(O)C(O)=C3)O2
56	4,77	5,57	2,57	2,64	OC1=CC(O)=CC2=C1CC(O)C(C3=CC=C(O)C(O)=C3)O2
57	4,25	4,61	4,76	8,61	C1[C@H]([C@H](OC2=CC(=CC(=C21)O)O)C3=CC(=C(C=C3)O)O)OC(=O)C4=CC(=C(C(=C4)O)O)O

No.	Fit Value		Relative Energy		SMILES
	14	39	14	39	
58	3,96	4,71	1,85	0,00	OC1=CC(O)=CC2=C1CC(O)C(C3=CC(O)=C(O)C(O)=C3)O2
59	3,97	3,50	7,43	4,93	O=C(O[C@@H]2Cc3c(O[C@@H]2c1cc(O)c(O)c(O)c1)cc(O)cc3O)c4cc(O)c(O)c(O)c4
60	3,74	4,83	1,07	0,08	C1=CC(=C(C=C1C=CC(=O)C2=C(C=C(C=C2)O)O)O)O
61	1,98	2,60	0,00	0,00	O=C(/C=C/C2=CC=CC=C2)C1=CC=CC=C1
62	1,65	3,12	4,21	2,96	O=C(C2=CC=CC=C2O)/C=C/C1=CC=CC(O)=C1
63	0,55	3,85	4,20	0,00	O=C(C2=CC=CC=C2O)/C=C/C1=CC=C(O)C=C1
64	3,54	3,91	0,00	0,00	O=C(C2=CC=C(O)C=C2O)/C=C/C1=CC=CC=C1
65	3,43	4,72	1,74	0,00	C1=CC(=C(C=C1C=CC(=O)C2=C(C=C(C=C2O)O)O)O)O
66	2,47	3,25	0,00	0,00	O=C(C2=CC=C(O)C=C2)/C=C/C1=CC=CC=C1
67	2,35	4,81	0,00	0,00	O=C(C2=C(O)C=C(O)C=C2)/C=C/C1=CC=C(O)C=C1
68	3,39	1,88	4,21	9,41	O=C(C2=CC=C(O)C=C2O)/C=C/C1=C(O)C=CC=C1
69	3,29	3,86	1,14	0,09	O=C(C2=CC(O)=CC=C2O)/C=C/C1=CC=C(O)C=C1
70	2,16	4,70	1,72	0,00	O=C(CCC1=CC=C(C=C1)O)C2=C(C=C(C=C2O)O)O
71	4,98	4,68	0,22	8,74	C1=CC(=C(C=C1C2=C(C=C3C(=CC(=CC3=[O+]2)O)O)O)O)O
72	4,30	4,75	7,64	9,64	OC1=C(C=C(O)C(C3=CC=C(O)C=C3)=[O]2)C2=CC(O)=C1
73	3,92	3,69	0,00	2,04	OC1=C(C=CC(C3=CC=C(O)C=C3)=[O]2)C2=CC(O)=C1
74	3,17	2,66	5,28	5,41	O=C1C2=C(O)C=C(O[C@H]3[C@H](O)[C@@H](O)[C@H](O)[C@@H](COC(C)=O)O3)C=C2OC=C1C4=CC=C(O)C=C4
75	2,63	3,20	7,87	6,05	O=C2C1=C(O)C=C(O)C=C1OC=C2C3=CC=C(OC)C=C3
76	2,44	3,18	0,00	0,00	C1=CC(=CC=C1C2=COC3=C(C2=O)C=CC(=C3)O)O
77	0,25	1,84	2,88	9,05	C1=CC(=CC=C1C2=COC3=C(C2=O)C=CC(=C3)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O)O
78	2,12	2,25	6,38	2,46	COC1=CC=C(C=C1)C2=COC3=CC(=CC(=C3C2=O)O)OC
79	2,26	2,39	9,80	2,07	COC1=CC=C(C=C1)C2=COC3=C(C2=O)C=CC(=C3)OC
80	1,88	3,14	9,45	1,94	O=C1C(C3=CC=C(OC)C=C3)=COC2=C1C=CC(O)=C2
81	2,90	2,22	0,00	0,00	O=C2C1=C(O)C=C(O)C=C1OC=C2C3=CC=C(O)C=C3
82	2,93	3,39	6,02	8,69	C1=CC(=CC=C1C2=COC3=CC(=CC(=C3C2=O)O)O)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O)O
83	2,32	3,47	9,37	9,36	COC1=C(C=C2C(=C1)C(=O)C(=CO2)C3=CC=C(C=C3)O)O
84	2,50	2,07	3,15	8,46	COC1=C(C=C2C(=C1)C(=O)C(=CO2)C3=CC=C(C=C3)O)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O
85	2,99	3,05	0,01	0,00	O=C1C(C3=CC=CC=C3)=COC2=C1C=CC(O)=C2
86	1,96	3,18	7,00	7,01	O=C1C(C3=CC=CC=C3)=COC2=C1C=C(OC)C(O)=C2
87	0,52	2,24	0,00	0,00	O=C2C1=CC=CC=C1OC=C2C3=CC=CC=C3

No.	Fit Value		Relative Energy		SMILES
	14	39	14	39	
88	3,04	2,48	5,18	6,22	O=C1C2=C(O)C=C(O[C@H]3[C@H](O)[C@@H](O)[C@H](O)[C@@H](COC(CC(O)=O)=O)O3)C=C2OC=C1C4=CC=C(O)C=C4
89	0,95	2,82	8,06	9,65	O=C2C1=C(O)C=C(OC)C=C1OC=C2C3=CC=C(O)C=C3
90	2,09	3,31	0,00	0,00	OC1=C(O)C=C(C(C(C3=CC=C(O)C=C3)=CO2)=O)C2=C1
91	0,80	2,68	0,00	0,29	OC1=CC=C(C(C(C3=CC=C(O)C=C3)=CO2)=O)C2=C1O
92	1,73	3,81	0,01	0,01	O=C1C(C3=CC(O)=C(O)C=C3)=COC2=C1C=CC(O)=C2
93	0,04	3,20	2,98	7,92	C1C(COC2=C1C=CC(=C2)O)C3=CC=C(C=C3)O
94	1,74	3,72	0,00	0,00	C1=CC2=C(C=C1O)OC3=C2C(=O)OC4=C3C=CC(=C4)O
95	3,58	4,38	0,06	0,31	OC(C=C2)=CC=C2/C=C/C1=CC(O)=CC(O)=C1
96	3,41	3,75	0,01	1,56	C1=CC(=C(C=C1C=C2C(=O)C3=C(O2)C=C(C=C3)O)O)O
97	2,00	2,48	0,00	0,00	C1=CC=C2C(=C1)C(=O)C3=CC=CC=C3O2

Additional explanation S2. 2D fingerprint modeling

A ROC plot is a graph of sensitivity (the ability of a model to avoid false negatives i.e. true positive rate) versus specificity (the ability of a model to predict true negatives i.e. false positive rate) for different cut-off values. A ROC score of 0.5 is random, and means that the model has no predictive value. A score near 1 means a sensitive and accurate model. The ROC scores of the fingerprint models created for this study were close to 1 which indicated excellent accuracy. The EstXVAUC, which is the ROC score from the leave-one-out cross-validation process, and the XV_2 and XV_3, which are the ROC scores from the 2-fold and 3-fold cross-validation process, presented fair values of above 0.7. These models were selected to perform "Random Y" cross-validation after 50 iterations. The value obtained from this process “P random”, is the probability that the predicted likelihood of activity is random. A value of 0.1 indicates a 10% chance that the model is random. From this test it is clear that the dumb descriptor model is 100% random, which is good as it is the negative control.

The abbreviations of the molecular descriptors FCFP, ECFP, LCFP, and SCFP can be explained as follows. The first letter represents the atom abstraction method. E (atom type) uses atom type, charge, and hybridization; F (functional class) uses H-bond donor, H-bond acceptor, positive ionizable, negative ionizable, halogen, and aromatic; L (AlogP code) uses AlogP category of atoms; S (Sybyl) uses sybyl atom types used in the Tripos Mol2 file format. The second letter represents the type of fingerprint to generate, in all here described cases extended-connectivity. The last two letters stand for fingerprint. The number behind

the fingerprint name describes the maximum diameter (in bond lengths) of the largest structure represented by the fingerprint.

Table S3. Selection of top model scores ROC plot hTAS2R14.

descriptor	ROC	P_random	EstXVAUC	XV_2	XV_3
FCFP_10	0.998	0.03	0.82	0.76	0.79
ECFP_10	0.998	0.10	0.80	0.74	0.79
LCFP_10	0.997	0.18	0.80	0.73	0.77
SCFP_10	0.996	0.06	0.78	0.73	0.76
Dumb	0.840	1.00	0.71	0.74	0.64

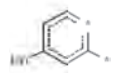
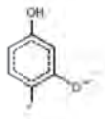
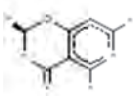
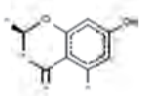
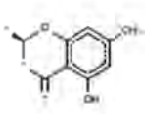
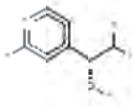
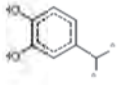
Table S4. Selection of top model scores ROC plot hTAS2R39.

descriptor	ROC	P_random	EstXVAUC	XV_2	XV_3
FCFP_12	0.996	0.15	0.73	0.75	0.77
ECFP_8	0.994	0.32	0.70	0.74	0.72
LCFP_8	0.996	0.27	0.68	0.70	0.69
LPFP_8	0.994	0.01	0.61	0.63	0.60
Dumb	0.893	1.00	0.73	0.65	0.71

Additional explanation S5. Key molecular features of 2D fingerprint models

The key molecular features found from the 2D- fingerprint models are shown in **Table S6-S9**. Each of the molecule fragments presents information regarding the type of feature: "G" for "good" followed by the ranking number (1 to 20 in this case), the fingerprint bit that is unique for each fingerprint, the number of molecules that were active in which the feature occurred, the total number of molecules in which the feature was present, and the Bayesian score.

Table S6. ECFP 10 Scitegic Bayesian Inference: good features from ECFP 10.

 G1: 477042227 13 good out of 13 Bayesian Score: 0.366	 G2: 658676414 10 good out of 10 Bayesian Score: 0.357	 G4: -58517464 6 good out of 6 Bayesian Score: 0.333	 G5: 1849099567 6 good out of 6 Bayesian Score: 0.333
 G6: 1275698324 6 good out of 6	 G7: -1925046727 6 good out of 6 Bayesian Score: 0.333	 G8: 1370869779 5 good out of 5	 G9: -529508685 5 good out of 5 Bayesian Score: 0.322

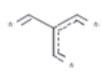
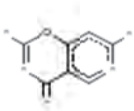
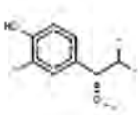
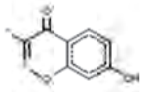
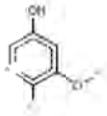
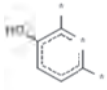
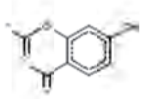
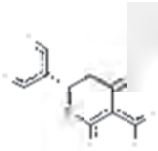
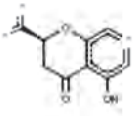
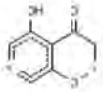
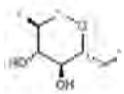
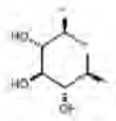
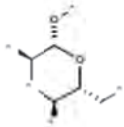
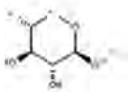
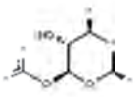
Bayesian Score: 0.333		Bayesian Score: 0.322	
 G10: -176483725 5 good out of 5 Bayesian Score: 0.322	 G11: -779557588 5 good out of 5 Bayesian Score: 0.322	 G12: -1901940684 5 good out of 5 Bayesian Score: 0.322	 G13: -877443398 5 good out of 5 Bayesian Score: 0.322
 G14: -1831055759 5 good out of 5 Bayesian Score: 0.322	 G15: 1644070299 26 good out of 28 Bayesian Score: 0.312	 G16: 2081690891 15 good out of 16 Bayesian Score: 0.311	 G17: -1756840804 4 good out of 4 Bayesian Score: 0.307
 G18: -764820435 4 good out of 4 Bayesian Score: 0.307	 G19: -1248472926 4 good out of 4 Bayesian Score: 0.307	 G20: -1341232669 4 good out of 4 Bayesian Score: 0.307	

Table S7. ECFP 10 Scitegic Bayesian Inference: bad features from ECFP 10.

 B1: 1811812564 0 good out of 4 Bayesian Score: -1.303	 B2: 1815582740 0 good out of 4 Bayesian Score: -1.303	 B3: 1405568809 0 good out of 4 Bayesian Score: -1.303	 B4: 1815173751 0 good out of 4 Bayesian Score: -1.303
 B5: 1426096942 0 good out of 4	 B6: -1409796893 0 good out of 4 Bayesian Score: -1.303	 B7: 305695353 0 good out of 4 Bayesian Score: -1.303	 B8: -2060414325 0 good out of 4 Bayesian Score: -1.303

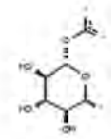
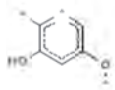
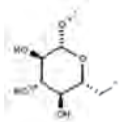
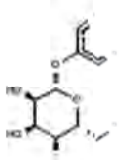
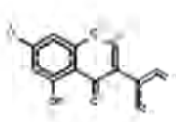
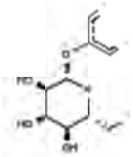
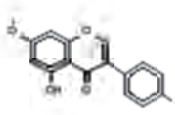
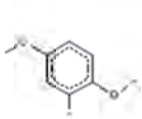
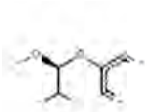
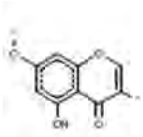
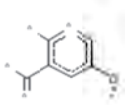
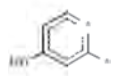
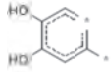
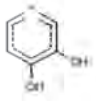
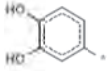
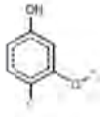
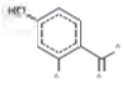
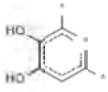
Bayesian Score: -1.303			
 B10: 1277224341 0 good out of 4 Bayesian Score: -1.303	 B11: -1433832737 0 good out of 4 Bayesian Score: -1.303	 B12: 1908828434 0 good out of 4 Bayesian Score: -1.303	 B13: -240665646 0 good out of 3 Bayesian Score: -1.102
 B14: 642771342 0 good out of 3 Bayesian Score: -1.102	 B15: -234090829 0 good out of 3 Bayesian Score: -1.102	 B16: 595500881 0 good out of 3 Bayesian Score: -1.102	 B17: -1687245953 0 good out of 3 Bayesian Score: -1.102
 B18: 966631805 0 good out of 3 Bayesian Score: -1.102	 B19: -975544103 0 good out of 3 Bayesian Score: -1.102	 B20: -1435824065 0 good out of 3 Bayesian Score: -1.102	

Table S8. ECFP 8 Scitegic Bayesian Inference: good features from ECFP 8.

 G1: 477042227 15 good out of 15 Bayesian Score: 0.183	 G2: -1364960748 13 good out of 13 Bayesian Score: 0.181	 G3: -101223435 12 good out of 12 Bayesian Score: 0.180	 G4: 1310213750 12 good out of 12 Bayesian Score: 0.180
 G7: 658676414 10 good out of 10 Bayesian Score: 0.177	 G8: 1375517620 9 good out of 9 Bayesian Score: 0.175	 G9: -1925046727 8 good out of 8 Bayesian Score: 0.172	 G10: -1553987716 7 good out of 7 Bayesian Score: 0.169

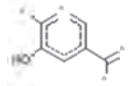
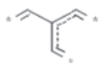
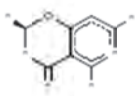
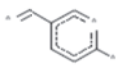
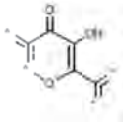
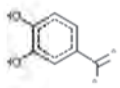
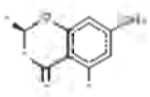
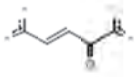
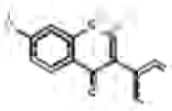
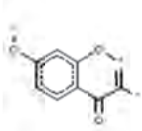
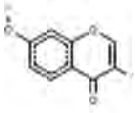
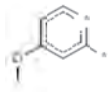
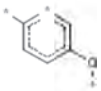
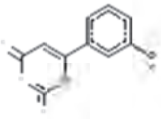
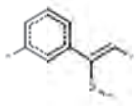
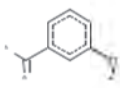
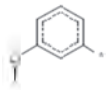
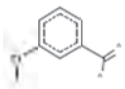
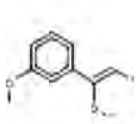
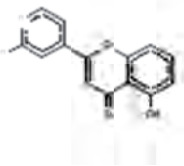
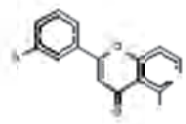
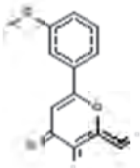
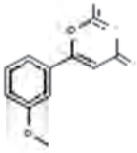
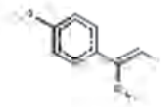
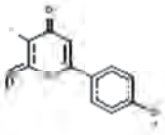
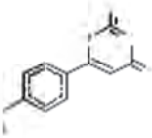
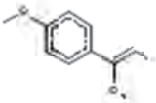
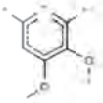
 G11: -2078659772 7 good out of 7 Bayesian Score: 0.169	 G12: -1831055759 7 good out of 7 Bayesian Score: 0.169	 G13: -176483725 7 good out of 7 Bayesian Score: 0.169	 G14: 1793888374 6 good out of 6 Bayesian Score: 0.166
 G15: -58517464 6 good out of 6 Bayesian Score: 0.166	 G16: 305957013 6 good out of 6 Bayesian Score: 0.166	 G17: 641508158 6 good out of 6 Bayesian Score: 0.166	 G18: 3221181 6 good out of 6 Bayesian Score: 0.166
 G19: 1849099567 6 good out of 6 Bayesian Score: 0.166	 G20: -52712319 6 good out of 6 Bayesian Score: 0.166		

Table S9. ECFP 8 Scitegic Bayesian Inference: bad features from ECFP 8.

 B1: 453543491 0 good out of 2 Bayesian Score: -0.972	 B2: -1611127761 0 good out of 2 Bayesian Score: -0.972	 B3: 1301997484 0 good out of 2 Bayesian Score: -0.972	 B4: 1760125606 1 good out of 4 Bayesian Score: -0.763
 B5: 425403071 3 good out of 8 Bayesian Score: -0.639	 B6: 338245611 0 good out of 1 Bayesian Score: -0.600	 B7: 127702438 0 good out of 1 Bayesian Score: -0.600	 B8: -566628975 0 good out of 1 Bayesian Score: -0.600

 B9: 536454864 0 good out of 1 Bayesian Score: -0.600	 B10: 1999165457 0 good out of 1 Bayesian Score: -0.600	 B11: -319036633 0 good out of 1 Bayesian Score: -0.600	 B12: 1078755483 0 good out of 1 Bayesian Score: -0.600
 B13: -1723293637 0 good out of 1 Bayesian Score: -0.600	 B14: 823848251 0 good out of 1 Bayesian Score: -0.600	 B15: 693911740 0 good out of 1 Bayesian Score: -0.600	 B16: 1859792422 0 good out of 1 Bayesian Score: -0.600
 B17: -672467221 0 good out of 1 Bayesian Score: -0.600	 B18: -897237392 0 good out of 1 Bayesian Score: -0.600	 B19: -309130633 0 good out of 1 Bayesian Score: -0.600	 B20: 1151345624 0 good out of 1 Bayesian Score: -0.600

Additional explanation S10. 3D pharmacophore modeling

The pharmacophore protocol created 10 hypotheses for each run. The models were analysed statistically, based on the confusion matrix and the ROC plot, or visually, by looking at ligand mapping and alignment. The confusion matrix gave information on the total number of positive and negative compounds included in the validation set, compared to the number of true positives, true negatives, false positive and false negatives as identified by the hypotheses. These numbers resulted in a value for sensitivity (the ability to retrieve actives, true positive rate) and a value for the specificity (the ability to discard inactives, true negatives rate). The favoured hypothesis was selected for a high sensitivity and a high specificity. Additionally, the Relative Operating Characteristics (ROC) curve was provided for each hypothesis, which resembles true positives rate against the false positives rate. The area under the curve represented the accuracy of the model: a value of '1' indicated a perfect model, which finds only true positives, whereas a value of '0.5'

represented a useless test, which found just as many true positives as false positives. The ROC curve was also used for the selection of the most optimal model. With the statistical scoring two or three 'good' models were selected, which were further analyzed graphically. The hypotheses were viewed side by side, to find redundancy and to cluster hypotheses. Alignment of the ligands to the model was checked for chemical meaningfulness and to check whether important features were identified within the hypothesis. The most active ligands had to fit the pharmacophore in a reasonable manner. Tools used to analyse the visual scoring of the models were the ligand/pharmacophore mapping, the ligand profiling and the heat map. If the hypothesis were not satisfactory, the ligands or the settings were changed to improve the hypothesis.

Table S11. Confusion matrix of pharmacophore models for hTAS2R14.

Validation with Known Actives/Inactives								
Pharmacophore	Total Actives	Total Inactives	True Positives	True Negatives	False Positives	False Negatives	Sensitivity	Specificity
Pharmacophore_1	21	23	15	13	10	6	0.71429	0.56522
Pharmacophore_2	21	23	15	13	10	6	0.71429	0.56522
Pharmacophore_3	21	23	16	13	10	5	0.76190	0.56522
Pharmacophore_4	21	23	16	13	10	5	0.76190	0.56522
Pharmacophore_5	21	23	15	12	11	6	0.71429	0.52174
Pharmacophore_6	21	23	15	11	12	6	0.71429	0.47826
Pharmacophore_7	21	23	15	11	12	6	0.71429	0.47826
Pharmacophore_8	21	23	15	12	11	6	0.71429	0.52174
Pharmacophore_9	21	23	16	15	8	5	0.76190	0.65217
Pharmacophore_10	21	23	16	15	8	5	0.76190	0.65217

Table S12. Confusion matrix of pharmacophore models for hTAS2R39.

Validation with Known Actives/Inactives								
Pharmacophore	Total Actives	Total Inactives	True Positives	True Negatives	False Positives	False Negatives	Sensitivity	Specificity
Pharmacophore_1	27	14	25	9	5	2	0.92593	0.64286
Pharmacophore_2	27	14	25	8	6	2	0.92593	0.57143
Pharmacophore_3	27	14	24	9	5	3	0.88889	0.64286
Pharmacophore_4	27	14	25	9	5	2	0.92593	0.64286
Pharmacophore_5	27	14	25	11	3	2	0.92593	0.78571
Pharmacophore_6	27	14	25	11	3	2	0.92593	0.78571
Pharmacophore_7	27	14	25	11	3	2	0.92593	0.78571
Pharmacophore_8	27	14	25	5	9	2	0.92593	0.35714
Pharmacophore_9	27	14	25	7	7	2	0.92593	0.50000
Pharmacophore_10	27	14	25	5	9	2	0.92593	0.35714

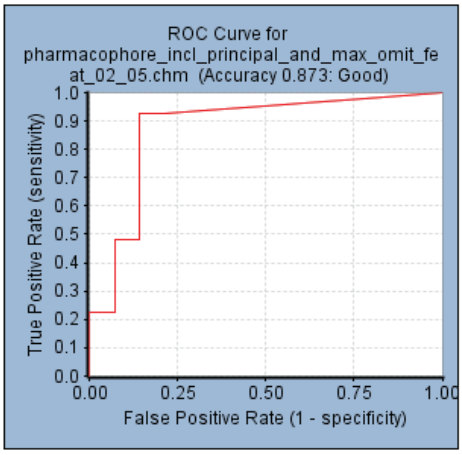


Figure S13. ROC plot for selected pharmacophore model for hTAS2R14.

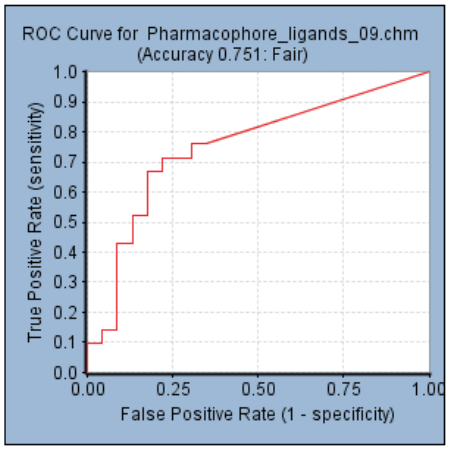


Figure S14. ROC plot for selected pharmacophore model for hTAS2R39.

Additional explanation S15. Heat map for the pharmacophore model for hTAS2R14

The heat map shows the mapping of the ligands to each of the pharmacophore features. If a feature maps to a feature in a ligand, the value is 1, if the feature is missed by a ligand, the value is 0. The row index indicates the row numbers of the ligands, which are separated in highly active and inactive ligands by the first column. A colour code is given to highlight the false negatives (yellow) and the false positives (red). All false negatives miss the feature HBD_4, which is the donor feature on the B-ring side. However, some of the ligands also miss the feature of the hydrophobic aromatic or the HBD_3, therefore the HBD_4 feature is not the only determinant for the presence of these false negatives. Another hypothesis following from the heat map is that the two donor features are equally relevant for discrimination between the active and inactive ligands. The aromatic features and the acceptor feature do not have a strong discriminator function, but they play a role in ligand alignment.

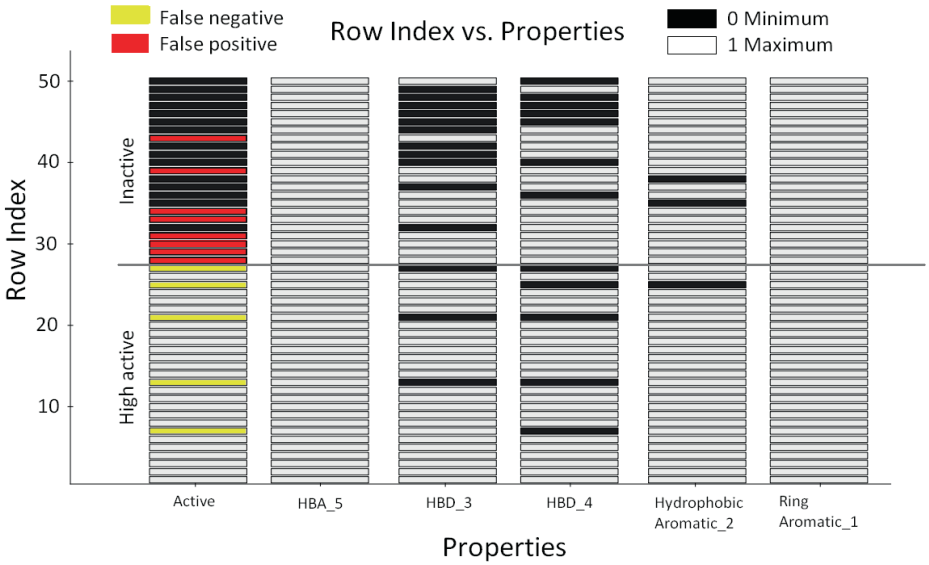


Figure S16. Heat map for the pharmacophore model of hTAS2R14, showing the activity of the ligands and the mapping of ligands to the 5 different features. HBA_5 is the hydrogen bond acceptor, HBD_3 is the first hydrogen bond donor, HBD_4 is the second hydrogen bond donor (at the B-ring side), Hydrophobic Aromatic_2 is the Hydrophobic Aromatic feature (at the B-ring side) and Ring Aromatic_1 is the Ring Aromatic feature.

Chapter 4

6-Methoxyflavanones as bitter taste receptor blockers for hTAS2R39

Based on: Wibke S.U. Roland, Robin J. Gouka, Harry Gruppen, Marianne Driesse, Leo van Buren, Gerrit Smit, Jean-Paul Vincken. 6-Methoxyflavanones as bitter taste receptor blockers for hTAS2R39. ***Accepted***

ABSTRACT

Many (dietary) bitter compounds, ***e.g.*** flavonoids, activate bitter receptor hTAS2R39 in cell-based assays. Several flavonoids, amongst which some flavanones, are known not to activate this receptor. As certain flavanones are known to mask bitter taste sensorially, flavanones might act as bitter receptor antagonists. Fourteen flavanones were investigated for their potential to reduce activation of hTAS2R39 by epicatechin gallate (ECG), one of the main bitter compounds occurring in green tea. Three flavanones showed inhibitory behavior towards the activation of hTAS2R39 by ECG: 4'-fluoro-6-methoxyflavanone, 6,3'-dimethoxyflavanone, and 6-methoxyflavanone (in order of decreasing potency). The 6-methoxyflavanones also inhibited activation of hTAS2R14 (another bitter receptor activated by ECG), though to a lesser extent. Dose-response curves of ECG at various concentrations of the full antagonist 4'-fluoro-6-methoxyflavanone and wash-out experiments indicated reversible insurmountable antagonism. The same effect was observed for the structurally different agonist denatonium benzoate, suggesting a non-competitive orthosteric mechanism.

INTRODUCTION

Even though bitter taste can be appreciated in some food products, such as beer, coffee, dark chocolate and red wine ***(1)***, in most cases bitterness in food is unwanted and efforts are taken to reduce bitter taste ***(2)***. One approach for masking bitter taste is the use of so-called bitter receptor blockers, which inhibit the taste receptor activation caused by the bitter compound. On the human tongue, bitterness is perceived by human bitter taste receptors (hTAS2Rs, TAS2Rs or T2Rs). The ***in vitro*** activation of these hTAS2Rs by bitter compounds has been studied intensively during the last decade. For 21 of the 25 the bitter receptors, an agonist, or in some cases dozens of agonists, have been identified ***(3, 4)***. On the contrary, bitter receptor antagonists are still quite rare.

The small molecule (4-(2,2,3-trimethylcyclopentyl)butanoic acid (or GIV 3727) has been reported as inhibitor of six bitter taste receptors ***(5)***. It was able to decrease the sensory perception of bitter aftertaste of the sweeteners acesulfame K and saccharin, as well as the activation of hTAS2R31 and hTAS2R43, the bitter receptors activated by these two compounds. Another compound, the decreased bitter receptor activation of which could be linked to sensory perception, was ***p***-(dipropylsulfamyl)benzoic acid (better known as probenecid). It has been reported to inhibit activation of hTAS2R16, hTAS2R38, and hTAS2R43, and to suppress the bitter taste perception of salicin in sensory tests ***(6)***. It has been reported that a compound can act as an agonist towards one subset of bitter receptors, whereas it can act as an antagonist towards another subset of bitter receptors. This has been described for the two sesquiterpene lactones 3β-hydroxydihydrocostunolide (3HDC) and 3β-hydroxypelenolide (3HP) ***(7)***.

Recently, a pharmacophore model for maskers of the bitter taste of caffeine has been developed ***(8)***. This pharmacophore was docked into a homology model of hTAS2R10 (one of the bitter receptors activated by caffeine). Docking of the two substances enterolactone and enterodiol predicted their bitterness modulating activities, which could be confirmed by sensory tests. Docking was also applied for the compound GIV 3727 in a model of hTAS2R31, and the presence of a single binding pocket was reasoned ***(5)***, in which both agonist and antagonist can bind.

Apart from ***in vitro*** studies on bitter receptor blocking, several molecules are reported to mask bitter taste ***in vivo (2)***, amongst which the flavanones homoeriodictyol, its Na-salt, and eriodictyol. They reduced the bitter taste of different chemical classes of bitter molecules up to 40 % with unknown mechanism ***(9)***. Their sensorial bitter masking effect has not been proven to be caused by inhibition of bitter taste receptor activation. Two other flavanones (sakuranetin and 6-methoxysakuranetin) have been described as antagonists for hTAS2R31 ***(10)***. Hence, flavanones seem to be of importance in reduction of bitter taste and bitter taste receptor activation.

The human bitter taste receptor hTAS2R39 seems to be a bitter receptor for dietary compounds, as many agonists are dietary compounds, such as thiamine (vitamin B1),

quinine ***(3)*** used in tonic water, catechins from green tea ***(11)***, wine tannin precursors ***(12)***, small peptides from casein hydrolysates ***(13)*** and cheese ***(14)***, isoflavones from soy bean ***(15)***, and many other flavonoids from several plant sources ***(16)***. Hence, it is of interest to identify a bitter blocker for this receptor. It is likely that an antagonist might have similar structural elements to an agonist in order to fit into the same binding pocket. In our previous study on (iso)flavonoid agonists of hTAS2R39, several of the compounds tested, amongst which flavanones, did not activate the bitter receptor despite structural similarity to active compounds ***(16)***. The aim of the present study was to investigate whether these and other flavanones could act as antagonists towards hTAS2R39.

MATERIALS AND METHODS

Materials

Compounds tested were obtained from Extrasynthese (Genay, France), Indofine Chemical Company (Hillsborough, NJ, USA), Interbioscreen (Moscow, Russia), and Sigma-Aldrich (Steinheim, Germany). Each compound was dissolved in DMSO (Sigma-Aldrich) to a 100 mM stock concentration. Trypan blue solution (0.4 % w/v) and isoproterenol were purchased from Sigma-Aldrich.

Tyrode's buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM Hepes, pH 7.4) with 0.5 mM probenecid (Sigma-Aldrich) was used for dilution of compound-DMSO stock solutions and for calcium imaging assays. The presence of probenecid in the buffer did not lead to inhibition of hTAS2R14 or hTAS2R39. All compounds were tested for autofluorescence and toxic effects on the cells used at a concentration of 1 mM as described before ***(15)***.

Expression of hTAS2R39 and hTAS2R14 in HEK293 cells

For functional expression of the human bitter taste receptor hTAS2R39, HEK293 T-Rex Flp-In cells (Invitrogen, San Diego, CA, USA) were used, stably expressing the chimeric G-protein α-subunit G 16-gust44 (cloned into pcDNA4 (Invitrogen)) ***(17)*** and the human bitter receptor genes for hTAS2R39 (cloned into pcDNA5/FRT (Invitrogen)). The 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 ***(18)*** with exception of the HSV-tag), in order to improve membrane targeting of the receptor protein. The same procedure was applied for stable expression of hTAS2R14. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and 10 % (v/v) tetracycline-free FBS (both Lonza, Verviers, Belgium) supplemented with blasticidin (5 ***μ***g/mL), geneticin (400 ***μ***g/mL) and hygromycin (100 ***μ***g/mL) (all from Invitrogen). Cells were grown and maintained at 37 °C and 5 % (v/v) CO_2.

Monitoring bitter receptor activation by intracellular calcium release

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 $7*10^3$ cells in 100 **µ**L/well and cultured for 24 h. Transcription of the receptors was induced by adding 0.25 µg/mL doxycycline (Sigma-Aldrich). Cells were induced for 24 h and then loaded with the calcium-sensitive fluorescent dye Fluo-4-AM (2.5 **µ**M, Invitrogen), 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).

Receptor activation or inhibition was measured via intracellular Ca^{2+} release ***(19)*** in a FlexStation II 384 or FlexStation III (Molecular Devices Corporation, Sunnyvale, CA, USA) by measuring fluorescence (excitation 485 nm / emission 520 nm) for either 90 s or 240 s at 37 °C. Two methods of compound administration were applied: simultaneous and stepwise addition of potential antagonist and agonist. The first 17 s before compound addition were used for baseline determination. For the simultaneous method, agonist and potential antagonist were pre-mixed, administered after 17 s, and fluorescence was measured for in total 90 s. For the stepwise method, after 17 s, the potential antagonist was added, fluorescence was measured until 120 s, and after 120 s the agonist was added and measured for another 120 s, in total 240 s. Non-induced cells, which did not express the taste receptor, were measured in parallel to verify specificity of receptor activation. All experiments were conducted in duplicate on two or more different days.

Calcium assay data processing

Data processing was done as reported previously ***(15)***. In brief, SoftMax Pro 5.4 software (Molecular Devices Corporation) was used to plot the fluorescence signals. The fluorescence value ($\Delta F/F_0$), representing receptor activity, was calculated by subtracting the baseline fluorescence (F_0) prior to loading from the maximum fluorescence (F) after compound addition, divided by the signal of the baseline in order to normalize background fluorescence ***(20)***. Dose-response curves were established as non-linear regression curves using Graph Pad Prism (version 4 for Windows, Graph Pad Software, San Diego, CA, USA). Half-maximal effective concentrations (EC_{50}) and half-maximal inhibitory concentrations (IC_{50}) were calculated. Error bars reflect the standard error of the mean (SEM). Statistical analysis was performed in Graph Pad Prism (one-way ANOVA at 5% risk level, followed by Bonferroni's post hoc test).

Investigation of inhibitory behavior of flavanones

Measuring dose-response curves of ECG on hTAS2R39 under the conditions used in this study revealed an EC_{80} concentration of 200 µM. Screening for hTAS2R39 inhibition by flavanones was performed with simultaneous application of agonist (200 µM ECG) and putative antagonist (250 µM flavanone). Inhibition was indicated when $\frac{\Delta F/F_0\ (agonist+flavanone)}{\Delta F/F_0\ (agonist)} < 1$. In case of indicated inhibition, flavanones were applied at different concentrations, in order to test for dose-dependent inhibition, at the EC_{80} concentration of the agonist. Another agonist of hTAS2R39, denatonium benzoate, was used in inhibition experiments at 1.7 mM (EC_{80}). hTAS2R14 inhibition was tested with 640 µM ECG or 70 µM genistein as agonists, representing their respective EC_{80} concentrations.

To determine whether inhibition was specific for the bitter taste receptor, the effect of the antagonists was tested on the β2-adrenergic receptor agonist isoproterenol (50 µM). To this end, the antagonists were applied at ~IC_{50} concentrations (100 µM 4'-fluoro-6-methoxyflavanone, 500 µM 6,3'-dimethoxyflavanone, and 500 µM 6-methoxyflavanone). To distinguish between reversible and irreversible inhibition, washout experiments were performed. Cells were stimulated with 200 µM ECG in the absence and in the presence of each antagonist (~IC_{50} concentrations), washed with Tyrode's buffer (80 ***µ***L/well), and again stimulated with 200 µM ECG.

RESULTS

Identification and characterization of hTAS2R39 inhibitors

Epicatechin gallate (ECG) (**Figure 1A**), one of the main bitter compounds in green tea ***(11)***, was chosen as agonist of hTAS2R39. In a previous study we identified nine flavanones as agonists of hTAS2R39, whereas three other flavanones did not activate this receptor ***(16)***. As some flavanones have been reported as bitter blockers ***(9, 10)***, it was investigated whether the three inactive flavanones, as well as other flavanones, might have antagonistic properties towards hTAS2R39. Fourteen flavanones (**Table 1**) were screened for their ability to reduce the activation of hTAS2R39 by ECG.

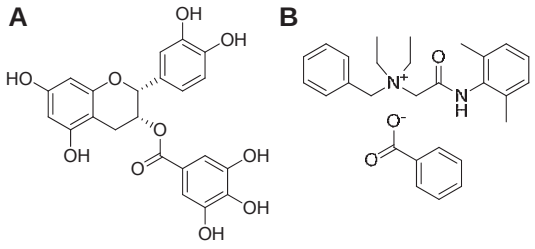
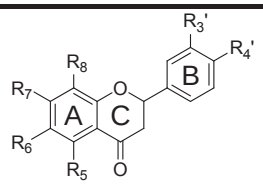


Figure 1. Chemical structures of hTAS2R39 agonists epicatechin gallate (ECG) (**A**) and denatonium benzoate (**B**).

Table 1. Flavanones tested for reduction of activation of hTAS2R39 by ECG.



	R_5	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$
6-Chloro-4-methylflavanone (**1**)	H	Cl	H	H	H	CH_3
8-Chloro-4-methylflavanone (**2**)	H	H	H	Cl	H	CH_3
6,3'-Dimethoxyflavanone (**3**)	H	OCH_3	H	H	OCH_3	H
Eriodictyol[a] (**4**)	OH	H	OH	H	OH	OH
Flavanone[b] (**5**)	H	H	H	H	H	H
4'-Fluoro-6-methoxyflavanone (**6**)	H	OCH_3	H	H	H	F
Homoeriodictyol[a] (**7**)	OH	H	OH	H	OCH_3	OH
6-Hydroxyflavanone (**8**)	H	OH	H	H	H	H
7-Hydroxyflavanone (**9**)	H	H	OH	H	H	H
4'-Hydroxyflavanone[b] (**10**)	H	H	H	H	H	OH
6-Methoxyflavanone[b] (**11**)	H	OCH_3	H	H	H	H
7-Methoxyflavanone (**12**)	H	H	OCH_3	H	H	H
4'-Methoxyflavanone (**13**)	H	H	H	H	H	OCH_3
Sakuranetin[c] (**14**)	OH	H	OCH_3	H	H	OH

[a] activation of hTAS2R39 in a previous study ***(16)***, but it was selected for testing as antagonist due to ability to reduce bitter taste perception in sensory tests ***(9)***.
[b] no activation of hTAS2R39 in a previous study ***(16)***.
[c] reported as antagonist of hTAS2R31 ***(10)***

Inhibition was indicated when the ratio between ECG response in the presence of a flavanone and the ECG response in the absence of a flavanone was <1 (**Figure 2**). Three compounds showed reduction of ECG responses on hTAS2R39: 6,3'-dimethoxyflavanone (**3**), 4'-fluoro-6-methoxyflavanone (**6**), and 6-methoxyflavanone (**11**). They were further investigated by two different ways of compound addition: simultaneous and stepwise addition. Simultaneous addition of agonist and antagonist to the receptor reflects the situation of ideal blocker application in food products. Stepwise addition of agonist and antagonist is commonly applied in pharmaceutical research when examining pharmacodynamics of receptor-antagonist interaction ***(21)***.

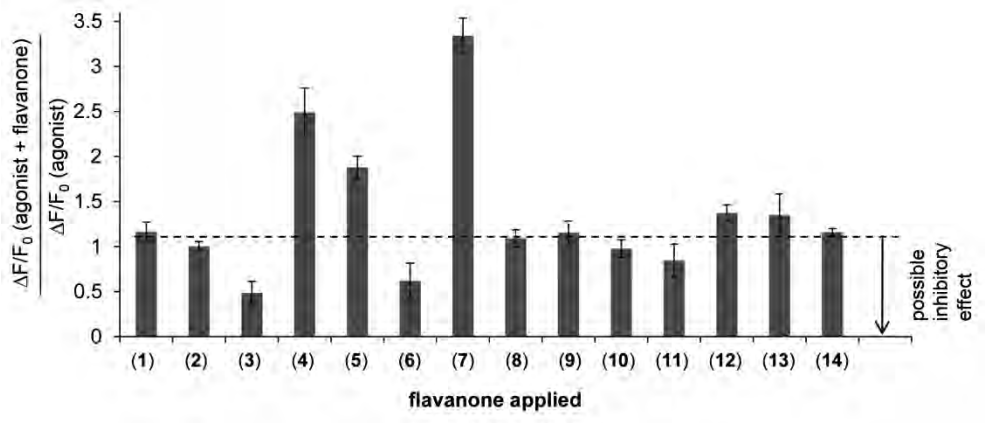


Figure 2. Screening of flavanones for reduction of ECG response on hTAS2R39. Values <1 indicate possible inhibitory effect.

The compound 4'-fluoro-6-methoxyflavanone (**6**) showed inhibitory activity towards ECG on hTAS2R39 both after simultaneous (**Figure 3A**) and after stepwise addition (**Figure 3B**). Application of (**6**) prior to addition of ECG led to 100 % receptor blocking (**Figure 3B**). For this full receptor blocker, the half-maximal inhibitory concentration (IC_{50}) was 102 µM. An overview of inhibition thresholds and IC_{50} values is given in **Table 2**. When (**6**) was added simultaneously with ECG, it had a lower inhibition threshold than when added in the stepwise way. Upon simultaneous addition, a maximal signal reduction of 65 % was reached at 63 µM, and further signal reduction could not be observed due to non-specific signals of the compound itself. The same holds for the simultaneous addition of 6,3-dimethoxyflavanone (**3**) (**Figure 3C**), where a maximal signal reduction of 55 % was reached at 500 µM. Due to increasing non-specific signals of (**3**), the full efficacy upon simultaneous addition could not be established. As shown in **Figure 3D**, a maximal reduction of ~85 % at 1000 µM was reached by (**3**) after stepwise addition. In contrast to (**6**) and (**3**), the compound 6-methoxyflavanone (**11**) showed negligible inhibitory activity against ECG on hTAS2R39 when applied simultaneously (**Figure 3E**), whereas it showed inhibitory activity when applied stepwise (ca. 50 % reduction of activation at 500 µM) (**Figure 3F**). When investigating the inhibitory behavior of the compounds identified, it thus became clear that the way of antagonist addition influenced the efficacy of the antagonist.

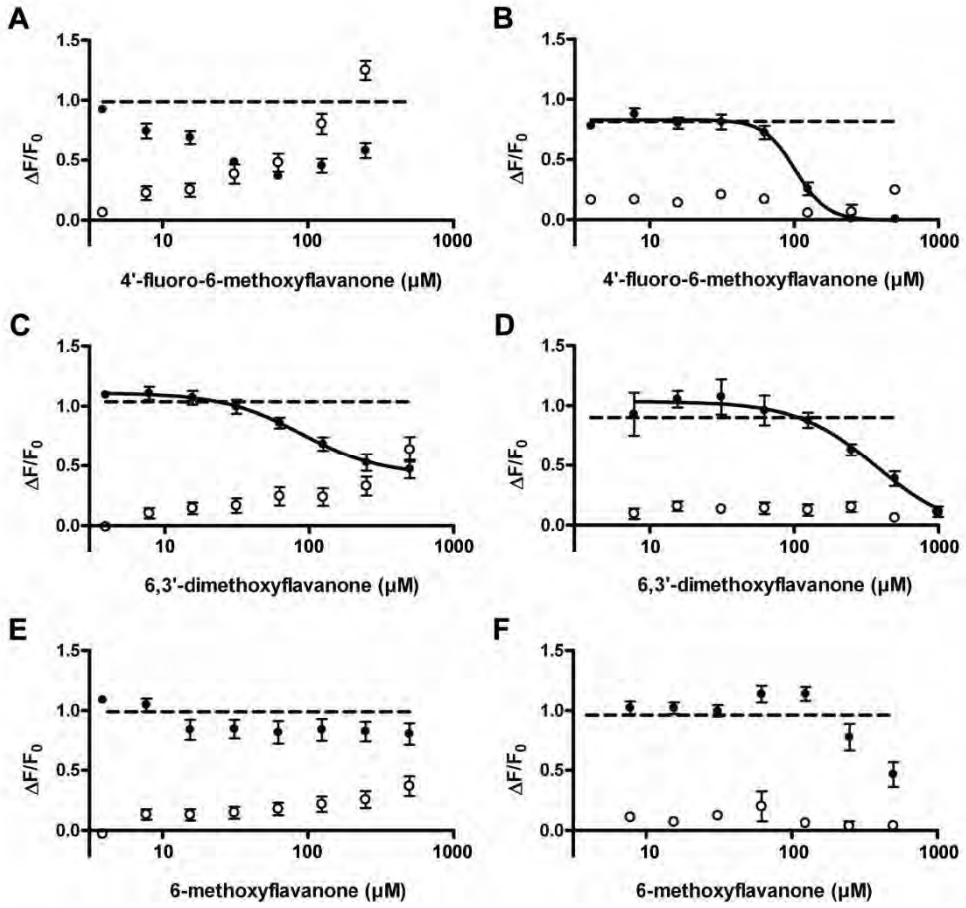


Figure 3. Inhibition of response of 200 μM ECG (---) on hTAS2R39 (induced (●), non-induced (○)) by 4'-fluoro-6-methoxyflavanone (**6**) after simultaneous addition (**A**) and stepwise addition (**B**), by 6,3'-dimethoxyflavanone (**3**) after simultaneous addition (**C**) and stepwise addition (**D**), and by 6-methoxyflavanone (**11**) after simultaneous addition (**E**) and stepwise addition (**F**).

Next, it was investigated whether the activation of hTAS2R39 by another agonist could also be reduced by the inhibitors identified. Denatonium benzoate (**Figure 1B**) was selected as well known agonist of hTAS2R39, which is different from ECG, in terms of structure and activation concentrations. Dose-response curves of denatonium benzoate were measured, and an EC_{50} of 711 μM, and an EC_{80} of 1.7 mM were established (data not shown). **Figure 4** shows the inhibitory behavior of 4'-fluoro-6-methoxyflavanone (**6**) towards 1.7 mM denatonium benzoate on hTAS2R39. The same trends as for ECG combined with antagonists were observed. The receptor activation of denatonium benzoate was reduced upon simultaneous addition of (**6**) up to a concentration of 63 μM (not further

reduced due to increasing non-specific signals), leading to maximal signal reduction of 58 %. Stepwise application of (**6**) led to 100 % receptor blocking, as already seen with ECG. The IC_{50} was calculated to be 55 µM. The results of all antagonists applied with denatonium benzoate are summarized in **Table 2**. Due to the fact that structurally different agonists were inhibited in a similar manner, we conclude that the reduced receptor response was not achieved by interaction between agonist and inhibitor, but by actual receptor antagonism.

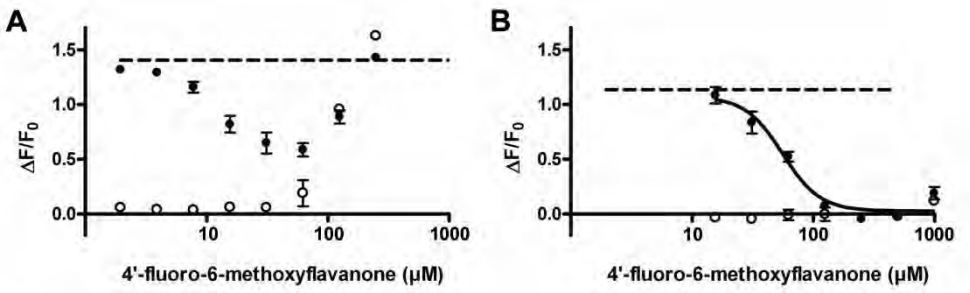


Figure 4. Inhibition of response of 1.7 mM denatonium benzoate (---) on hTAS2R39 (induced (●), non-induced (○)) by 4'-fluoro-6-methoxyflavanone (**6**) after simultaneous addition (**A**) and stepwise addition (**B**).

Table 2. Thresholds and IC_{50} values of 6-methoxyflavanones for inhibition of hTAS2R39 responses towards 200 µM ECG and 1.7 mM denatonium benzoate.

	hTAS2R39	Simultaneous		Stepwise	
		Threshold (µM)	IC_{50} (µM)	Threshold (µM)	IC_{50} (µM)
ECG (200 µM)	6-Methoxyflavanone	n.b.	n.b.	250	479
	6,3-Dimethoxyflavanone	63	282	125	407
	4'-Fluoro-6-methoxyflavanone	8	32	63	102
Denatonium b. (1.7 mM)	6-Methoxyflavanone	n.b.	n.b.	500	n.d.
	6,3-Dimethoxyflavanone	8	89	63	240
	4'-Fluoro-6-methoxyflavanone	8	22	32	55

n.b., no blocking; n.d., not determined

Specificity of hTAS2R39 inhibitors

It was investigated whether the antagonists identified specifically inhibit hTAS2R39, or also hTAS2R14, as many flavonoids behave similarly towards these two receptors ***(16)***. As agonist for hTAS2R14, ECG was used. **Figure 5** shows that no blocking of hTAS2R14 occurred upon simultaneous application of 4'-fluoro-6-methoxyflavanone (**6**) and ECG

(here at 640 µM, EC_{80} on hTAS2R14). The increase of signal at increasing concentrations of (**6**) is of non-specific nature, which can also be seen in the increase of response of non-induced cells, in which the bitter receptor is not expressed. Upon stepwise application, an inhibitory effect was observed. The three methoxyflavanones were also tested with genistein, another agonist of hTAS2R14, at 70 µM (EC_{80}). The results were similar to the results obtained with ECG. An overview of inhibition thresholds and IC_{50} values on hTAS2R14 is given in **Table 3**. It is remarkable that none of the three methoxyflavanones blocked hTAS2R14, when applied simultaneously with one of the agonists.

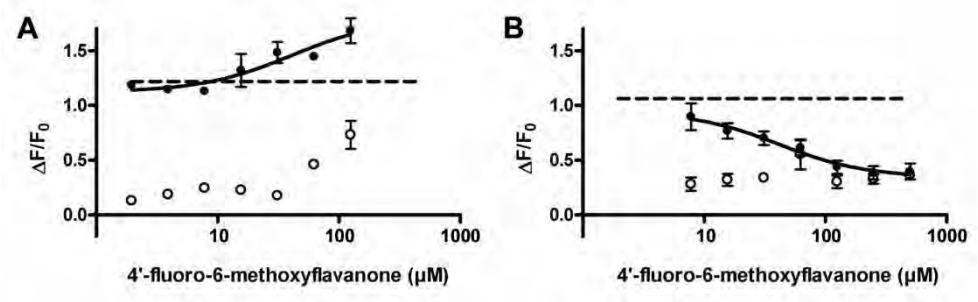


Figure 5. Inhibition of response of 640 µM ECG (---) on hTAS2R14 (induced (●), non-induced (○)) by 4'-fluoro-6-methoxyflavanone (**6**) after simultaneous addition (**A**) and stepwise addition (**B**).

Table 3. Thresholds and IC_{50} values of 6-methoxyflavanones for inhibition of hTAS2R14 responses towards 640 µM ECG and 70 µM genistein.

	hTAS2R14	Simultaneous		Stepwise	
		Threshold (µM)	IC_{50} (µM)	Threshold (µM)	IC_{50} (µM)
ECG (640 µM)	6-Methoxyflavanone	n.b.	n.b.	250	447
	6,3-Dimethoxyflavanone	n.b.	n.b.	125	~250
	4'-Fluoro-6-methoxyflavanone	n.b.	n.b.	< 8	79
Genistein (70 µM)	6-Methoxyflavanone	n.b.	n.b.	500	741
	6,3-Dimethoxyflavanone	n.b.	n.b.	n.d.	n.d.
	4'-Fluoro-6-methoxyflavanone	n.b.	n.b.	32	~500

n.b., no blocking; n.d., not determined

In order to further investigate the specificity of the antagonists identified towards taste receptors, isoproterenol responses were measured. Inhibition of isoproterenol response would indicate non-specific inhibition of β2-adrenergic receptors, endogenous to HEK293 cells. The results (**Figure 6**) show that the isoproterenol responses were not reduced and therewith the inhibition is concluded to be specific for taste receptors.

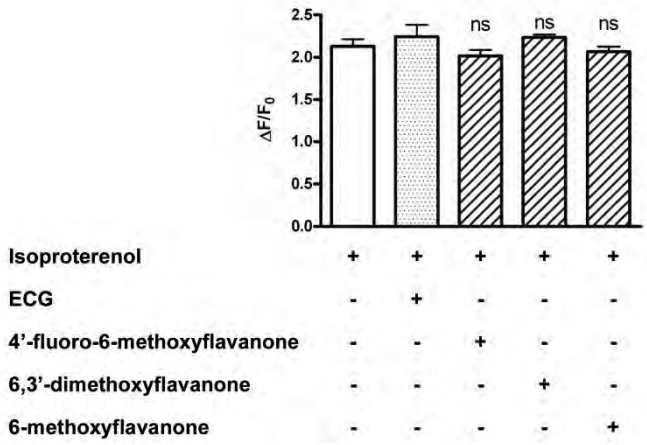


Figure 6. Isoproterenol responses upon application with buffer, ECG, 4'-fluoro-6-methoxyflavanone (**6**), 6,3'-dimethoxyflavanone (**3**), and 6-methoxyflavanone (**11**). n.s., not significant.

Pharmacological characterization of 4'-fluoro-6-methoxyflavanone

Due to full elimination of agonistic responses by 4'-fluoro-6-methoxyflavanone (**6**) on hTAS2R39, this molecule seemed to be the most effective antagonist identified in this study. The mechanism of antagonism was further clarified by measuring dose-response curves of ECG (**Figure 7A**) and denatonium benzoate (**Figure 7B**) in the presence of various concentrations of (**6**). Two effects were observed upon increasing antagonist concentrations: the dose-response curves shifted to the right, and the signal amplitudes decreased. The dose-response curves for the inhibition of ECG and denatonium benzoate showed the same pattern. EC_{50} values at all antagonist concentrations were calculated and are given in **Table 4**.

To distinguish between reversible and irreversible inhibition, washout experiments were performed. In **Figure 7C** it can clearly be seen that the inhibition was reversible.

Table 4. EC_{50} values of ECG and denatonium benzoate in the presence of various concentrations of 4'-fluoro-6-methoxyflavanone (**6**) on hTAS2R39.

	Concentration of 4'-fluoro-6-methoxyflavanone			
	0 µM	**50 µM**	**100 µM**	**200 µM**
ECG	128 µM	156 µM	501 µM	781 µM
Denatonium benzoate	659 µM	1.09 mM	3.36 mM	6.94 mM

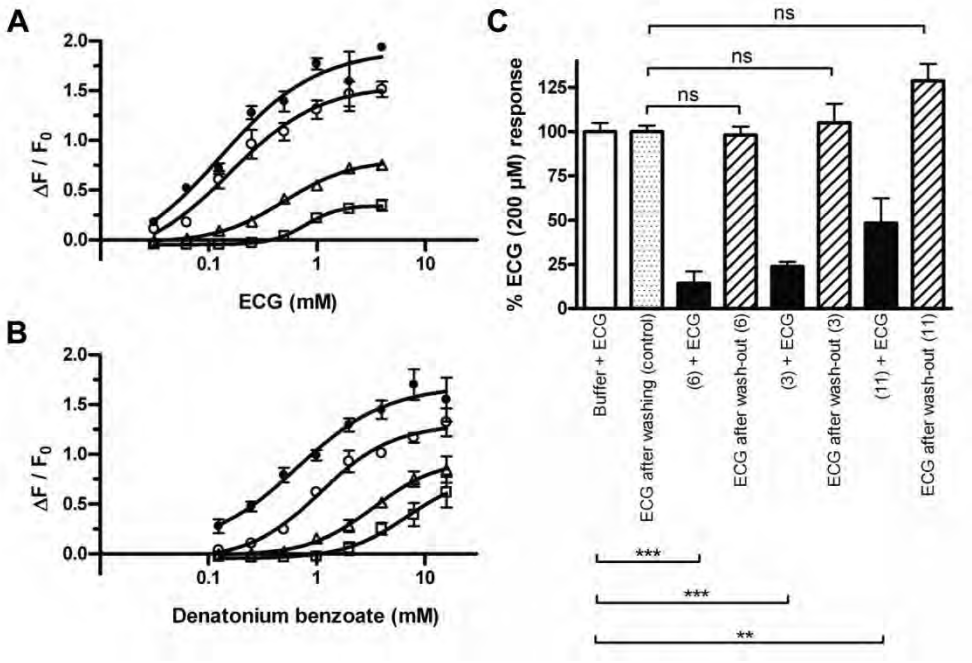


Figure 7. Dose-response curves for epicatechin gallate (ECG) (●) (**A**) and denatonium benzoate (●) (**B**) on hTAS2R39, and their modification by increasing 4'-fluoro-6-methoxyflavanone (**6**) concentrations (○ 50 µM, Δ 100 µM, □ 200 µM). Wash-out experiments (**C**). Cells were stimulated with 200 µM ECG in the absence (open bars) and in the presence (filled bars) of 100 µM 4'-fluoro-6-methoxyflavanone (**6**), or 500 µM 6,3'-dimethoxyflavanone (**3**), or 500 µM 6-methoxyflavanone (**11**), washed with Tyrode's buffer, and again stimulated with 200 µM ECG (hatched bars; control: grey bar). Significance of signal reduction is indicated by *** ($p \leq 0.001$), ** ($p \leq 0.01$), and n.s. (not significant, $p > 0.05$).

DISCUSSION

In this paper we describe, to our knowledge for the first time, the identification of antagonists for hTAS2R39. For hTAS2R39, 4'-fluoro-6-methoxyflavanone (**6**), 6,3'-dimethoxyflavanone (**3**), and 6-methoxyflavanone (**11**) were identified as antagonists (in decreasing order of potency), amongst which (**6**) fully eliminated the agonistic response. This was observed both for the bitter tea flavonoid ECG and for the synthetic bitter compound denatonium benzoate. The activation of hTAS2R14, another bitter receptor recognizing ECG ***(16)***, was also inhibited by the three flavanones, though to a lesser extent.

In view of the fact that the application of (**11**) and (**3**) did not lead to full inhibition of the ECG signal on hTAS2R39, the question arises whether these two compounds are antagonists or partial agonists. They were tested for hTAS2R39 agonism as well, but none

of them activated the receptor (data not shown). Hence, they probably act as real antagonists.

Structural requirements for hTAS2R39 antagonists

Several flavanones similar to the antagonists identified did not show inhibitory activity towards bitter receptor hTAS2R39. It turned out that only flavanones with a methoxy group on the 6-position of the A-ring, and various B-ring configurations were able to act as antagonists of hTAS2R39, as flavanone (**5**) (substitution of flavanone crucial for inhibition), 6-hydroxyflavanone (**8**) (methoxy-substitution crucial for inhibition), 4'-methoxyflavanone (**13**) (A-ring methoxylation crucial for inhibition), and 7-methoxyflavanone (**12**) (6-position crucial for inhibition) did not show inhibitory activity. Additionally, the compound 6-methoxyflavone was unable to inhibit hTAS2R39 activation (data not shown), which indicated that absence of a double bond in the C-ring is essential for inhibition.

Amongst the antagonists identified for hTAS2R39, the difference in substitution of the B-ring determined the blocking potency. Compound (**11**), which is unsubstituted on the B-ring, showed poor blocking behavior compared to (**6**) and (**3**). It might be speculated that size, electronegativity and/or electron withdrawing effect of the B-ring substituent influences the potency of antagonists.

Pharmacological characterization of 4'-fluoro-6-methoxyflavanone

Due to full elimination of agonistic responses by 4'-fluoro-6-methoxyflavanone (**6**) on hTAS2R39, this molecule was investigated further with respect to a possible antagonistic mechanism. Two effects were observed when increasing 4'-fluoro-6-methoxyflavanone concentrations: the dose-response curves shifted to the right, and the signal amplitudes decreased. These phenomena suggest that it can be classified as insurmountable antagonist ***(22)***. This curve pattern can be an indication for three different mechanisms: (i) irreversible antagonism, (ii) reversible non-competitive orthosteric antagonism, in which an equilibrium is not reached, and (iii) reversible insurmountable allosteric antagonism ***(22)***. Washout experiments (**Figure 7C**) clearly showed that the ECG responses after washing-out the antagonist were similar to the ECG responses prior to application of the antagonist. It can thus be concluded that the interaction was reversible. Next, in order to distinguish between (ii) and (iii), the antagonistic behavior towards another, structurally different, agonist was investigated. If the antagonist would be unable to inhibit a structurally different agonist, it would strongly suggest allosteric antagonism ***(22)***. As can be seen in **Figure 7B**, the synthetic hTAS2R39 agonist denatonium benzoate was also inhibited by 4'-fluoro-6-methoxyflavanone and exhibited a curve pattern almost alike that by ECG upon different blocker concentrations. Analogous results for two structurally different agonists might on

the one hand indicate orthosteric antagonism, but on the other hand they do not completely exclude allosteric antagonism ***(22)***.

For hTAS2R46, hTAS2R31, hTAS2R43 ***(23)***, hTAS2R16 ***(24)***, and hTAS2R38 ***(25)***, docking simulations into homology models, validated by site-directed mutagenesis, have predicted the presence of a single binding pocket in the respective bitter receptors. Furthermore, the mechanism of antagonism of GIV3727 on hTAS2R31 was described as orthosteric, insurmountable antagonism ***(5)***, supported by docking the antagonist into the same binding pocket as the agonist. In contrast, one study suggests allosteric antagonism as mechanism for inhibition of hTAS2R16 and hTAS2R38 by probenecid ***(6)***. No information has been reported yet on the binding pocket of hTAS2R39. As the majority of studies suggests the presence of a single binding pocket in different bitter receptors, an orthosteric mechanism seems most likely for explaining our observations.

Application of bitter receptor blockers

For application of blockers in food products, several requirements should be met. (i) The blocker should be functional at a low dose. Therefore, an antagonist that has to be applied in equimolar or higher quantity to the agonist, is not efficient. (ii) In order to block the bitter taste of dietary compounds, for practical reasons, it is necessary that a bitter receptor blocker is also functional when applied simultaneously with the bitter compound. Therefore, compounds like (**3**) and (**6**) seem to be more suitable than (**11**). (iii) In order to achieve a sensorial effect, blocking of all bitter receptors activated by one compound is desirable. A blocker, that can only inhibit hTAS2R39, but not hTAS2R14, might be too specific to effectively reduce bitterness of ECG. On the other hand, it is not known yet whether these two receptors have an equally important role in the mouth. (iv) The blocker should preferably be of natural origin, and should be available in sufficient quantity. We could not find any natural source of the three inhibitors described in this study, and assume that they are only available synthetically. (v) Only compounds that are known as safe for consumption are of interest for food applications. Due to unknown safety of 4'-fluoro-6-methoxyflavanone, we abstained from sensory tests, which might confirm the function as bitter taste blocker ***in vivo***. As not all criteria are met by the blockers discovered in the present study, they might not be applicable to food products. Nevertheless, a 6-methoxy substituent on the A-ring of a flavanone has been identified as important for inhibition of hTAS2R39, which might form the basis for other, more suitable, blockers.

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Chapter 5

Evaluation of the bitter-masking potential of food proteins for EGCG by a cell-based human bitter taste receptor assay and binding studies

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ABSTRACT

Epigallocatechin gallate (EGCG) has been ascribed to several health benefits, but its bitter taste influences the liking of products with high concentrations of this compound. β-Casein, in particular, and several gelatins are known as strong binders of EGCG, contrary to β-lactoglobulin. The current study aimed at relating the EGCG-binding characteristics of those proteins, and their food-grade equivalents, to their effects on reducing bitter receptor activation by EGCG *in vitro* and their bitter-masking potential *in vivo*. Also in the bitter receptor assay, β-casein showed the strongest effect, with a maximum reduction of hTAS2R39 activation of about 93%. A similar potency was observed for Na-caseinate. β-Lactoglobulin had little effect on bitter receptor activation, as expected based on its low binding affinity for EGCG. The bitter-masking potential of Na-caseinate was confirmed *in vivo* using a trained sensory panel. β-Lactoglobulin also slightly reduced EGCG bitter perception, which could not be directly related to its binding capacity. The bitter receptor assay appeared to be a valid tool to evaluate *in vitro* the efficacy of food proteins as complexing agents for bitter-masking.

INTRODUCTION

Epigallocatechin gallate (EGCG) is known to be the most abundant catechin in green tea (ca. 60 % of the total catechins) and has been ascribed to several beneficial health effects (e.g. anticarcinogenic and cardioprotective effects) *(1)*. With respect to taste, tea catechins are known to be astringent and bitter *(2)*. The mechanism of astringency perception is not yet fully defined, but can be partially attributed to the interaction of EGCG with salivary proteins. Astringency seems sensorially coupled with bitterness, although, compared to the latter, it has been usually reported as a secondary attribute in time-intensity experiments *(3, 4)*.On the human tongue, bitter compounds are perceived by bitter taste receptors, referred to as hTAS2Rs, which are part of the family of G-protein coupled receptors *(5)*. To date, 21 hTAS2Rs out of the 25 known have identified agonists *(6, 7)*. Amongst these, hTAS2R39 has been associated with taste perception of green tea catechins *(8)*. As evaluated *in vitro* with hTAS2R39, EGCG has a two times lower EC_{50} value (181.6 µM) compared to its non-galloylated equivalent epigallocatechin (EGC; EC_{50} = 395.5 µM). This difference was confirmed *in vivo* by a higher perceived bitterness for EGCG *(2, 8)*.

Effective health benefits against cardiovascular and metabolic diseases have been associated with a daily intake of green tea containing 200-300 mg of EGCG *(9)*. Food products with high concentrations of EGCG may have off-tastes and consequently low consumer acceptance *(10)*. Various approaches to modulate bitterness of bioactive compounds in functional foods have been described, such as the use of sweeteners, blockers for bitter taste receptors and complexation with other compounds. In the latter approach, cyclodextrins are the most commonly used carriers while other carriers (e.g. proteins) are seldomly reported *(10-13)*. A typical example of off-taste reduction in food is the addition of milk to tea, which has been linked to the interaction of tea catechins with milk proteins *(3)* without impairing their bioavailability *(14)*. Milk proteins have also been suggested as carriers for bioactive compounds and, in particular, thermally-induced β-lactoglobulin-EGCG complexes *(12, 15)*.

In our previous work *(16)*, we investigated the potential of food proteins as carriers for flavonoids. Based on affinities and binding capacities measured, β-casein and gelatins, in particular fish gelatin, were found to be the most promising carriers for EGCG. One necessary condition for the applicability of those complexes in food is their effective reduction in bitter taste perception of EGCG. Bitter receptor activation by flavonoids can be evaluated *in vitro* by a cell-based receptor assay *(8, 17)*. To our knowledge, the present study is the first report using such a setup to evaluate the reduction in activation of bitter receptors by EGCG after forming complexes with proteins. This primary approach can help to predict the outcome of sensory panels. The aim of the present study was to correlate EGCG-binding of pure β-casein, food-grade caseinates and several gelatins to the potential of these proteins for reducing bitterness perception of EGCG. This was first tested *in vitro*

using a cell-based bitter receptor assay and then *in vivo* with a trained sensory panel, in order to evaluate the applicability of those complexes in foods.

MATERIALS AND METHODS

Materials

Bovine β-casein (≥98% of total protein), bovine β-lactoglobulin (≥90% of protein), solid fish gelatin (Gelatin F1) from cold water fish skin, and gelatin type B (Gelatin B1) from bovine skin (75 bloom) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The protein contents of the two gelatins were estimated to be ≥90% as detailed in a previous study *(16)*. Food-grade EGCG (≥94%), Sunphenon 90LB (>80% catechins including ~50% EGCG), and food-grade calcium and sodium caseinates (protein content ≥90%, N×6.38) were kindly provided by DSM Nutritional Products (Delft, The Netherlands), Taiyo GmbH (Filderstadt, Germany) and DMV International (Veghel, The Netherlands), respectively. Bovine Vinoferm® gelatin powder (≥85% of protein; N×5.55), Vinoferm® gelatin liquid (20% (w/v) gelatin (supplier information)) and Vinoferm® Isinglass (fish gelatin, 2% (w/v) protein (supplier information)) were food-grade and purchased from Brouwland (Beverlo, Belgium). Throughout this study, these gelatins are referred to as gelatin B2, gelatin B3, and gelatin F2, respectively. Food-grade BioPURE - β-lactoglobulin™ (≥90% of total protein) was kindly provided by Davisco Food International (Eden Prairie, MN, USA). Water for *in vitro* tests was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Water (Spa Reine, Spadel Group, Brussels, Belgium) for *in* vivo tests was obtained from a local supermarket. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Assessment of binding of proteins to EGCG by ultrafiltration (UF assay)

Determination of binding parameters of food-grade proteins for EGCG (UF assay – method 1). All samples were prepared in a 50 mM sodium phosphate buffer, pH 7.0. Protein stock solutions (0.2 mM) were prepared freshly before each experiment. For both caseinates, an average molecular mass of 23.3 kDa was calculated from the protein composition in bovine milk *(18)*. Gelatins were prepared at a concentration equivalent to 0.2 mM β-casein (i.e. 4.72 mg/mL). Similarly, a stock solution of EGCG (6 mM) was used to obtain a range of dilutions between 0 and 6 mM. EGCG-protein mixtures were prepared and the binding affinities of each protein towards EGCG measured using an ultrafiltration microtiter plate setup (Ultracel 10, Millipore, Cork, Ireland) as described previously *(16)*.

The protein-bound and free fractions of EGCG at each concentration tested were calculated and plots of the bound fraction versus the concentration of free EGCG were used to determine the binding parameters. For each binding curve obtained, a linear regression

was used on the initial linear increase (R2>0.8) in order to estimate the binding affinity (K) of the compounds. A maximal binding capacity (Rmax) was derived from the plateau value or the highest bound fraction observed at high phenolic compound/protein molar ratios. Binding parameters were reported as mean ±standard deviation (SD) of two replicates.

*Determination of [EGCG]free with increasing concentrations of various proteins (UF assay – method 2)*An EGCG stock solution (0.5 mM) and solutions of proteins with concentrations ranging from 0.013 to 0.2 mM (EGCG-to-protein molar ratios from 2.5 to 40) were prepared in a similar way as described above. The concentration of free EGCG remaining in the mixtures after incubation was determined using an ultrafiltration microtiter plate setup as described previously *(16)*.

In vitro assessment of hTAS2R39 activation by intracellular calcium release

Activation of bitter receptors was investigated by the release of intracellular Ca2+, using a fluorescent calcium dye *(19)*. The expression of hTAS2R39 in HEK293 cells and the detailed procedure for monitoring its activation were performed as reported elsewhere *(17)*.

All samples were prepared in Tyrode's buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM Hepes, pH7.4). EGCG stock solution (1 mM) was prepared freshly before each experiment. Similarly, stock solutions of proteins (0.8 mM) were used to obtain a concentration range from 0.006 to 0.8 mM. Protein-EGCG complexes were made in a microtiter plate by mixing protein solutions 1:1 with EGCG solutions. Controls were made by mixing EGCG with buffer without proteins. The microtiter plate was incubated at room temperature under constant shaking (300 rpm, 10 min).

Next, the complexes were loaded (ratio 1:1 (v/v)) in a microtiter plate containing the cells (final concentrations of EGCG of 0.25 mM and of protein between 0 and 0.2 mM) and evaluated for their potential to activate bitter receptor hTAS2R39 at 37 °C with a FlexStation II 384 (Molecular Devices Corp., Sunnyvale, CA, USA) for 90 s as described elsewhere *(17)*. Prior to the addition of the complexes to the cells, the baseline signal was determined in the first 17 s. Then, fluorescence signals (excitation 485 nm/emission 520 nm) were measured until 90 s. As negative control, non-induced cells, which do not express the hTAS2R receptor, were always measured in parallel. Additionally, a dose-response curve of EGCG was determined in the same way by measuring concentrations of EGCG up to 1 mM without proteins. Measurements were performed at least in duplicates on two or more different days.

Sensory analysis

Panelists. The panel consisted of 13 persons (10 males and 3 females), which were part of a larger sensory panel familiar to bitterness rating and selected within Unilever R&D

(Vlaardingen, The Netherlands) for their ability to taste and rank bitterness. Panelists could join each session on a voluntary basis and participated in at least one of the sessions described below. Panelists were trained to taste bitterness and rate it on a scale from 1 to 10 using a known reference (Sunphenon 90 LB). Sunphenon 90 LB was sensorially close to EGCG as it was mostly bitter with a low astringency (supplier information) *(2)*. The ratings reported were relative to this EGCG-rich reference and not to pure EGCG. No specific training was conducted to discriminate between astringency and bitterness.

Sample preparation. Proteins (10 g/L) and EGCG (1 g/L) stock solutions were prepared freshly before each experiment. The compounds were dissolved in water (Spa Reine), known for its neutral pH and low content in minerals. EGCG was mixed 1:1 with each protein solution and incubated at room temperature for at least 10 min. 15 mL of each sample was poured into a yellow cup. The colored cups were used to limit the visual perception of the differences between samples (e.g. slight haze or color) with or without proteins and thus to limit its possible impact on the choice and rating by the panelists. Additionally, three solutions of Sunphenon 90 LB were prepared: Two references known to the panelists (0.3 g/L and 0.8 g/L) and one unknown to the panelists (0.4 or 0.5 g/L). The former were used as a calibration for the panelists prior to and throughout each session. The latter was used by the panelists to check the accuracy of their rating at the beginning of each session.

Selection of suitable food-grade proteins for quantitative sensory analysis. A preliminary session was organized to select food-grade proteins with the highest potential for bitterness reduction. Volunteers from the panel (n=8) were allowed to taste a known sample with only EGCG (0.5 g/L) and rate it against the references. Then, each protein-EGCG sample was tasted, described individually and subsequently discussed with the other panelists. The group rating and the most recurrent descriptors were used to select the most suitable proteins for further experiments.

2-Alternative Forced Choice (2-AFC) test. EGCG complexed with Na-caseinate and β-lactoglobulin were evaluated in duplicate against a control EGCG sample without proteins. The experiment was conducted on two different days with 12 panelists and 6 of them were present on both days (n=36 per sample). Pairs of samples were all provided at once to each panelist. In between samples, panelists were instructed to rinse their mouth with water or milk, and to eat a piece of cucumber or plain cracker. For each pair, panelists had to indicate which sample was the most bitter and to rate the two samples on a scale from 0 to 10 as described above.

Ranking test. EGCG (1 g/L) was mixed 1:1 with 4 different concentrations of Na-caseinate (2, 5, 10, and 15 g/L) and prepared as described above. Panelists were provided with a series of 5 samples in duplicate (13 panelists, n=26 per sample) and were instructed to rank them in order of increasing bitterness and rate them on a scale from 0 to 10 as described above. Series included one control containing only EGCG (0.5 g/L) and no

proteins. The instructions given to the panelists for rinsing their mouth between samples were the same as for the 2-AFC test.

Data processing and statistical analysis

For hTAS2R39 activation data, SoftMax Pro 5.4 software (Molecular Devices Corp.) was used to plot fluorescence signals. Data processing for the activation curve of hTAS2R39 by EGCG was performed as described previously *(17)*. Similarly, for EGCG-protein complexes, 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 compounds, divided by the signals of the baseline to normalize to background fluorescence *(17)*. Besides the response of induced cells, also the response of non-induced cells was measured as negative control for every compound at every concentration on the same plate. In cases that a non-specific signal occurred with $\Delta F/F_0 > 0.25$, the corresponding concentration of the protein was not considered for further calculations. Response of non-induced cells was subtracted from its corresponding response of induced cells at all valid concentrations. The activation and decrease of receptor activation were expressed as percentages relative to the maximum response measured (i.e. EGCG control) and plotted versus the protein concentration. Data were reported as the mean value of the replicates and error bars represented the standard error of the mean (SEM).

The dose-response curve of hTAS2R39 by EGCG was fitted with nonlinear regression curves in Graph Pad Prism (version 4 for Windows, Graph Pad Software, San Diego, CA, USA). The sigmoidal dose-response curve model with variable slope corresponded to the following equation:

$$\frac{\Delta F}{F_0} = B + \frac{(T-B)}{1+10^{(LogEC_{50}-Log[EGCG])-H}} \qquad \text{(equation 1)}$$

with B, the bottom plateau value; T, the top plateau value; $LogEC_{50}$, the Log([EGCG]) value at which the response is halfway between B and T; and H, the Hill slope or steepness of the curve. Best-fit parameters for the activation curve of hTAS2R39 by EGCG were as follows: B = 0.107, T = 1.531, $LogEC_{50}$ = -3.793, and H = 1.976.

The aforementioned best-fit parameters and the dose-response curve equation were used to predict the receptor activation ($\Delta F/F_0$) which should be observed based on the concentration of free EGCG measured in the UF assay – method 2. The theoretical receptor activation was plotted as percentage of reduction of activation versus the protein concentration using the following equation:

$$\%\, reduction\ activation = \left(1 - \frac{\left(\Delta F/F_0\right)_i}{\left(\Delta F/F_0\right)_{250}}\right) \times 100 \qquad \text{(equation 2)}$$

With $\left(\Delta F/F_0\right)_i$, the theoretical receptor activation at $[EGCG]_{free}$ = i (in µM), and

$\left(\Delta F/F_0\right)_{250}$, the theoretical receptor activation at $[EGCG]_{free}$ = 250 µM. The data were reported as the mean value of the replicates and error bars represented the SEM.

Averages and confidence intervals (95%) from the 2-AFC and ranking tests were calculated. Significance ($p<0.05$) for the 2-AFC test was determined based on a minimum number of correct judgments for paired difference using a statistical table reported elsewhere *(20)*. Significance ($p<0.05$) for the ranking test was determined by Kramer's rank sum test *(21)*.

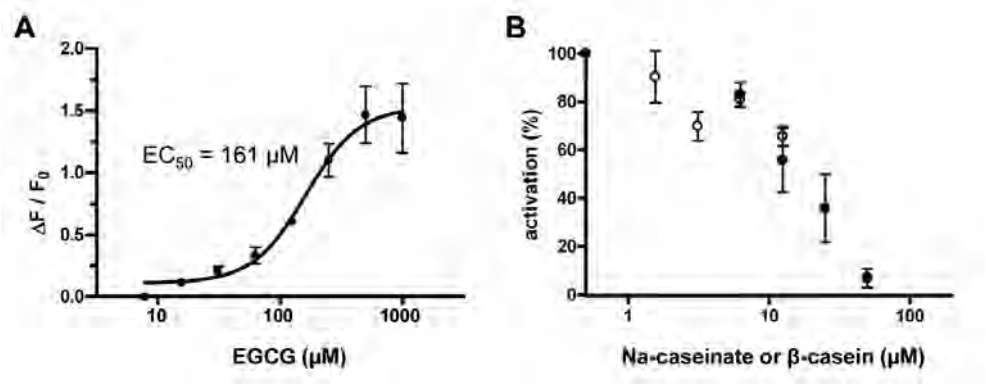

Figure 1. Dose-response curve of hTAS2R39 stimulated with EGCG (**A**) and example of receptor activation by EGCG (250 µM) complexed with increasing concentrations of β-casein (●) or Na-caseinate (○) (**B**).

RESULTS

Reduction of activation of bitter receptor hTAS2R39 by complexing EGCG to proteins

Based on a previous study on common food proteins binding EGCG *(16)*, β-casein, β-lactoglobulin, gelatin B1 and gelatin F1 were selected and tested for their potential to reduce the activation of the bitter receptor hTAS2R39 by EGCG in a cell-based assay. The test was conducted at a concentration of EGCG of 250 µM, which was about the EC_{70} value (EC_{50} = 161 µM, **Figure 1A**) and provided sufficient signal to clearly observe the effect of the proteins, as illustrated in **Figure 1B**. The decrease of hTAS2R39 activation by EGCG with the various proteins tested is reported in **Figure 2**. Maximum reductions of receptor activation (as percentages of reduction of activation from control EGCG without protein) for protein-EGCG complexes are summarized in **Table 1**.

Table 1. Comparison of the bitterness reduction potential of proteins evaluated ***in vitro*** and ***in vivo*** (based on preliminary experiment) at an EGCG-to-protein molar ratio of 5.

Protein	Grade	% Reduction of activation (cell assay)	% Reduction of activation (UF assay)[c]	Reduction of rating (***in vivo***)[d]
β-Casein	analytical	93.3 (±5.3)	72.8 (±2.9)	n.a.
Na-Caseinate	food	34.3 (±4.1)[a]	51.9 (±0.9)	3
Ca-Caseinate	food	n.d.	49.2 (±0.5)	3.5
β-Lactoglobulin	analytical	-[b]	5.8 (±2.9)	n.a.
β-Lactoglobulin	food	-[b]	9.0 (±0.5)	1
Gelatin B1	analytical	23.0 (±8.0)[a]	18.3 (±0.8)	n.a.
Gelatin B2	food	n.d.	n.d.	2.5
Gelatin B3	food	n.d.	n.d.	1.5
Gelatin F1	analytical	46.0 (±2.6)[a]	30.6 (±6.5)	n.a.
Gelatin F2	food	n.d.	n.d.	n.a.[e]

[a]percentage of the highest measurable protein concentration in the bitter receptor assay
[b]no clear trend in reduction of receptor activation detected
[c]percentages calculated based on concentrations of unbound EGCG in UF assay – method 2
[d]reduction of ratings and percentages calculated using a bitterness score of 7 for the EGCG reference
[e]strong sour taste overruled bitter taste
n.a., not applicable; n.d., not determined

Amongst the four tested proteins, β-casein showed the strongest concentration-dependent reduction of the receptor activation by EGCG, with a decrease of 93.3 (±5.3)% at the highest measurable protein concentration applied (i.e. 50 μM). β-Lactoglobulin did not show a clear effect on decreasing the receptor activation, and relatively high variations between replicates were observed. Gelatin F1 was found to have the second strongest reduction of the receptor activation by EGCG (maximum receptor activation decrease measured of 46.0 (±2.6)%), whereas its maximum decrease was reached at a lower protein concentration than with β-casein. Gelatin B1 did not reduce the activation of hTAS2R39 by EGCG by more than 23.0 (±8.0)% in the measurable protein concentration range, indicating that it had low potential for masking bitterness. The slight trend of decreasing receptor activation with increasing protein concentrations observed for gelatins B1 and F1, contrary to the clear trend observed for β-casein, might be caused by the turbidity of these samples, affecting the accuracy of the measurements. In fact, the presence of insoluble aggregates might have affected the loading volumes on the cells and interfered with fluorescence readings. The observations made for gelatins suggest that a larger proportion of EGCG remained able to interact with the bitter receptor when applying gelatins compared to β-casein.

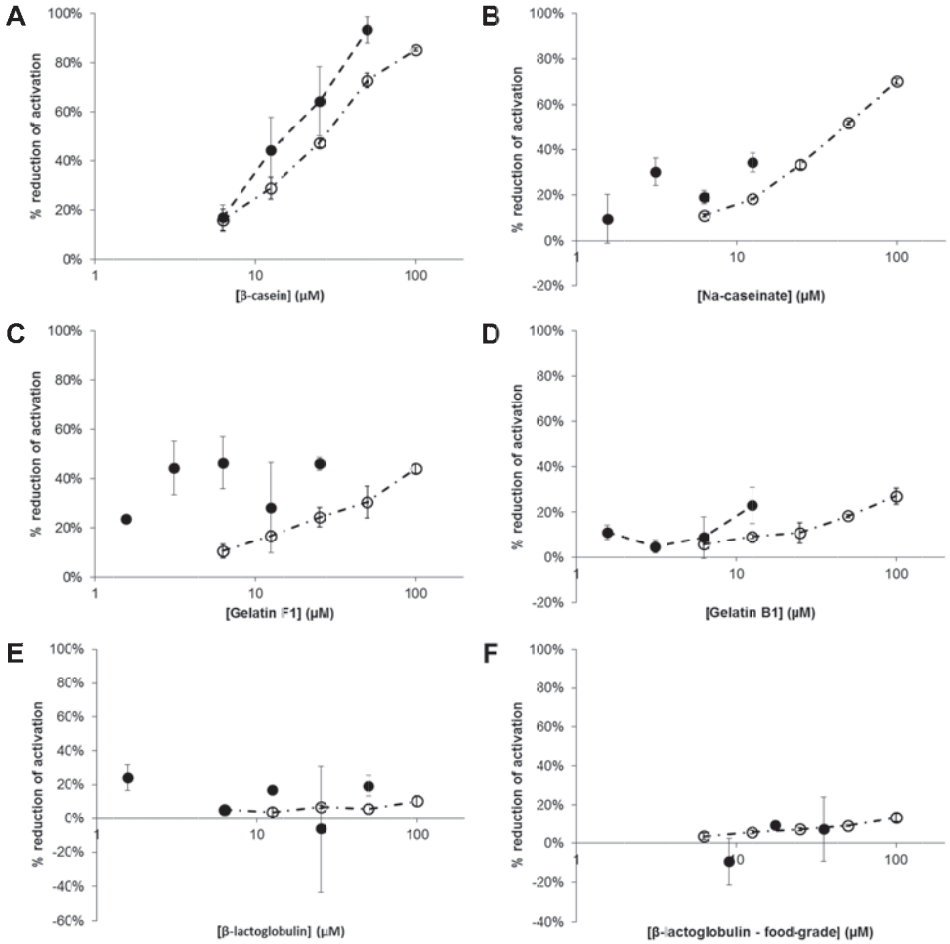

Figure 2. Comparison of percentages of reduction of hTAS2R39 activation by EGCG by various proteins measured experimentally with the bitter receptor assay (●) and predicted with data from the UF assay – method 2 (○) at constant concentration of EGCG (250 µM). (**A**) β-casein, (**B**) Na-caseinate, (**C**) gelatin F1, (**D**) gelatin B1, (**E**) analytical-grade β-lactoglobulin, (**F**) food-grade β-lactoglobulin.

Relationship between hTAS2R39 activation and binding characteristics of EGCG to analytical-grade proteins

The binding affinity (K) and maximal binding capacity (R_{max}) of the aforementioned proteins for EGCG were determined in a previous study *(16)* and are summarized in **Table 2**. β-Casein and gelatin F1 had similar affinities for EGCG, both about two times and ten times higher than the ones measured with gelatin B1 and β-lactoglobulin, respectively. Those affinities were found to be sufficient to have a strong effect on the reduction of activation of hTAS2R39 (**Figure 2A and 2C**). The limited effect of β-lactoglobulin on the

reduction of receptor activation in the bitter receptor assay is thought to be linked to its low affinity for EGCG. Even though gelatin B1 had an intermediate affinity for EGCG, it had a limited effect on the receptor activation by EGCG, suggesting that a minimum affinity is required for a significant reduction of receptor activation. Binding affinity seemed to be a more important factor than R_{max} as gelatin B1 had a higher R_{max} than β-casein, but only showed a limited effect on decreasing receptor activation.

A second ultrafiltration method (UF assay – method 2) was used to mimic the conditions of the bitter receptor assay (i.e. constant [EGCG] and variable [protein]). The concentrations of free EGCG measured in the UF assay – method 2 were used to predict the percentage of reduction of receptor activation using equations 1 and 2, and compared to the experimental data obtained. β-Casein was used to evaluate the accuracy of this approach (**Figure 2A**). The data derived from the UF assay – method 2 and the bitter receptor assay were in good agreement, with an underestimation of only 10-20% for the theoretical values compared to the experimental values.

In contrast to β-casein, the theoretical and experimental values showed clear discrepancies for gelatin F1 (**Figure 2C**). As mentioned earlier, interferences due to aggregates could explain this observation. In the case of gelatin B1 (**Figure 2D**), a limited amount of experimental data points could be matched, but similar trends between the theoretical and experimental values were observed. The theoretical percentages of reduction of receptor activation calculated for gelatin F1 are two times lower than those for β-casein although their previously reported affinities for EGCG are similar (**Table 2**) *(16)*. This shows that affinity is not the only parameter for an efficient reduction of receptor activation, although it might be a good first indicator. For example, the flexibility of β-casein and its ability to form micelles which could entrap EGCG might be advantageous characteristics compared to the more rigid structure of gelatins *(16, 22, 23)*. Summarizing, the bitter receptor assay was in good agreement with the ultrafiltration assay when applying a protein with a good binding capacity, such as β-casein. Discrepancies, however, could be observed when using proteins forming insoluble aggregates, such as gelatins, or having a low binding capacity, such as β-lactoglobulin.

In the present study, β-casein was confirmed as promising carrier for EGCG in food as its previously reported high binding affinity and capacity for EGCG could be linked to an effective reduction of bitter receptor activation by EGCG.

Table 2. Binding characteristics (affinity, K; maximum binding capacity, R_{max}) of analytical-grade (taken from previously published data ***(16)***) and food-grade proteins (current study) with EGCG by UF assay at pH 7.0, 25°C.

Analytical-grade proteins	K (10^3 M^{-1})	R_{max} (mol/mol)	R_{max} (g/100g)	Food-grade protein equivalents	K (10^3 M^{-1})	R_{max} (mol/mol)	R_{max} (g/100g)
β-Casein	45.0(±7.2)	19.6(±4.9)	38.1(±9.6)	Na-Caseinate	22.8(±0.5)	12.8(±0.4)	24.7(±0.7)
				Ca-Caseinate	24.8(±0.5)	12.7(±0.0)	24.7(±0.1)
β-Lactoglobulin	4.5(±2.3)	6.6(±3.7)	16.5(±9.2)	β-Lactoglobulin	3.6(±0.3)	7.5(±0.3)	14.6(±0.5)
Gelatin B1	25.4(±4.1)	31.6(±2.1)	57.8(±3.8)	Gelatin B2	29.2(±0.6)	16.9(±0.4)	32.8(±0.8)
				Gelatin B3	6.9(±1.1)	9.8(±1.0)	19.1(±2.0)
Gelatin F1	53.3(±1.8)	57.5(±1.8)	43.9(±1.4)	Gelatin F2	7.8(±0.8)	10.8(±0.7)	21.1(±1.3)

Efficiency of food-grade protein ingredients to complex EGCG

Highly purified proteins are not commonly used in the food industry and are, in most cases, not commercially available as food ingredients. Hence, the analytical-grade proteins were replaced by common food ingredients containing these proteins (e.g. β-casein replaced by caseinates). These food-grade proteins were evaluated for their binding potential for EGCG by the UF assay and their binding parameters were compared to the ones of their equivalent analytical-grade proteins as summarized in **Table 2**. A higher concentration of proteins as used in the current assay (i.e. 100 µM) did not seem to influence the binding affinity as found for β-casein ((45.0 ±7.2) ×103 M-1 at 25 µM versus (43.3 ±2.2) ×103 M-1 at 100 µM). Ca-Caseinate and Na-caseinate were considered as an acceptable replacement for β-casein with good potential for further application. The lower values obtained for the binding parameters of caseinates compared to β-casein might be related to their more heterogeneous protein composition, with a ratio αS1:αS2:β:κ of about 11:3:10:4, on a molar basis *(18)*. In fact, it has been shown that α-casein had a two times lower binding affinity for EGCG compared to β-casein *(24)*.

Na-Caseinate was tested for its capacity to reduce hTAS2R39 activation by EGCG (**Figure 1B**). Although only a narrower range of protein concentration could be measured due to non-specific signals, Na-caseinate showed a similar trend in receptor activation decrease compared to β-casein at protein concentrations between 1.5 and 6 µM. Theoretical percentages of reduction of receptor activation were calculated for Na-caseinate using the UF assay – method 2 and were similar to those obtained for β-casein (**Figure 2B**), with a maximum reduction of 70.1 (±1.2)% at a protein concentration of 100 µM (85.3 (±0.6)% for β-casein). Theoretical and experimental values at Na-caseinate concentrations of 6 and 12.5 µM were in good agreement. Ca-Caseinate showed the same theoretical potential of reduction of receptor activation compared to Na-caseinate using the UF assay – method 2, with a maximum reduction of 72.0 (±0.9)% (data not shown). Hence, Na-caseinate and Ca-caseinate were confirmed as acceptable food-grade alternatives to analytical-grade β-casein for reducing hTAS2R39 activation by EGCG.

Gelatin B2 showed similar binding potential compared to the model protein gelatin B1, whereas gelatins B3 and F2 displayed lower binding affinities compared to their analytical equivalents. This difference compared to the model proteins could be due to variations in the characteristics of the gelatin samples (e.g. amino acid composition, average molecular mass). Food-grade and analytical-grade β-lactoglobulins had similar low binding affinities (**Table 2**). As shown in **Figure 2** and **Table 1**, the weak binding properties of both β-lactoglobulins to EGCG also did not result in a significant effect in reduction of hTAS2R39 activation. This was also shown by the UF assay – method 2 with a maximum value in reduction of receptor activation below 15% calculated for both proteins (**Figure 2E** and **2F**).

Sensory analysis of EGCG complexed with proteins

As summarized in **Table 3**, various food-grade proteins were compared in a preliminary sensory experiment for their potential to reduce bitterness of EGCG at a protein-to-EGCG mass ratio of 10, which was equivalent to an EGCG-to-protein molar ratio of about 5 using the molecular mass of β-casein. Ca-Caseinate and Na-caseinate had similar effects on the taste of EGCG with a reduction of EGCG bitterness rating by 3.5 and 3 units, respectively. The relatively transparent appearance of Na-caseinate was preferred over the white color of Ca-caseinate in water for further investigation as it offers fewer limitations for applications (e.g. in clear beverages). β-Lactoglobulin had the least effect on the bitterness reduction of EGCG (1 unit). Therefore, it was selected as a negative control for further experiments. The three gelatin samples generally had unpleasant off-tastes, especially gelatin F2. In addition, gelatins B2 and F2 formed visible aggregates with EGCG at the molar ratio used. Taken together, gelatins were considered as unsuitable for further sensory tests and applications as bitter masking compounds.

Table 3. Sensorial comparison of EGCG (0.5 g/L) complexed with various food-grade proteins (5 g/L).

Protein	pH in water[a]	Rating	Aspect	Taste attributes (other than bitter)
EGCG control	6.3	7	clear	-
Ca-Caseinate	6.9	3.5	turbid (milk-like)	milky, astringent
Na-Caseinate	6.9	4	slightly turbid	milky, slightly metallic
Gelatin B2	6.0	5.5	visible aggregates	astringent, burned, strong off-taste
Gelatin B3	4.9	4.5	slightly turbid	off-taste
Gelatin F2	2.6	n.a.[b]	visible aggregates	very sour
β-Lactoglobulin	6.3	6	clear	slight off-taste

[a]pH of EGCG-protein complexes after incubation; [b]n.a., not applicable, strong sour taste overruled the bitter taste

In a 2-AFC test, β-lactoglobulin and Na-caseinate significantly reduced the bitter taste of EGCG by 1.4 ±0.4 and 2.3 ±0.5 units, respectively (**Figure 3**). The effect of Na-caseinate on EGCG bitterness perception was in accordance with the expectations based on the reduction of hTAS2R39 activation by Na-caseinate (**Figure 2B**). A significant, although lower, effect of β-lactoglobulin on bitterness of EGCG was not expected as only a limited effect was observed in a preliminary sensory session (**Table 3**) and also in the *in vitro* assays (**Figure 2D**).

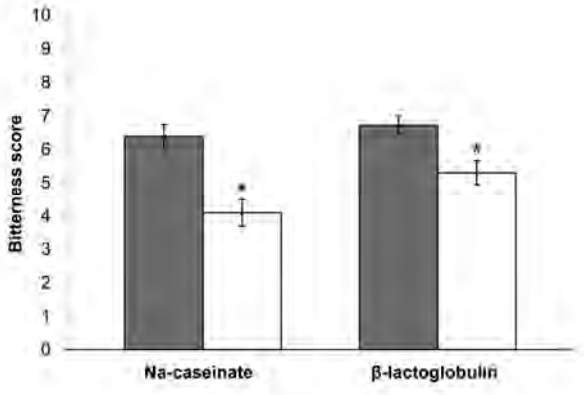

Figure 3. Comparison of perceived bitterness of EGCG, free (grey bars) or complexed with Na-caseinate or β-lactoglobulin (transparent bars), in a 2-AFC sensory test. (*) significant difference ($p<0.05$).

In a ranking test with increasing concentrations of Na-caseinate, it appeared that the lowest bitterness score (~4) was already reached at a concentration of 0.25 % (w/v) of Na-caseinate (**Figure 4A**). This observation concurs with a sensory study on olive oil phenolics binding Na-caseinate, for which a minimum bitterness score was reached with 1% (w/v) protein and did not decrease further with increasing protein concentration *(25)*. In the current study, 0.25% (w/v) Na-caseinate resulted in a bitterness reduction of 3 units compared to the EGCG reference, and the reduction of bitterness ratings reported at lower EGCG-to-protein molar ratios remained between 3 and 3.5 units (**Figure 4B**). As shown in **Figure 4B**, the reduction of bitterness ratings measured *in vivo* at EGCG-to-protein molar ratios of 10 and 25 followed the trend of the theoretical percentages for the reduction of receptor activation calculated from the UF assay – method 2. At molar ratios lower than 10, however, a plateau was observed *in vivo* while *in vitro* theoretical values predicted a continuously increasing percentage of reduction of hTAS2R39 activation.

DISCUSSION

In vitro prediction of bitterness reduction compared to *in vivo* sensory analysis

According to the *in vitro* assays conducted in this study (**Figure 2**), the efficacy in reducing bitterness of EGCG of the food-grade proteins tested should be ranked as follows: Ca-caseinate/Na-caseinate ≥ gelatins > β-lactoglobulin. This order was in line with our first sensory experiment (**Table 3**), confirming the applicability of our *in vitro* approach for screening the potential of food proteins for bitter masking.

A 2-AFC test demonstrated a decrease of perceived bitterness of EGCG when complexed to Na-caseinate by 2.3 units. The effect of Na-caseinate on the intrinsic bitterness of EGCG *in vitro* at the same EGCG-to-protein molar ratio (i.e. 5) was calculated to be a decrease of 50% based on the concentration of free EGCG after binding measured by ultrafiltration and related to the activation curve of hTAS2R39 by EGCG. Although our *in vivo* and *in vitro* results match well, it should be noted that the *in vitro* assay does not take account of actors other than hTAS2R39 in the mouth environment, such as other hTAS2Rs being activated by EGCG (although their response will also be modulated by complexation of EGCG to protein) and salivary proteins which might interact with EGCG and disturb the binding equilibrium. The intrinsic bitterness is calculated under the assumptions that hTAS2R39 is the main bitter receptor sensing EGCG, that complexes remain stable in the mouth, and that the slight off-taste of Na-caseinate does not influence bitterness ratings. In addition, a stronger effect of β-lactoglobulin on EGCG bitterness was observed *in vivo* compared to the *in vitro* experiments. This effect is unlikely to have resulted from its binding to EGCG, nor from a direct interaction of β-lactoglobulin with the hTAS2R39 receptor, as suggested for another protein *(26)*. An indirect effect due to the interaction of β-lactoglobulin with the buccal environment could have interfered with the bitter perception (e.g. interaction with saliva and buccal cells) *(27, 28)*.

In this study, a maximum reduction in bitterness of EGCG was achieved at 0.25% (w/v) of Na-caseinate, although our *in vitro* assay predicted a continuous decrease in receptor activation with increasing protein concentrations (**Figure 4B**). This concurs with a model proposed by Pripp *et al.*, which predicted a minimum bitterness reached at 0.5% (w/v) Na-caseinate for olive oil phenolics, assuming a binding affinity of 10^5 M^{-1}.*25* The bitter receptor assay complemented with ultrafiltration appears as an appropriate tool to evaluate the efficacy of a given macromolecule as a bitter-masking ingredient, although it tends to overestimate its potential (**Figure 4B**). Discrepancies between *in vitro* and *in vivo* evaluation of bitterness have already been reported. For example, higher threshold concentrations and EC_{50} values for bitter hop compounds were found in a sensory test compared to the taste receptor assay, whereas the ranking in order of potency for the compounds was the same *(29)*.

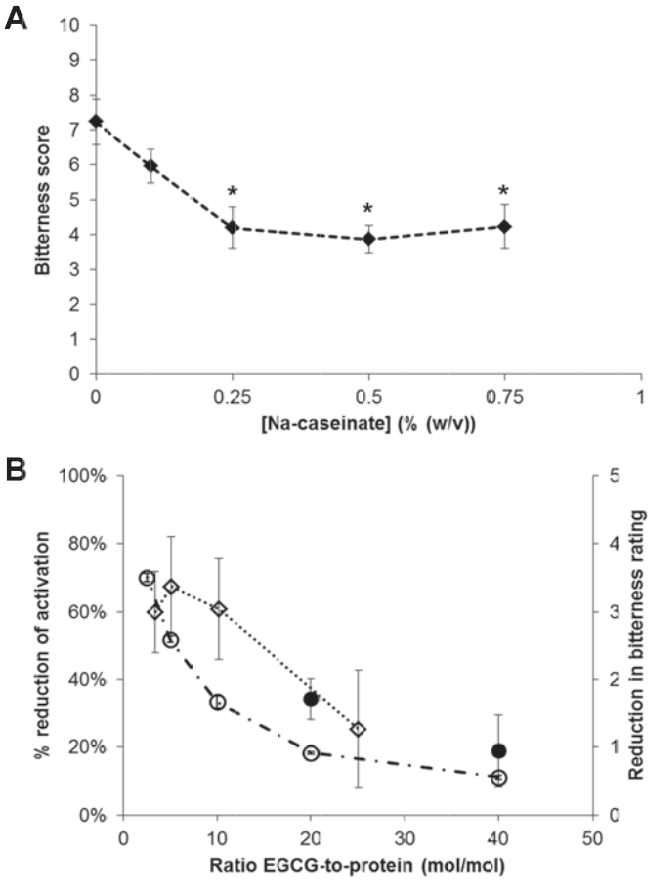

Figure 4. Dose-response curve for perceived bitterness of EGCG (0.5 g/L) with increasing concentration of Na-caseinate ((*) significant difference ($p<0.05$)) (**A**) and comparison of the reduction of bitterness ratings ***in vivo*** (◇) (right y-axis) with percentages of reduction of hTAS2R39 activation by EGCG with Na-caseinate measured experimentally with the bitter receptor assay (●) and predicted with data from the UF assay (○) (left y-axis) (**B**).

Bitter receptor assay as a tool to study bitterness masking by complexing agents

It has been shown that a cell-based bitter receptor assay can be a valuable tool to predict the intrinsic bitterness of food-related components *(8, 17, 29)*. In the present study, we report for the first time its potential in evaluating the modulation of the intrinsic bitterness of bitter tastants, e.g. EGCG, by combining them with complexing agents, such as proteins. Despite some limitations related to the range of protein concentrations that can be used, or the influence of turbidity on the measurement, challenging hTAS2R39 with a combination of bitter tastant and protein allowed a rapid identification of good candidates for complexing,

such as β-casein. Proteins were ranked for their efficacy for reducing receptor activation as follows, β-casein > gelatin F1 ≈ Na-caseinate > gelatin B1 > β-lactoglobulin. This ranking was in good agreement with findings from a complementary ultrafiltration assay relating the concentration of free EGCG with increasing protein concentration to hTAS2R39 activation.

Provided that the hTAS2R activated by the bitter compound of interest is known, the bitter receptor assay seems to be promising for the discovery of bitter-masking agents. It has been applied in several instances for high-throughput screening for so-called bitter blockers, i.e. compounds that act antagonistically on the bitter receptor of interest *(30, 31)*. These blockers are thought to be rather specific in reducing bitterness, although their suggested promiscuity (i.e. the bitter blocker inhibits several hTAS2Rs) *(31)*, or their potential agonistic behavior on other bitter receptors, might compromise this idea *(32)*. Besides, some bitter compounds have been described to activate more than one bitter receptor, which might call for more than one blocker for a particular bitter tastant *(6, 17, 29)*. Therefore, it might be advantageous to use a more generic approach for masking bitterness, e.g. by applying complexing agents, such as food proteins, which do not act directly at the receptor. We have now shown that the cell-based bitter receptor assay can be used as a tool to study such complexing agents, given that the protein is able to bind a significant amount of the bitter tastant.

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Chapter 6

General Discussion

As described in the *General Introduction* of this thesis, (iso)flavonoids are present in many food raw materials and food products. Also, they are additionally incorporated in functional food products as bioactive compounds in order to enhance their nutritional value. Due to contradictory statements on the bitter taste of some (iso)flavonoids in literature, and the unknown taste properties of other (iso)flavonoids, a systematic investigation of intrinsic bitterness and structure-activity relationships of these compounds was performed using receptor assays. To this end, the research described in this thesis started with (i) identification of the bitter receptor(s) activated by soy isoflavones, and (ii) investigation of structural requirements for (iso)flavonoids to activate the bitter receptors identified. A subsequent aim was the investigation of debittering strategies on receptor level by (iii) identification of bitter receptor antagonists, and (iv) complexation of flavonoids with food proteins.

This chapter discusses the findings presented in this thesis, addresses prospects and limitations of the bitter receptor cell assay, and compares taste evaluation by sensory tests, receptor assays and modeling. Furthermore, it evaluates strategies for bitter taste reduction, and applies the findings to soy products and tea.

(ISO)FLAVONOIDS AS DIETARY hTAS2R AGONISTS

Determination of hTAS2Rs activated by soy isoflavones

The first hypothesis of this Ph.D. research implied that compounds causing bitterness in soy products can be assigned to bitter taste receptors. As no consensus existed in literature about the impact of several isoflavones on bitterness, an objective tool to determine intrinsic bitterness of isoflavones was needed in order to identify the target molecules for modifying bitter taste of soy products. **Chapter 2** elaborates on the investigation of bitter receptor activation by soy isoflavones, as they are most frequently reported as responsible for bitterness in soy products. All 25 hTAS2Rs were screened for activation by genistein, genistin, acetyl genistin and malonyl genistin. At screening concentrations, genistein was the only compound activating bitter taste receptors, namely hTAS2R14 and hTAS2R39. Besides the predominant genistein form, also daidzein, daidzin, glycitein and glycitin were screened for activation of all 25 bitter receptors (not reported in **Chapter 2**), but at screening concentrations (400 µM), no receptor was clearly activated (data not shown). Investigation of dose-response behavior of all soy isoflavones mentioned towards activation of hTAS2R14 and hTAS2R39 revealed that also soy isoflavones other than genistein activated these two receptors, though at concentrations above the screening concentrations. It can, therefore, not be excluded that bitter receptors other than hTAS2R14 and hTAS2R39 might be activated by soy isoflavones as well. To conclude, the first hypothesis could be accepted, and hTAS2R14 and hTAS2R39 were identified as bitter receptors activated by soy isoflavones, with different impact of aglycones and glucosides.

Based on sensory tests, there was no consensus in literature about the impact on bitterness of genistein, daidzein, genistin, daidzin, and malonyl isoflavones They were all reported as bitter *(1-4)*, but while converting isoflavone glucosides enzymatically into their aglycones was suggested as method to reduce bitterness and astringency by one author *(5)*, another author reported increasing objectionable taste after β-glucosidase treatment, caused by aglycones *(6)*. The results presented in **Chapter 2** enable us to clarify the potential bitterness of soy isoflavones from the more objective receptor point of view. On hTAS2R39, the order of intrinsic bitterness was determined genistein > acetyl genistein > genistin / malonyl genistin / glycitin / glycitein / daidzein. hTAS2R14 was only activated by aglycones (genistein >> glycitein / daidzein). It is not known yet, which of the two receptors hTAS2R14 and hTAS2R39 is more important in the mouth, and whether the joint activation of two bitter taste receptors can lead to an additive effect in the mouth. Even without knowledge about this, it is expected that the aglycone genistein has a much larger contribution to bitter taste than daidzein and genistein glucosides, and therefore, probably represents the key bitter compound amongst soy isoflavones. This conclusion is discussed in **Chapter 2** in the context of intrinsic bitterness thresholds and isoflavone concentrations in different soy products.

Molecular signature of dietary (iso)flavonoids activating hTAS2R14 and hTAS2R39

The second hypothesis of this thesis research implied that bitter isoflavonoids and flavonoids hold molecular signatures involved in bitter receptor activation. This was investigated by comparing several isoflavonoids in their behavior towards hTAS2R14 and hTAS2R39 (**Chapter 2**), and was extended to a large set of flavonoids (**Chapter 3**). This allowed a systematic investigation of the influences of (iso)flavonoid backbones and substitutions. Owing to the large number of possible structural variations, molecular signatures responsible for bitter receptor activation were not easily recognizable. Therefore, different molecular modeling approaches were applied. In the end, molecular features of (iso)flavonoids with distinct relative spatial orientation were identified as signatures responsible for activation of hTAS2R14 and hTAS2R39. This allowed discrimination between active and inactive (iso)flavonoids. The substitution pattern of (iso)flavonoid aglycones was shown to be of higher importance for bitter receptor activation than the backbone structure. Therewith, the second hypothesis could be accepted, and we were able to precise the molecular signature involved.

Approximately 70 (iso)flavonoids revealed to be agonists of one or both bitter receptors hTAS2R14 and hTAS2R39 (**Chapter 3**). The identification of such a large number of agonists can be ascribed to the systematic investigation of substitution patterns and backbone variation of the (iso)flavonoids. So far, an approach like this has scarcely been reported in literature *(7)* and has never been applied with such a large set of

structurally similar compounds. Mostly, structurally different bitter compounds have been tested.

As discussed in **Chapter 3**, pharmacophore modeling revealed, which common signatures underlay binding of (iso)flavonoids to hTAS2R14 and -39. Therefore, it might be better to describe tuning breadth of bitter receptors in terms of the number of molecular signatures recognized by the receptor, instead of in terms of the number of receptor agonists. In this way, a collection of molecules with similar signature will only count as one with respect to tuning breadth, and tuning breadth is less likely to be overestimated.

Taste properties of dietary (iso)flavonoid sources

Besides looking at structural variation, it is of great interest to look at the occurrence in food products of the compounds exhibiting the highest intrinsic bitterness. The dietary isoflavonoids identified as bitter receptor agonists occur mainly in soybeans and soy products, amongst which fermented products, such as tempeh *(8)* (an Indonesian soybean cake), are especially rich in the aglycones identified. The isoflavonoid activating hTAS2R14 and hTAS2R39 with the lowest threshold (4 µM and 8 µM, respectively) was genistein, the aglycone of the predominant soy isoflavone form. The dietary flavonoids identified as bitter receptor agonists occur in various kinds of honeys, fruits, vegetables, herbs and roots (licorice). Some compounds identified with very low bitterness thresholds are present in many food sources *(9-11)* (*e.g.* kaempferol and luteolin), whereas others are quite rare *(9-11)* (*e.g.* morin and pinocembrin), or have disappeared from our diet in modern days *(12)* (*e.g.* silibinin).

Several dietary sources of (iso)flavonoids have been reported as bitter before, whereas other dietary sources, containing (iso)flavonoids identified as intrinsically bitter in **Chapter 3**, are not known to be bitter (*e.g.* strawberries (source of morin and pelargonidin) *(9-11)*, licorice root (source of isoliquitirigenin) *(13)*, and oregano (source of apigenin, lutelin, pinocembrin, and naringenin). This is probably caused by the low concentrations of aglycones, or by naturally occurring masking compounds, such as sweet compounds (*e.g.* high sugar content in fruits and occurrence of intensely sweet triterpenoids in licorice root *(14)*, and the intense aroma of herbs. Products containing exceptionally high amounts of aglycones are typically tea and fermented soy products, which are discussed further on in this chapter.

(Iso)flavonoid glycosides have scarcely been investigated, although they are often the predominant (iso)flavonoid form in foods. So far, the only hTAS2R activation reported for flavonoid glycosides is the activation of hTAS2R39 by four isoflavone glucosides (reported in **Chapter 2**, published as *(15)*) and the activation of hTAS2R7 by malvidin-3-glucoside *(16)*. Therefore, a next step of interest would be the investigation of more flavonoid glycosides for hTAS2R activation, although this is currently limited by the poor commercial availability of these compounds.

Possible impact of hTAS2R39 on food perception

With the identification of (iso)flavonoids as bitter receptor agonists in this thesis, the number of dietary bitter taste receptor agonists for hTAS2R14 and hTAS2R39 is elevated enormously. The number of agonists for hTAS2R14 was doubled, which is largely contributing to the spectrum of dietary agonists for hTAS2R14. More than two thirds of all hTAS2R39 agonists have been newly reported in this thesis, of which the majority is of dietary origin. Dietary agonists have been reported for 16 of the 25 bitter taste receptors. Amongst them, a few receptors are activated by numerous representatives of the same chemical class, *e.g.* sesquiterpene lactones (mainly hTAS2R46, hTAS2R10 and hTAS2R14) *(17)*, peptides (mainly hTAS2R1, hTAS2R4, and hTAS2R39) *(18-21)*, hop acids (hTAS2R1, hTAS2R14, and hTAS2R40) *(22)*, steviosides (hTAS2R4 and hTAS2R14) *(23)*, and flavonoids (mainly hTAS2R14 and hTAS2R39) (this thesis, **Chapters 2 and 3** and *(16)*). In contrast to niche compounds, peptides and flavonoids are ubiquitously present in many food products consumed in our daily diet. This makes hTAS2R39 a potentially important receptor for sensing bitter compounds in foods, although its contribution in the mouth (e.g. density in comparison to other hTAS2Rs) is still not known. Furthermore, no functional polymorphisms for this receptor are known to result in different sensitivities towards certain bitter compounds, as reported for *e.g.* hTAS2R16, hTAS2R38, and hTAS2R43 *(24-26)*. This could mean that everyone should be able to perceive the bitter taste of (iso)flavonoids.

PROSPECTS AND LIMITATIONS OF THE BITTER RECEPTOR CELL ASSAY

In this thesis, it has been demonstrated that the bitter receptor assay is an appropriate tool not only for identification of bitter receptor agonists (**Chapter 2 and 3**) and antagonists (**Chapter 4**), as reported before, but also for identification of reduced receptor activation by complexing agents (**Chapter 5**). So far, bitter receptor assays have been used with relatively small molecules only. We have shown now that this cell assay can also be applied in combination with macromolecules. The applicable concentrations were, however, limited to ~1 g/L for most proteins investigated.

Some compounds reported in **Chapter 2 and 3** could not be characterized due to non-specific signals. The receptor assay in its widely used form appeared to be inappropriate to some bitter compounds. This will be discussed below based on results for soy saponins, another class of bitter compounds, not earlier reported in this thesis.

Screening bitter receptors for activation by soy saponins

In soybeans, not only isoflavones have been reported as bitter, but also saponins. Soybean saponins are triterpenoid glycosides, consisting of an aglycone backbone (called sapogenin

or sapogenol) and one or two glycosidic chains consisting of two or three units *(27)*. Directed measures against the bitter taste of soy products might be rationally taken when also the target receptor for saponins is known. Therefore, we tried to identify the bitter taste receptor(s) responsible for these molecules. To this end, a bitter receptor screening was performed with a soy saponin concentrate[1] and with the purified major soy saponin Bb (**Figure 1,** isolated according to *(27)*), following the same experimental procedures as outlined in **Chapter 2**. Results of screening for bitter receptor activation by saponin Bb are shown in **Figure 2**. The results show non-specific signals without clear distinction between signals from induced and non-induced cells. Similar curves were obtained for the soy saponin concentrate (data not shown), and at other concentrations. Cell permeabilization (determined by ATP test) and influence of micelle formation ability (determined by surface tension measurements) of saponins could be excluded as reasons for the phenomena observed, as cells were vital at all concentrations used and results were independent of critical micelle concentration (data not shown).

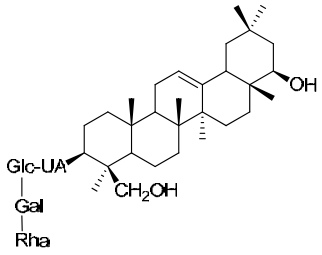

Figure 1. Structure of the major soy saponin, saponin Bb. Glc-UA, glucuronic acid; Gal, galactose; Rha, rhamnose.

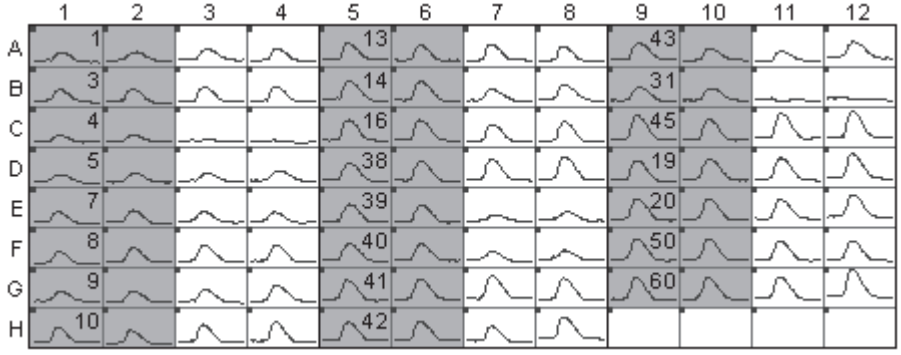

Figure 2. Calcium release signals of HEK293 cells stably expressing hTAS2Rs (except hTAS2R 46 and 30 (formerly hTAS2R47)) and $G\alpha_{16}$-gust44, stimulated with 130 µM soy saponin Bb. Non-induced cells (receptor not expressed) are measured as control (depicted with grey background).

[1] Organic Technologies, Coshocton, OH, USA

In order to investigate whether the G-proteins incorporated in the cell lines employed might have influence on the non-specific signals, wild type HEK293 cells were stimulated with soy saponins as well. Stimulation of wild type HEK293 cells by soy saponins did not lead to a calcium release signal, whereas the non-induced HEK293 cells stably expressing $G\alpha_{16}$-gust44 (not expressing bitter receptors) did (**Figure 3**).

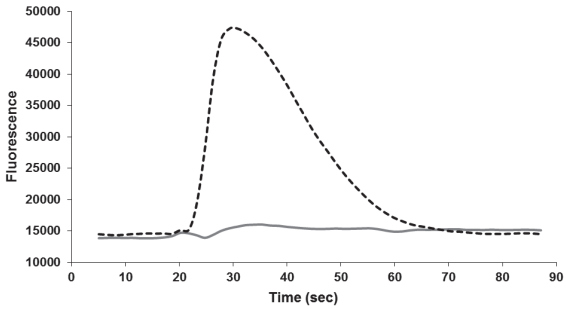

Figure 3. Calcium release signals of wild type HEK293 cells (grey line) versus non-induced HEK293 cells stably expressing $G\alpha_{16}$-gust44 (dashed black line), stimulated with 125 µM soy saponins.

$G\alpha_{16}$ is not present in wild type HEK293 cells. It was introduced into HEK293 cells, due to its capacity to activate an easy-measurable Ca^{2+} signaling pathway, as explained in the *General Introduction* of this thesis. The results shown in **Figure 3** indicated that the promiscuous $G\alpha_{16}$ might bind to endogenous GPCRs in HEK293 cells. Saponins might thus activate endogenous GPCRs, leading to non-taste receptor specific signals. Alternatively, direct activation of G-proteins by saponins might explain our observations. Direct activation of G-proteins by amphiphilic bitter compounds has been reported before *(28)*. Consequently, the bitter receptor assay in this setting might not be suitable for detection of bitter receptor activation by saponins. We speculate that the fact that not all 25 hTAS2Rs have been deorphanized yet, and that the target receptor for several bitter compounds has not been identified yet, might find its origin, amongst others, in the possibility that certain bitter compounds are not compatible with the cell assay employed, similar to our observations with saponins.

In order to develop an assay suitable for soy saponins, and possibly other compounds leading to non-specific signals, we suggest one of the following methods. Of most of these methods it still remains to be established whether they can indeed function as an alternative. (i) Expression of specific G-proteins in HEK293 cells. Due to its presence in native taste receptor cells, $G\alpha_{gustducin}$ might be used. As coupling of $G\alpha_{gustducin}$ (a $G\alpha_i$ protein) to bitter receptors might not result in intracellular Ca^{2+} release in a cell assay system, Ca^{2+} release has to be triggered by the $\beta\gamma$-subunit via the $PLC\beta_2$-IP_3/DAG pathway. We suggest the use of the native taste receptor cell G-proteins $G\beta_3$ and $G\gamma_{13}$, as it is unknown whether the β- and γ-G-proteins naturally occurring in HEK293 cells are able to interact with $G\alpha_{gustducin}$.

(ii) Use of cells naturally containing $G\alpha_{gustducin}$, such as HuTu-80 cells *(29)*, or native taste receptor cells, as recent advances have been reported in culturing these cells for a long period of time *(30)*. (iii) Use of another signalling method, different from Ca^{2+} measurement by fluorescence, ***e.g.*** measuring GTPγS-binding to $G\alpha_{gustducin}$ in insect membranes, as applied previously to bitter receptor assays by *(31, 32)*, or measuring bioluminescence resonance energy transfer (BRET) as reported for umami receptors *(33)*.

Despite the fact that no hTAS2R for soy saponins was identified under the conditions used in the receptor assay, it might be speculated which bitter receptor they activate. Saponins (which are triterpenoids), have structural characteristics in common with the steroid glycoside H.g.-12 (agonist of hTAS2R14 and hTAS2R7) *(34)*, steviol glycosides (agonists of hTAS2R14 and hTAS2R4) *(23)*, and with sesquiterpene lactones (agonists of mainly hTAS2R46, hTAS2R10 and hTAS2R14) *(17, 35)*. Therefore, one of these hTAS2Rs might be a candidate for activation by saponins. In additional experiments, not described before in the previous chapters of this thesis, we tested other triterpenoids, not occurring in soybean, but structurally very similar to the backbone of soy saponin Bb. These were the sapogenin aglycones betulinic acid, betulin, oleanolic acid, and ursolic acid[2] (structures shown in **Figure 4**), for their ability to activate hTAS2R7, hTAS2R10 and hTAS2R14. We found activation of hTAS2R14 by betulin, betulinic acid, and oleanolic acid, activation of hTAS2R7 by betulin, oleanolic acid, and ursolic acid, and no activation of hTAS2R10 (data not shown). These findings indicate that our assumption was reasonable, and likely the glycosidic chain of soy saponins hampered detection in bitter receptor assays. However, glycosylation does not hamper recognition by bitter receptors per se, as the glycosidic chain(s) of H.g.-12 and steviol glycosides do not seem to impair bitter receptor activation *(23, 34)*.

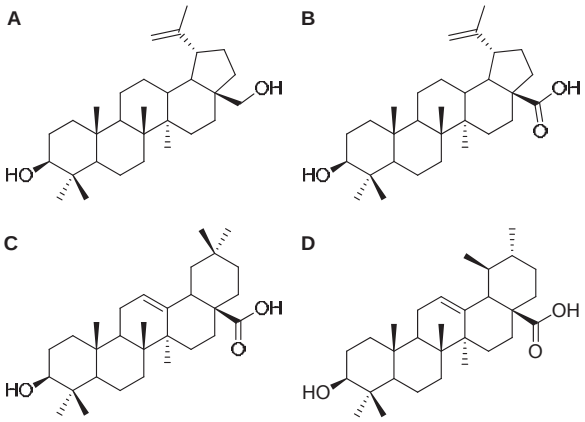

Figure 4. Structures of betulin (**A**), betulinic acid (**B**), oleanolic acid (**C**), and ursolic acid (**D**).

[2] all from Sigma Aldrich, Steinheim, Germany

ASSESSMENT OF ANTAGONISTIC PROPERTIES OF (ISO)FLAVONOIDS

In the third hypothesis of this Ph.D. research, it was assumed that flavanones have the ability to block (iso)flavonoid bitter receptors at the molecular level. This hypothesis could be partially accepted, but also has to be partially rejected. The flavanones homoeriodictyol and eriodictyol, reported in literature as masking agents against bitter taste perception of caffeine *(36)*, were not identified as antagonists, but as agonists of hTAS2R14 and hTAS2R39. Nevertheless, they might act as antagonists on another bitter receptor activated by caffeine (hTAS2R7, -10, -14, -43, and -46 *(17, 35)*). Amongst a set of 14 flavanones, we identified three flavanones as receptor blockers (**Chapter 4**). These three flavanones had a methoxy substituent on position 6 of the A-ring in common.

In **Chapter 3**, several of the compounds tested did not or poorly activate hTAS2R14 and/or hTAS2R39. It is likely that an antagonist might have similar structural elements to an agonist in order to fit into the same binding pocket. Therefore, additionally to the results presented for flavanones in **Chapter 4**, more (iso)flavonoids were tested for their ability to act as antagonists towards hTAS2R14 and/or hTAS2R39. All compounds reported in **Chapter 3** with a threshold of 250 µM or 500 µM, or with no activation detected on hTAS2R14 or hTAS2R39, were selected. They were applied together with genistein on hTAS2R14 or ECG on hTAS2R39, following the experimental procedures outlined for flavanones in **Chapter 4**. This resulted in the identification of apigeninidin chloride (**Figure 5A**), able to reduce activation of hTAS2R14 by genistein by 29 %, and of tricetin (**Figure 5B**) able to reduce activation of hTAS2R39 by ECG by 30 %. Based on the structural characteristics of these two compounds, similar compounds were selected and screened, resulting in the additional identification of luteolinidin chloride (**Figure 5C**) and 7,3',4',5'-tetrahydroxyflavone (**Figure 5D**). Luteolinidin chloride decreased the activation of hTAS2R14 by genistein by 36%, and 7,3',4',5'-tetrahydroxyflavone decreased the activation of hTAS2R39 by ECG by 22%.

Although all four compounds were able to reduce bitter receptor activation during simultaneous application with the respective agonist, further investigations of these compounds indicated that they were not suitable as bitter receptor blockers. The two deoxyanthocyanidins, apigeninidin chloride and luteolinidin chloride, showed signal reduction of non-induced cells (bitter receptors not expressed), by the same trend as signal reduction of induced cells (bitter receptors expressed). This indicated that these deoxyanthocyanidins did not act as hTAS2R antagonists, but reduced fluorescence by another mechanism. The two flavanones tricetin and 7,3',4',5'-tetrahydroxyflavone were able to activate hTAS2R39 at high concentrations, therewith acting as partial agonists on hTAS2R39. Their ability to reduce activation of hTASR39 at sub-threshold concentrations was not sufficient enough to comply with the requirements for a good receptor blocker. In conclusion, it was shown that not only some flavanones, but also other flavonoids can reduce bitter receptor activation, though no high potential compounds were part of the set

tested. Additionally, it was found that not every reduction of bitter receptor activation necessarily implies antagonism.

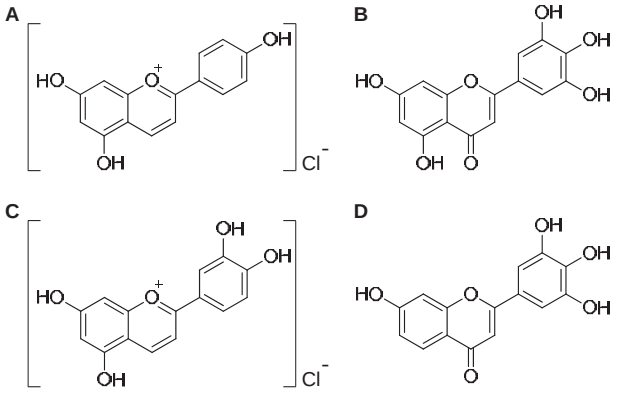

Figure 5. Structures of apigeninidin chloride (**A**), tricetin (**B**), luteolinidin chloride (**C**), and 7,3',4',5'-tetrahydroxyflavone (**D**).

When comparing structure-activity relationships of (iso)flavonoids regarding their behavior as inactive compounds, as bitter receptor agonists, and as bitter receptor antagonists, two additional conclusions can be drawn based on the results presented in **Chapter 3**, **Chapter 4** and here (**Chapter 6**). (i) The substitution pattern of (iso)flavonoids is of higher importance for bitter receptor activation than the backbone structure. To illustrate this conclusion with one example, seven out of eight (iso)flavonoids with a 5,7,3',4'-tetrahydroxy substitution pattern activated hTAS2R14 and hTAS2R39. The behavior of the eighth compound could not be determined due to non-specific signals. Except for differences in potency, this behavior was independent of their backbone structure. Moreover, most (iso)flavonoid subclasses comprised of active as well as inactive constituents, meaning that the backbone structure is not crucial for receptor activation, as long as the correct substitution pattern is present. (ii) In case of bitter receptor antagonists, the substitution pattern as well as backbone structure revealed to be crucial for functionality. This was seen for 6-methoxyflavanones, as flavonoids comprising an -OCH_3 group on position 6 were only antagonists, when they included a flavanone backbone, and flavanones were only antagonists of hTAS2R39 when they were methoxylated on position 6.

SENSORY TESTS, RECEPTOR ASSAYS AND MODELING COMPARED FOR EVALUATION OF BITTER TASTE

Testing for bitter compounds and bitter taste maskers

The use of the bitter receptor assay enabled us to identify (iso)flavonoid bitter receptors, and to compare and characterize almost 100 (iso)flavonoids regarding their intrinsic bitterness, receptor activation thresholds and EC_{50} values (**Chapter 3**). With the knowledge of the target bitter receptors and the identified bitter receptor agonists, we were able to identify three antagonists of hTAS2R39 (**Chapter 4**). Although we were not able to test their functionality as bitter blockers in the mouth, due to unknown safety for human consumption, their identification created insight into structural requirements to inhibit hTAS2R39. This might lead to other blockers, suitable for food applications. The bitter receptor assay is a high throughput method, sometimes limited by poor solubility and non-specific signals of the test compounds, but without safety concerns. The findings just described would not have been possible to achieve by the use of sensory tests. Sensory tests have a low throughput, they are laborious, and it is difficult to find panel members for tasting unpleasant compounds. Their outcomes can be highly variable, depending on the sensory protocol, genetic profile of the panellists, and environmental and physiological conditions. Most importantly, only food-grade compounds, safe for human consumption (GRAS), can be used. A complicating factor for bitterness detection by sensory tests is that bitterness and astringency are difficult to distinguish. On the other hand, only sensory tests can establish the real taste perception, as receptor assays cannot account for interactions of bitter compounds with saliva, or cross-modal interactions. The bitter receptor assay can thus be seen as a filter to reduce the number of candidate compounds to the most potent ones. In this way, the services of sensory panels can be minimized to evaluate the impact of compounds of high interest.

In **Chapter 5** we demonstrated a successful combination of the advantages of bitter receptor assays and sensory tests. We studied the ability of the bitter compound EGCG to bind to various proteins (in ultrafiltration assays), investigated whether this phenomenon could be translated to reduced availability of EGCG for activation of hTAS2R39 (in bitter receptor assays), and selected the protein with the highest ability to reduce bitter receptor activation. The best protein, Na-caseinate, was tested in sensory tests together with EGCG, and we could show that the *in vitro* findings were in agreement with actual perception *in vivo*. Therewith, the last hypothesis of this thesis, namely that bitter taste reduction for dietary flavonoids by complexation with food proteins can be predicted by the use of *in vitro* assays, was accepted. However, not all *in vitro* and *in vivo* observations were in full agreement with each other, as slight bitter taste reduction by use of β-lactoglobulin was not predicted by *in vitro* assays. This bitterness reduction was most likely not caused by complexation, but by interactions between β-lactoglobulin and the buccal environment, which cannot be accounted for in the bitter receptor assays.

Consensus and discrepancy between receptor assays and sensory tests

Findings of *in vitro* bitter receptor activation do not necessarily imply that exactly the same effect will be observed sensorially. This will be discussed here by means of two examples.

In the bitter receptor assays, catechin and epicatechin activated hTAS2R39 and hTAS2R14. No difference was observed between the potency of these two stereoisomers (activation thresholds on hTAS2R39: 250 µM, activation thresholds on hTAS2R14: 500 µM) (**Chapter 3**). However, in sensory studies, various outcomes have been reported. In two sensory studies, bitterness of epicatechin (bitterness threshold 800 µM *(37)* or 930 µM *(38)*) was perceived as slightly stronger than that of catechin (bitterness threshold 1000 µM) *(37, 38)*. In another sensory study, the bitterness of epicatechin was perceived as equal to that of catechin at low concentrations, but as significantly stronger than that of catechin with increasing concentrations *(39)*. It should be mentioned that other bitter receptors than hTAS2R14 and hTAS2R39 have been identified for epicatechin at high concentrations, namely hTAS2R4 (threshold 2 mM) and hTAS2R5 (threshold 1 mM) *(16)*. Thus, epicatechin was probably perceived as more bitter than catechin in sensory tests, because more bitter receptors are activated by it. This would explain the findings of *(39)* at increased concentrations. Caution should be taken in the interpretation of these data, as catechin has not been tested on hTAS2R4 and hTAS2R5, and might be an agonist as well. Furthermore, catechin and epicatechin might differ in binding with salivary proteins or mucus layers in the mouth, which can lead to lower bitter perception in the mouth than expected based on the outcomes of bitter receptor assays. An effect like this has been demonstrated before as reason for discrepancy between intrinsic bitterness and bitterness perception of hop acids *(22)*. Altogether, the attribute of catechin and epicatechin both being bitter, was consistent *in vitro* and *in vivo*. Furthermore, it can be concluded that the intensity of bitterness perception becomes difficult to predict by receptor assays when there are several bitter receptors involved, as it is not known yet whether some bitter receptors play a more important role for bitter perception than others.

Two compounds showing noticeably different effects in bitter receptor assays and sensory tests are eriodictyol and homoeriodictyol. In **Chapter 3**, they are identified as agonists of hTAS2R14 and hTAS2R39. In contrast, they are reported to act as bitter taste maskers in sensory tests *(36)*. However, their mechanism of bitterness masking has not been established yet. One explanation for this discrepancy between receptor assay and sensory perception could be that they are agonists on hTAS2R14 and hTAS2R39, but antagonists (not yet identified) on other bitter receptors, and their antagonistic properties overrule their agonistic properties. The ability of compounds to act as agonist on one subset of hTAS2Rs and as antagonists on another subset of hTAS2Rs has been demonstrated before *(40)*.

The use of modeling for identification of bitter compounds and bitter taste maskers

Chapter 3 describes the behavior of many structurally similar (iso)flavonoids towards hTAS2R14 and hTAS2R39. Subsequently, it describes the development of fingerprint and pharmacophore models predicting whether they activate the respective bitter receptor, or not. The question arises whether modeling could replace the measurements with cell assays or sensory tests. The models developed in **Chapter 3** were suitable for a defined type of molecules only, and not applicable to bitter molecules in general. Within their structural limitations, they are suitable to make predictions on whether an (iso)flavonoid is likely to activate hTAS2R14 or hTAS2R39. Development of quantitative structure activity relationship (QSAR) models was not successful, as the bitter receptor thresholds of (iso)flavonoid never differed by more than three orders of magnitude. Therefore, no prediction could be made on the intensity of intrinsic bitterness, but only on the activation or absence of activation. The trend in food industry to fortify products with potentially health promoting ingredients, such as (iso)flavonoids, might lead to introduction of bitter taste. By means of model-aided predictions, the potential of various (iso)flavonoids to introduce bitterness when used for food fortification can thus be accessed before product development starts. This can save time and money, and limit laborious sensory tests to a minimum.

Pharmacophore modeling is also of interest as a strategy to identify new bitter blockers. It has been shown before that pharmacophore modeling is a valid approach to identify new bitter blockers, especially when it is combined with docking into the active site of the target receptor *(41)*. For the bitter receptor blockers described in **Chapter 4,** pharmacophore modeling and docking into the active site of hTAS2R39, might enable discovery of new receptor blockers as well. Nevertheless, it might be necessary to extend our current data set of three antagonists with more molecules for this approach.

Despite the fact that there can be some discrepancies between bitter receptor assays and sensory tests, it can be concluded that bitter receptor assays are a useful tool for high throughput identification of bitter receptors and consequently identification of compounds able to reduce their activation. This can be aided by modeling, especially in order to understand structural requirements involved in receptor agonism or antagonism. In the end, the most promising findings should be validated by sensory tests.

EVALUATION OF STRATEGIES FOR BITTER TASTE REDUCTION

In this thesis, two strategies of bitter taste reduction were investigated: blocking of bitter taste receptors (**Chapter 4**) and complexation of bitter flavonoids with food proteins (**Chapter 5**). In the first case, bitter taste receptor blockers were identified, which reduced the activation of hTAS2R39 to a large extent. Unfortunately, we were not able to perform sensory tests with the bitter receptor blockers identified, due to unknown safety of the

compounds identified. In the second case, we were able to show that reduced activation of bitter receptors by addition of food proteins led to a real reduction of bitter perception, as both in the bitter receptor assay and in sensory experiments the reduction was observed. In the following, the two bitterness reduction strategies will be discussed, followed by an overview of their specifications in **Table 1**.

Additional to the results presented in **Chapter 5**, two other bitter compounds, ECG and EC, were tested together with food proteins in bitter receptor assays. The effect of complex formation between bitter compounds and food proteins on reduction of bitter receptor activation was investigated. An example (**Figure 6**) is shown for β-casein, as it was the most effective food protein to reduce EGCG signals. It can be seen that addition of β-casein to ECG resulted in similar effects as for EGCG. On the other hand, activation of hTAS2R39 by the structurally related non-galloylated EC was not reduced by β-casein. This result can be explained by the low binding affinity of EC for β-casein *(42)*, leading to weak complexes. It has been reported that complex formation between caseins and flavonoids is favored by the presence of rotatable bonds and four or more aromatic rings in the flavonoid *(43)*. Therefore, whereas it works for galloylated flavonols, a debittering strategy using complexation with food proteins is probably not suitable for monomeric (iso)flavonoids and glycosylated (iso)flavonoids containing less than four aromatic rings.

An alternative for molecules unable to engage in complexation with food proteins, is the use of other agents suitable for food applications, such as β-cyclodextrin (E 459). This complexing agent can form molecular inclusion complexes with hydrophobic molecules and is quite broadly applicable when size and hydrophobicity requirements of the included molecule are met. Complexation of catechin with β-cyclodextrin has been reported as effective in reduction of bitterness in sensory tests *(44)*.

The main advantage of complexation with food proteins is the use of a compound naturally present in food products. This might provide high consumer acceptability and no difficulties with labelling. Quantities needed for complexation of EGCG with *e.g.* β-casein have been shown to be realistic for application in food *(42)*. The EGCG-β-casein or caseinate complex is functional during consumption, has a short residence time in the mouth, and it can be expected that the complexes will fall apart in the stomach, therewith not altering the absorption of EGCG. Furthermore, via complexation of EGCG and food proteins not only bitter taste is masked, but also astringency is probably affected. Reduced astringency of catechins has been reported for other complexing agents, like sodium carboxymethyl cellulose and β-cyclodextrin *(44, 45)*.

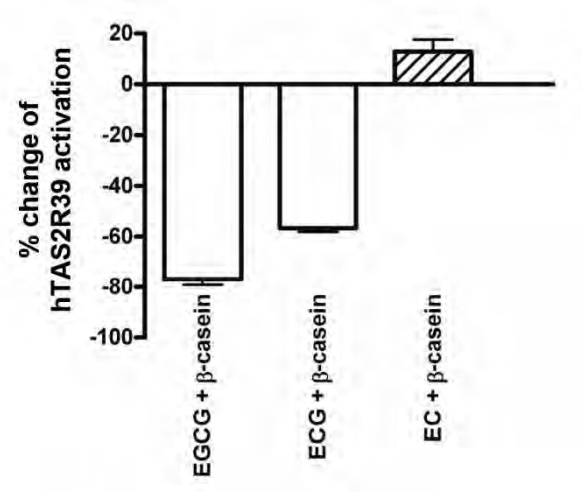

Figure 6. Change of hTAS2R39 activation by EGCG, ECG, and EC, after complexation with β-casein, following the same methodology as described in Chapter 5.

In **Chapter 3**, we reported ECG as agonist of at least two bitter receptors, hTAS2R39 and hTAS2R14. In order to fully eliminate bitter taste of ECG by an antagonist, all receptors activated by ECG have to be inhibited. In **Chapter 4** we reported the identification of hTAS2R39 antagonists, which were able to decrease bitter receptor activation when applied simultaneously with the bitter compound. The same compounds also acted as hTAS2R14 antagonists, though only after stepwise administration. For food applications, a receptor blocker is only suitable, when it is functional in simultaneous administration with the bitter compound. Therefore, blockers that are only functional when applied prior to the bitter compound, are not suitable. Although bitter taste by ECG might not be eliminated by receptor blockers with these properties, inhibition of one of the two bitter receptors might already lead to a decrease of bitter taste. Moreover, a bitter compound activating several bitter receptors can be debittered by the use of one complexing agent, whereas several receptor blockers might be necessary to achieve this if the blocker specificity is narrower than the agonist specificity. On the other hand, the advantage of bitter receptor blockers is that they are applicable independently from the molecular structure of the bitter receptor agonist, in contrast to debittering by complexation, which is dependent on the molecular structure of the bitter compound. Prior to application, sensory panels should evaluate complexing agent or receptor blocker for both their bitterness reduction ability, and, furthermore, for their own taste profile, in order to avoid possible aftertastes.

Table 1. Comparison of properties of complexing agents and receptor blockers.

Characteristics	Complexation with food proteins	Bitter receptor blockers
suitable independent of bitter agonist	no	yes[a]
suitable independent of bitter receptor	yes	no
receptor specific	no	yes
naturally present in food	yes	possible[b]
persisting effect on the tongue	no[c]	possible[b,d]
affecting also astringency	yes[c]	no

[a] if orthosteric antagonist, [b] not reported yet, [c] assumption, [d] if irreversible antagonist

Altogether, the use of bitter receptor blockers seems to be suitable for molecules of which the target receptor is known. Complexation with food proteins seems to be suitable for larger molecules, and might for these be broader applicable than receptor blockers, as the effect of complexation is independent from the number of receptors activated by one bitter compound. Food systems containing both non-complexable smaller bitter compounds and complexable larger bitter compounds, might, therefore, be most effectively debittered by a combination of the two methods.

APPLICATION OF DEBITTERING STRATEGIES TO SOY PRODUCTS AND TEA

Debittering strategies for soy

Application of the debittering strategies targeted for single compounds in complex food matrices, such as soy products, is difficult to predict, due to possible interactions of the bitter blockers or food proteins with other compounds present. In this paragraph it is assumed that such interactions do not hamper accessibility of bitter compounds by debittering agents.

Application of the two debittering strategies described in this thesis to soy products can only be predicted for isoflavones. The use of food proteins for complex formation with isoflavones is not promising, due to low predicted affinity between food proteins and phenolic compounds comprising of less than four aromatic rings *(43)*. The use of receptor blockers that block hTAS2R39, and to some extent hTAS2R14, as reported in **Chapter 4**, seems a better option, as isoflavone glucosides activate hTAS2R39, and the less abundant aglycones activate hTAS2R14 and hTAS2R39.

Due to the high variation of isoflavone compositions reported for soybean varieties and soy products *(46-48)*, two alternative strategies to control bitterness of soy isoflavones should be considered as well: cultivar selection and processing. Applying the information about intrinsic bitterness gained with the bitter taste receptor assays (**Chapter 2**) to the isoflavone composition of soy products upon processing and amongst cultivars *(46-48)*, results in two main conclusions: (i) Intrinsic bitterness of soybean isoflavones cannot be

reduced by transformation into other isoflavone forms due to processing, it can only be enhanced. Heating of soybean isoflavones under wet conditions leads mainly to the transformation of natively most abundant malonyl glucosides *(4)* into unsubstituted glucosides (by deesterification) *(4, 48)*, not changing intrinsic bitterness, due to the same threshold of hTAS2R39 activation by genistin compared to malonyl genistin. Heating of soybean isoflavones under dry conditions leads mainly to the transformation of malonyl glucosides into acetyl glucosides (by decarboxylation) *(48, 49)*, and can thus implement increasing intrinsic bitterness, due to four times lower threshold of hTAS2R39 activation by acetyl genistin compared to malonyl genistin. Soaking of soybeans in water leads to modest formation of aglycones *(6, 48)*, while fermentation of isoflavones leads to major formation of aglycones *(48, 50)*. It will thereby drastically enhance intrinsic bitterness, at least for the dominating genistein form. (ii) As genistein forms show the highest intrinsic bitterness, a relatively lower bitterness is expected from soybean varieties low in genistein forms, and richer in daidzein and glycitein forms. Although not common, existence of cultivars with these characteristics has been shown *(51, 52)*. Decreasing bitterness of soy isoflavones by heating or enzymatic conversion of glucosides into aglycones is highly unlikely. If debittering of soy products can be achieved by processing, it might be caused by certain losses of isoflavones into the processing water during boiling *(53)*, or by other soy constituents, such as saponins, but not by transformation of isoflavones. The effect of saponins on bitterness of soy products, in addition to isoflavones, remains to be established.

In conclusion, the proposed debittering strategy for soy products is the use of bitter receptor blockers against intrinsically bitter isoflavones, and control of genistein occurrence by selection of cultivars low in genistein forms, and/or by limitation of processing resulting in formation of acetyl glucosides and aglycones.

Effect of debittering strategies on tea

In **Chapter 3**, we identified hTAS2R39 (as *(54)*) and hTAS2R14 (not reported before) as tea catechin bitter receptors. Next, we presented solutions on how to reduce bitterness of catechins at receptor level by bitter receptor blockers (although not food-grade) (**Chapter 4**), or on receptor and sensorial level by complexation with food proteins (**Chapter 5**). The question arises whether these measures are sufficient to reduce bitterness in tea.

In green tea, the bitter compound present in by far the highest concentrations, and additionally exhibiting the highest bitterness, is EGCG. The remaining less bitter compounds caffeine, theobromine, and other catechins, are present in much lower concentration than EGCG. Due to the key impact of EGCG, complexation of EGCG by food proteins might be sufficient to largely reduce bitterness in green tea.

The question whether bitter taste in black tea and oolong tea can be reduced by one of the two debittering methods applied in this thesis, is more complex. Due to the low amount of catechins, and the presence of thearubigins and theaflavins (oxidation products of

catechins), the answer to this question might dependent on the unclear taste effect of thearubigins and theaflavins. If they do not contribute to bitterness, as reported by *(55)*, the bitter compounds are narrowed down to catechins and caffeine / theobromine, like in green tea. If thearubigins and theaflavins contribute to bitterness, as reported by *(56)*, it should be investigated if they can be complexed with food proteins, or if they can activate bitter taste receptors, in order to identify the target receptor. As mentioned above, complex formation between caseins and flavonoids is favored in the presence of rotatable bonds and four or more rings in the flavonoid *(43)*. Due to the presumed oligomeric character of thearubigins and theaflavins, complexation with food proteins seems thus likely. Therefore, also for oolong and black tea, complexation seems to be a good approach to reduce bitter taste.

In conclusion, the choice of debittering strategies depends on the molecular structure of the bitter food compounds, as exemplified for soybean products and tea. Therefore, each food product needs its own tailor-made debittering solution.

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Summary

Many flavonoids and isoflavonoids have been associated with beneficial health effects. Therefore, consumption of (iso)flavonoid-rich food products, and enrichment of foods with (iso)flavonoids is becoming increasingly popular. However, several (iso)flavonoids have been reported as bitter. Consequently, their incorporation in (or fortification of) foods can introduce (or enhance) bitterness. Hence, debittering strategies are demanded.

Some (iso)flavonoids have unknown taste properties, as they have never been incorporated in high levels in food products. For other (iso)flavonoids, contradictory findings on bitterness have been made in sensory tests. Therefore, objective tests are necessary to identify which (iso)flavonoids contribute to bitterness of a food product. An objective tool to study bitterness is a cell-based bitter taste receptor assay. Twenty-five different bitter taste receptors (hTAS2Rs) occur on the human tongue, each of which has been introduced in a separate human embryonic kidney (HEK)293 cell line. With these, the "intrinsic bitterness" of a compound can be investigated *in vitro*. Intrinsic bitterness is the capacity of a compound to activate bitter taste receptors, uncoupled from cross-modal interactions and interactions with salivary proteins and oral mucosa. The aim of this research was to study the intrinsic bitterness of a large set of (iso)flavonoids and to investigate structural requirements for (iso)flavonoids to activate the bitter receptors identified. A subsequent aim was the investigation of different debittering strategies by the use of the bitter receptor assay.

Chapter 1 provides an overview of flavonoids and isoflavonoids with respect to their structural classification, sensorial properties and occurrence as dietary compounds. Taste perception and the mode of action of bitter taste receptors are introduced. The measurement of bitter receptor activation *in vitro* is explained, as well as strategies to reduce bitter receptor activation, and bitter taste in general. A state-of-the-art overview of all 25 bitter taste receptors is given with respect to known agonists and antagonists.

The aim of **Chapter 2** 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 µM on hTAS2R14 and hTAS2R39, and EC_{50} values of 29 and 49 µM, respectively. Besides, the behavior of structurally similar isoflavonoids was investigated. Although the two receptors are not closely related, the results for hTAS2R14 and hTAS2R39 were similar towards most isoflavonoid aglycones. Glucosylation of isoflavones seemed to inhibit activation of hTAS2R14, whereas four of five glucosylated isoflavones were agonists of hTAS2R39, namely glycitin, genistin, acetyl genistin, and malonyl genistin. A total of three hydroxyl substitutions of the A- and B-rings of the isoflavonoids seemed to be more favorable for receptor activation than less hydroxyl groups. The concentration of the trihydroxylated genistein in several soy foods exceeds the bitter receptor threshold values determined, whereas those of other soy isoflavones are around or below their respective threshold values. 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.

In **Chapter 3**, the intrinstic bitterness of (iso)flavonoids, which can hamper their use as food bioactives, was investigated further. The effect of a large set of structurally similar (iso)flavonoids on the activation of bitter receptors hTAS2R14 and hTAS2R39 was tested, and their structural requirements to activate these receptors were predicted. In total, 68 compounds activated hTAS2R14 and 70 compounds activated hTAS2R39, amongst which 58 ligands were overlapping. Their activation threshold values varied over a range of three log units between 0.12 and 500 μM. Ligand-based 2D-fingerprint and 3D-pharmacophore models were created to detect structure activity relationships. The 2D-models demonstrated excellent predictive power in identifying bitter (iso)flavonoids and discrimination from inactive ones. The structural characteristics for an (iso)flavonoid to activate hTAS2R14 and hTAS2R39 were determined by 3D-pharmacophore models to be composed of two (for hTAS2R14) or three (for hTAS2R39) hydrogen bond donor sites, one hydrogen bond acceptor site, and two aromatic ring structures, of which one had to be hydrophobic. An additional hydrogen bond donor feature for hTAS2R39 ligands indicated the possible presence of another complementary acceptor site in the binding pocket, compared to hTAS2R14. Hydrophobic interaction of the aromatic feature with the binding site might be of higher importance in hTAS2R14 than in hTAS2R39. Together, this might explain why OH-rich compounds showed different behavior towards the two bitter receptors. The combination of *in vitro* data and different *in silico* methods created a good insight in activation of hTAS2R14 and hTAS2R39 by (iso)flavonoids and provided a powerful tool in prediction of their potential bitterness. By understanding the "bitter motif", introduction of bitter taste in functional foods enriched in (iso)flavonoid bioactives might be avoided.

Bitter receptor hTAS2R39 is activated by many different classes of bitter compounds, amongst which (iso)flavonoids. Nevertheless, several flavanones are known to mask bitter taste sensorially, and not all flavanones reported in **Chapter 3** activated hTAS2R39. For that reason, in **Chapter 4**, fourteen flavanones were investigated for their potential to reduce activation of hTAS2R39 by epicatechin gallate (ECG), one of the main bitter compounds present in green tea. Three compounds showed inhibitory behavior towards the activation of hTAS2R39 by ECG: 4'-fluoro-6-methoxyflavanone, 6,3'-dimethoxyflavanone, and 6-methoxyflavanone (in order of decreasing potency). The 6-methoxyflavanones also inhibited activation of hTAS2R14 (another bitter receptor activated by ECG), though to a lesser extent. Dose-response curves of ECG at various concentrations of the most potent antagonist 4'-fluoro-6-methoxyflavanone and wash-out experiments indicated reversible insurmountable antagonism. The same effect was observed for the structurally different agonist denatonium benzoate, suggesting a non-competitive orthosteric mechanism. The bitter receptor blockers identified might not be

applicable to food products. Nevertheless, they create insight into structural requirements, which might lead to other, more suitable, blockers.

Chapter 5 investigates another strategy to reduce bitterness, namely complexation of bitter flavonoids with food proteins. The binding characteristics of the bitter tea compound epigallocatechin gallate (EGCG) to purified food proteins, and their equivalent food-grade preparations, were related to their effects on reducing bitter receptor activation by EGCG *in vitro* and their bitter-masking potential *in vivo*. β-Casein, in particular, and several gelatins, are known as strong binders of EGCG, contrary to β-lactoglobulin. Also in the bitter receptor assay, β-casein showed the strongest effect, with a maximum reduction of hTAS2R39 activation of about 93%. A similar potency was observed for Na-caseinate, which was applied as food-grade alternative for β-casein. β-Lactoglobulin had little effect on bitter receptor activation, as expected based on its low binding affinity for EGCG. The bitter-masking potential of Na-caseinate was confirmed *in vivo* using a trained sensory panel. β-Lactoglobulin also slightly reduced EGCG bitter perception, which could not be directly related to its binding capacity. The bitter receptor assay appeared to be a valid tool to evaluate *in vitro* the efficacy of food proteins as complexing agents for bitter-masking.

Chapter 6 discusses the findings presented in this thesis, addresses prospects and limitations of the bitter receptor cell assay, presents additional results on testing (iso)flavonoids for possible antagonistic properties, and compares taste evaluation by sensory tests, receptor assays and modeling. Furthermore, it evaluates strategies for bitter taste reduction, and applies the findings to soy products and tea.

The systematic investigation of (iso)flavonoid aglycones showed that the substitution pattern of (iso)flavonoids is of higher importance for bitter receptor activation than the backbone structure. In case of bitter receptor antagonists, the substitution pattern as well as backbone structure revealed to be crucial for functionality. The bitter receptor assay was shown to be an appropriate tool not only for identification of bitter receptor agonists and antagonists, but also for identification of reduced receptor activation by complexing agents. Based on the findings of this thesis, it was concluded that complexation with food proteins is the most promising strategy to reduce bitter taste of flavonoids in tea. On the other hand, for soybean isoflavones, debittering by use of bitter receptor blockers seemed to be a promising debittering strategy. Alternatively to the use of receptor blockers, processing conditions (leading to low isoflavone aglycone formation) or raw material choice (i.e. cultivars low in genistein forms) were recommended. In conclusion, the choice of debittering strategies depends on the molecular structure of the bitter food compounds, as exemplified for soybean products and tea. Therefore, each food product seems to require its own tailor-made debittering solution.

Samenvatting

Veel flavonoïden en isoflavonoïden worden in verband gebracht met gezondheids-bevorderende effecten. Om deze reden wordt de consumptie van voedingsmiddelen rijk aan (iso)flavonoïden en het verrijken van producten met (iso)flavonoïden steeds populairder. Verschillende (iso)flavonoïden worden echter als bitter ervaren. Als gevolg hiervan kan het toevoegen van of verrijken met (iso)flavonoïden voedingsmiddelen bitter maken of de bitterheid verhogen. Er is daarom behoefte aan methoden om producten te ontbitteren.

Van sommige (iso)flavonoïden zijn de smaakeigenschappen onbekend, omdat ze nooit in hoge concentraties aan voedingsmiddelen zijn toegevoegd. Voor andere zijn tegenstrijdige resultaten gevonden in sensorisch onderzoek. Daarom zijn objectieve methoden nodig om te bepalen welke (iso)flavonoïden bijdragen aan de bitterheid van een product. Een objectief hulpmiddel om bitterheid te bestuderen is een cel-gebaseerde bitterreceptor-assay. Op de menselijke tong komen 25 verschillende bitterreceptoren (hTAS2Rs) voor, welke allemaal in een afzonderlijke HEK 293 cellijn zijn geïntroduceerd. Met behulp van deze cellijnen kan de “intrinsieke bitterheid” van een verbinding *in vitro* worden onderzocht. Intrinsieke bitterheid is de capaciteit van een verbinding om bitterreceptoren te activeren, losgekoppeld van cross-modale interacties en interacties met speekseleiwitten en mondslijmvlies. Het doel van dit onderzoek was het bestuderen van de intrinsieke bitterheid van een groot aantal (iso)flavonoïden en het identificeren van hun structurele eigenschappen, die het activeren van bitterreceptoren mogelijk maken. Daarnaast is door middel van de bitterreceptor-assay onderzocht welke verschillende strategieën er zijn voor ontbittering.

In **hoofdstuk 1** wordt een overzicht gegeven van flavonoïden en isoflavonoïden met betrekking tot hun structurele indeling, sensorische eigenschappen en voorkomen als dieetbestanddelen. Smaakperceptie en de werking van bitterreceptoren worden geïntroduceerd. Het *in vitro* meten van bitterreceptoractivatie wordt uitgelegd, evenals strategieën om bitterreceptoractivatie, en bittere smaak in het algemeen, te verminderen. Er wordt een actueel overzicht weergegeven van de bekende agonisten en antagonisten van alle 25 bitterreceptoren.

Het doel van **hoofdstuk 2** was het identificeren van de bitterreceptor(en) die de bittere smaak van het sojaisoflavon genisteïne herkennen. Screening van alle 25 humane bitterreceptoren maakte duidelijk dat genisteïne een agonist van hTAS2R14 en hTAS2R39 is. Drempelwaarden van genisteïne waren 4 en 8 µM voor hTAS2R14 en hTAS2R39, EC_{50} waarden waren respectievelijk 29 en 49 µM. Daarnaast werd het gedrag van structureel vergelijkbare isoflavonoïden onderzocht. Hoewel de twee receptoren hTAS2R14 en hTAS2R39 niet nauwverwant zijn, waren de resultaten met de meeste isoflavon aglyconen vergelijkbaar. Glucosylering van isoflavonen leek de activatie van hTAS2R14 te remmen, terwijl vier van de vijf geglucosyleerde isoflavonen, namelijk glycitine, genistine, acetylgenistine en malonylgenistine, agonisten waren van hTAS2R39. Een totaal aantal van drie hydroxylsubstituenten op de A- en B-ring van de isoflavonoïden leek meer

bevorderlijk voor receptoractivatie dan minder hydroxylgroepen. De concentraties van het trihydroxyl isoflavonoïd genisteïne in verschillende sojaproducten overschrijden de gevonden bitterreceptor drempelwaarden, terwijl die van andere soja-isoflavonen rond of onder hun respectievelijke drempelwaarden liggen. Ondanks zijn lage concentratie zou genisteïne een van de belangrijkste veroorzakers van bitterheid van sojaproducten kunnen zijn. Verder activeerden de bioactieve isoflavonoiden equol en coumestrol beide receptoren. Hetgeen suggereert dat hun sensorische invloed in acht moet worden genomen wanneer ze gebruikt worden als ingrediënt in voedsel.

In **hoofdstuk 3** werd de intrinsieke bitterheid van (iso)flavonoïden, wat hun toepassing als bioactieve ingrediënten kan bemoeilijken, verder onderzocht. Het effect van een grote set structureel vergelijkbare (iso)flavonoïden op de activatie van bitterreceptoren hTAS2R14 en hTAS2R39 werd onderzocht en hun structurele vereisten om deze receptoren te activeren werden voorspeld. 68 verbindingen activeerden hTAS2R14 en 70 verbindingen activeerden hTAS2R39, waarvan 58 liganden overlapten. Hun drempelwaarden voor activatie varieerden over een bereik van drie logeenheden tussen 0,12 en 500 µM. Ligand gebaseerde 2D-vingerafdruk en 3D-pharmocaphore modellen werden gemaakt om structuur-activiteit-relaties te vinden. De 2D modellen konden erg goed voorspellen of (iso)flavonoïden bitter zijn of niet. Met behulp van 3D-pharmacophoremodellen werd gevonden dat de structurele eigenschappen voor (iso)flavonoïden om hTAS2R14 en hTAS2R39 te activeren bestaan uit twee (voor hTAS2R14) of drie (voor hTAS2R39) waterstofbrugdonoren, één waterstofbrugacceptor en twee aromatische ringstructuren, waarvan één hydrofoob dient te zijn. Een additionele waterstofbrugdonor voor hTAS2R39 liganden duidt op de mogelijke aanwezigheid van nog een complementaire acceptorplaats in de bindingsplaats, vergeleken met hTAS2R14. Hydrofobe interactie van de aromatische eigenschap met de bindingsplaats zou in hTAS2R14 belangrijker kunnen zijn dan in hTAS2R39. Tezamen zou dit kunnen verklaren waarom OH-rijke verbindingen een verschillend effect op de twee bitterreceptoren lieten zien. De combinatie van *in vitro* data met verschillende *in silico* methoden heeft geleid tot een goed inzicht in de activatie van hTAS2R14 en hTAS2R39 door (iso)flavonoïden en bleek een krachtig hulpmiddel te zijn bij het voorspellen van hun potentiële bitterheid. Door het begrijpen van het “bitter motief” kan het introduceren van bittere smaak in functional foods verrijkt met (iso)flavonoïden mogelijk voorkomen worden.

Bitterreceptor hTAS2R39 wordt geactiveerd door veel verschillende klassen van bittere verbindingen, waaronder ook (iso)flavonoïden. Desalniettemin zijn er enkele flavanonen bekend die bittere smaak sensorisch maskeren, en niet alle in **hoofdstuk 3** genoemde flavanonen activeerden hTAS2R39. Om deze reden werden in **hoofdstuk 4** veertien flavanonen onderzocht op hun potentieel om de activatie van hTAS2R39 door epicatechine gallaat (ECG), een van de belangrijkste bittere verbindingen in thee, te verminderen. Drie verbindingen remden de activatie van hTAS2R39 door ECG: 4’-fluor-6-methoxyflavanone,

6,3'-dimethoxyflavanone en 6-methoxyflavanone (in volgorde van afnemende potentie). De 6-methoxyflavanonen remden ook de activatie van hTAS2R14 (een andere bitterreceptor die wordt geactiveerd door ECG), maar in mindere mate. Dosis-respons curves van ECG met verschillende concentraties van de meest potente antagonist 4'-fluoro-6-methoxyflavanone en wash-out experimenten wezen op omkeerbaar onoverkomelijk antagonisme. Hetzelfde effect werd gevonden voor de structureel verschillende agonist denatoniumbenzoaat, hetgeen een niet-competitief orthosterisch mechanisme suggereert. Hoewel de geïdentificeerde bitterreceptorblokkeerders mogelijk niet kunnen worden toegepast in levensmiddelen, geven ze inzicht in structurele vereisten welke tot andere, meer geschikte blokkeerders zouden kunnen leiden.

Hoofdstuk 5 onderzoekt een andere strategie om bitterheid te verminderen, namelijk complexatie van bittere flavonoïden met voedingseiwitten. De karakteristieken van de binding van de bittere theeverbinding epigallocatechine gallaat (EGCG) met gezuiverde voedingseiwitten en hun vergelijkbare food-grade preparaten, werden gerelateerd aan hun effecten op de reductie van bitterreceptoractivatie door EGCG *in vitro* en hun bittermaskeringspotentieel *in vivo*. Van vooral β-caseïne en van verscheidene gelatines is bekend dat het sterke binders van EGCG zijn, dit in tegenstelling tot β-lactoglobuline. Ook in de bitterreceptor-assay had β-caseïne het grootste effect met een maximale reductie van hTAS2R39 activatie van ongeveer 93%. Een vergelijkbare potentie werd gevonden voor Na-caseïnaat, welke als food-grade alternatief voor β-caseïne werd gebruikt. β-Lactoglobuline had weinig effect op bitterreceptoractivatie, zoals verwacht op basis van de lage bindingsaffiniteit voor EGCG. Het bittermaskerende effect van Na-caseïnaten werd *in vivo* bevestigd met behulp van een getraind sensorisch panel. β-Lactoglobuline verlaagde de bitterperceptie van EGCG ook enigszins, hetgeen niet direct gerelateerd kon worden aan de bindingscapaciteit. De bitterreceptor-assay bleek een goed hulpmiddel te zijn om *in vitro* de effectiviteit van voedingseiwitten als bittermaskeerder te onderzoeken.

Hoofdstuk 6 bediscussieert de in dit proefschrift beschreven resultaten, behandelt kansen en beperkingen van de bitterreceptor-cel-assay, beschrijft aanvullende resultaten aangaande onderzoek naar mogelijke antagonistische eigenschappen van (iso)flavonoïden en vergelijkt smaakevaluatie door middel van sensorisch onderzoek, receptor-assays en modelleren. Daarnaast worden strategieën voor de reductie van bittere smaak geëvalueerd en worden de gevonden resultaten toegepast op sojaproducten en thee.

Het systematische onderzoek van (iso)flavonoïde-aglyconen toonde aan dat het substitutiepatroon van (iso)flavonoïden belangrijker is voor het activeren van de bitterreceptoren dan de basisstructuur. In het geval van bitterreceptorantagonisten bleken zowel het substitutiepatroon als de basisstructuur cruciaal te zijn voor functionaliteit. Aangetoond werd dat de bitterreceptor-assay niet alleen een geschikt hulpmiddel is voor de identificatie van bitterreceptoragonisten en –antagonisten, maar ook voor het identificeren van verminderde receptoractivatie door complexerende verbindingen. Gebaseerd op de

resultaten in dit proefschrift wordt geconcludeerd dat complexatie van flavonoïden met voedingseiwitten de meest veelbelovende strategie is om hun bittere smaak in thee te verminderen. Voor isoflavonen uit soja bleek ontbittering door middel van bitterreceptorblokkeerders een veelbelovende strategie te zijn. Als alternatief voor het gebruik van receptorblokkeerders werden aangepaste verwerkingscondities (resulterend in een beperkte vorming van isoflavon aglyconen) en de keuze van grondstoffen (het gebruiken van cultivars met een lage concentratie van de verschillende vormen van genisteïne) aanbevolen. Geconcludeerd kan worden dat de keuze van geschikte ontbitteringsstrategieën afhankelijk is van de moleculaire structuur van de bittere verbindingen, zoals toegelicht is aan de hand van sojaproducten en thee. Op grond hiervan lijkt het dat elk voedingsmiddel een eigen, op maat gemaakte ontbitteringsstrategie vereist.

Zusammenfassung

Die Aufnahme von Flavonoiden und Isoflavonoiden wird häufig mit gesundheitsfördernden Effekten assoziiert. Aus diesem Grund besteht sowohl für den Verzehr von (iso-)flavonoid-reichen Lebensmitteln als auch für die Anreicherung von Lebensmitteln mit (Iso-) Flavonoiden wachsendes Interesse. Einige (Iso-)Flavonoide besitzen jedoch einen bitteren Geschmack. Daher kann ihre Zugabe (oder Anreicherung) Bitterkeit in Lebensmitteln verursachen (oder verstärken). Die Entwicklung von Entbitterungsstrategien ist daher nötig, um dieses Problem zu lösen.

Die Geschmackseigenschaften vieler (Iso-)Flavonoide sind unbekannt, da sie noch nie in höheren Konzentrationen in Lebensmitteln angewandt wurden. Für andere (Iso-) Flavonoide ergaben sensorische Tests widersprüchliche Ergebnisse bezüglich ihres bitteren Geschmacks. Objektive Tests sind notwendig, um zu identifizieren, welche (Iso-) Flavonoide zum bitteren Geschmack von Lebensmitteln beitragen können. Ein objektives Instrument für Bitterkeitsstudien ist ein zellbasiertes Bitterrezeptor-Assay. Auf der menschlichen Zunge befinden sich 25 verschiedene Bittergeschmacksrezeptoren (hTAS2Rs). Jeder dieser Rezeptoren wurde individuell in Zellkulturen (HEK 293) eingebracht. Mit diesen Bitterrezeptor enthaltenden Zellen kann die „intrinsische Bitterkeit“ eines Stoffes *in vitro* untersucht werden. Intrinsische Bitterkeit ist ein Maß für die Fähigkeit eines Stoffes, Bitterrezeptoren zu aktivieren, unabhängig von anderen sensorischen Wahrnehmungen und von Wechselwirkungen mit Speicheleiweißen und der Mundschleimhaut. Das Ziel der vorliegenden Arbeit war es, die intrinsische Bitterkeit einer Vielzahl von (Iso-)Flavonoiden zu untersuchen und Strukturvoraussetzungen für die Aktivierung von Bitterrezeptoren durch (Iso-)Flavonoide zu erforschen. Anschließend wurden verschiedene Entbitterungsstrategien mit Hilfe des Bitterrezeptor-Assays getestet.

Im **ersten Kapitel** wird eine Übersicht über Flavonoide und Isoflavonoide bezüglich ihrer Strukturklassifizierung, ihrer sensorischen Eigenschaften und ihres Vorkommens in Lebensmitteln gegeben. Außerdem werden Geschmackswahrnehmung und die Wirkungsweise von Bitterrezeptoren erklärt. Sowohl der Mechanismus von Bitterrezeptor-Aktivierung *in vitro* als auch Strategien zur Verminderung von Bitterrezeptor-Aktivierung und Bittergeschmack im Allgemeinen werden beschrieben. Es wird ein aktueller Überblick über alle 25 Bitterrezeptoren in Bezug auf bekannte Agonisten und Antagonisten gegeben.

Das Ziel der im **zweiten Kapitel** beschriebenen Studie war die Identifizierung der Bitterrezeptoren, die den bitteren Geschmack des Soja-Isoflavons Genistein erkennen. Das Screening aller 25 menschlichen Bitterrezeptoren ergab, dass Genistein einen Agonisten für hTAS2R14 und hTAS2R39 darstellt. Genistein-Wahrnehmungsschwellenwerte lagen bei 4 und 8 µM für hTAS2R14 und hTAS2R39. Die jeweiligen EC_{50}-Werte waren 29 und 49 µM. Neben Genistein wurden außerdem Isoflavonoide mit einer genistein-ähnlichen Struktur untersucht. Obwohl die zwei Rezeptoren nicht eng verwandt sind, konnten ähnliche Resultate für hTAS2R14 und hTAS2R39 für die meisten Isoflavonoid-Aglykone erzielt werden. Für hTAS2R39 konnten vier von fünf glykosylierten Isoflavonen (Glycitin,

Genistin, Acetyl-Genistin, Malonyl-Genistin) als Agonisten identifiziert werden, wohingegen die Aktivierung von hTAS2R14 durch Glykosylierung von Isoflavonen verhindert zu werden schien. Eine dreifache Hydroxylierung des A- und B-Rings eines Isoflavonoids schien für eine Rezeptor-Aktivierung günstiger zu sein als eine geringere Hydroxylierung. Die Konzentration des dreifach-hydroxylierten Genistein liegt in vielen soja-basierten Lebensmitteln über dem in dieser Studie bestimmten Grenzwert. Die Konzentrationen anderer Soja-Isoflavone liegen dicht bei oder unter ihren Wahrnehmungsgrenzwerten. Trotz seiner niedrigen Konzentration könnte Genistein daher einer der Hauptverursacher des Bittergeschmacks von Soja-Produkten sein. Da auch die bioaktiven Isoflavonoiden Equol und Coumestrol beide Rezeptoren aktivierten, sollte vor ihrem Einsatz in Lebensmitteln ihr möglicher sensorischer Einfluss in Betracht gezogen werden.

Im **dritten Kapitel** wird die intrinsische Bitterkeit, die den Einsatz von (Iso-) Flavonoiden als bioaktive Lebensmittelinhaltsstoffe einschränken könnte, genauer untersucht. Eine große Anzahl von (Iso-)Flavonoiden mit einander ähnelnden Strukturen wurde bezüglich ihres Effekts auf die Bitterrezeptoren hTAS2R14 und hTAS2R39 untersucht und die strukturellen Voraussetzungen für die Aktivierung dieser Rezeptoren wurden vorausgesagt. Insgesamt aktivierten 68 Stoffe hTAS2R14 und 70 Stoffe hTAS2R39, wovon 58 Liganden identisch waren. Die Aktivierungsschwellenwerte lagen bis zu drei logarithmische Einheiten auseinander, nämlich zwischen 0.12 und 500 μM. Ligand-basierte 2D-Fingerprint- und 3D-Pharmakophor-Modelle wurden entwickelt, um Struktur-Aktivitäts-Beziehungen bestimmen zu können. Die 2D-Modelle zeigten hervorragende Voraussageeigenschaften für die Identifizierung von bitteren (Iso-) Flavonoiden und unterschieden diese von inaktiven (Iso-)Flavonoiden. Mit Hilfe der 3D-Pharmakophor-Modelle konnte für hTAS2R14- und hTAS2R39-aktivierende (Iso-) Flavonoidmoleküle bestimmt werden, welche Elemente sie aufweisen müssen, nämlich: entweder zwei (hTAS2R14) oder drei (hTAS2R39) Wasserstoffdonoren, einen Wasserstoffakzeptor und zwei aromatische Ringstrukturen, wovon mindestens ein Ring hydrophobe Eigenschaften aufweisen muss. Ein zusätzliches Wasserstoffdonor-Element für hTAS2R39 (verglichen mit hTAS2R14) wies auf die mögliche Anwesenheit eines weiteren, komplementären Akzeptor-Elementes im aktiven Zentrum von hTAS2R39 hin. Hydrophobe Wechselwirkungen zwischen dem aromatischen Element und dem aktiven Zentrum könnten für hTAS2R14 von höherer Bedeutung sein als für hTAS2R39. Zusammengenommen können die beiden letzten Eigenschaften erklären, warum OH-reiche Stoffe sich gegenüber diesen zwei Bitterrezeptoren anders verhalten. Die Kombination von *In-vitro*-Daten und verschiedenen *In-silico*-Methoden erlaubten eine gute Einsicht in die Aktivierung von hTAS2R14 und hTAS2R39 durch (Iso-)Flavonoide und stellten ein leistungsstarkes Instrument für die Voraussage ihrer möglichen Bitterkeit dar. Durch das Begreifen des „Bitter-Motives“ könnte das Einbringen eines bitteren Geschmacks in funktionelle Lebensmittel durch (Iso-)Flavoinoide verhindert werden.

Der Bitterrezeptor hTAS2R39 kann durch viele verschiedene Klassen von Bitterstoffen, worunter auch (Iso-)Flavonoide fallen, aktiviert werden. Jedoch sind auch einige Flavanone bekannt, die einen bitteren Geschmack maskieren können und nicht alle im **dritten Kapitel** beschriebenen Flavanone aktivierten hTAS2R39. Aus diesem Grund wurde im **vierten Kapitel** für 14 Flavonone untersucht, ob sie die Aktivierung von hTAS2R39 durch Epicatechingallat (ECG), einem der Hauptbitterstoffe in grünem Tee, reduzieren können. Drei der getesteten Stoffe zeigten einen hemmenden Effekt auf die Aktivierung von hTAS2R39 durch ECG: 4'-Fluoro-6-methoxyflavanon, 6,3'-Dimethoxyflavanon, und 6-Methoxyflavanon (in abnehmender Wirkungsstärke). Die getesteten 6-Methoxyflavanone hemmten auch die Aktivierung von hTAS2R14 (einem anderen Bitter-Rezeptor, der auch durch ECG aktiviert wird), jedoch in geringerem Maße. Sowohl die bei verschiedenen Konzentrationen des wirksamsten Antagonisten 4'-Fluoro-6-methoxyflavanon gemessenen Dosis-Wirkungs-Kurven von ECG als auch Waschexperimente wiesen auf reversiblen unüberwindbaren Antagonismus hin. Da ein vergleichbarer Effekt auch für den strukturell anders aufgebauten Agonisten Denatoniumbenzoat beobachtet werden konnte, kann von einem nichtkompetitiven orthosterischen Mechanismus ausgegangen werden. Die hier identifizierten Bitterrezeptor-Blocker können wahrscheinlich nicht in Lebensmitteln angewandt werden. Sie tragen jedoch zu einem besseren Verständnis von strukturellen Voraussetzungen für Bitterrezeptor-Blocker bei und können bei der Identifizierung von für den Einsatz in Lebensmitteln geeigneten Blockern helfen.

Im **fünften Kapitel** wird eine andere Strategie zur Reduzierung von Bitterkeit untersucht: die Komplexbildung von bitteren Flavonoiden mit in Lebensmitteln vorkommenden Eiweißen. Der bittere Teeinhaltsstoff Epigallocatechingallat (EGCG) wurde bezüglich seiner Bindungseigenschaften zu aufgereinigten Lebensmitteleiweißen und ihren entsprechenden Zubereitungen in Lebensmittelqualität untersucht. Diese Bindungseigenschaften wurden sowohl zu reduzierter Bitterrezeptor-Aktivierung *in vitro* als auch zu ihren Bitterkeit-Maskierungseigenschaften *in vivo* in Beziehung gesetzt. Im Gegenteil zu β-Laktoglobulin sind vor allem β-Kasein und einige Gelatinearten für ihre starke Bindungsneigung mit EGCG bekannt. Auch im Bitterrezeptor-Assay zeigte β-Kasein die stärksten Bindungseigenschaften mit einer maximalen Abnahme der Aktivierung von hTAS2R39 von ungefähr 93%. Eine vergleichbare Wirkung konnte für Na-Kaseinat, die Alternative zu β-Kasein in Lebensmittelqualität, beobachtete werden. Wie bereits durch seine niedrige Bindungsaffinität zu EGCG zu erwarten, hatte β-Laktoglobulin einen geringen Effekt auf die Bitterrezeptor-Aktivierung. Das Bittermaskierungspotential von Na-Kaseinat konnte *in vivo* durch ein sensorisch geschultes Expertenpanel bestätigt werden. Auch β-Laktoglobulin konnte die Bitter-Wahrnehmung leicht vermindern. Dieses Ergebnis konnte nicht direkt den Bindungskapazitätsergebnissen zugeordnet werden. Das Bitterrezeptor-Assay konnte als hilfreiches *In-vitro*-Instrument für die Einschätzung der

Wirkungskraft von Lebensmitteleiweißen als Komplexbildner zu Bittermaskierungszwecken eingesetzt werden.

Im **sechsten Kapitel** werden sowohl die in der vorliegenden Arbeit beschriebenen Ergebnisse als auch die Möglichkeiten und Einschränkungen des Bitterrezeptor-Zell-Assays diskutiert. Außerdem werden weitere Testergebnisse zu möglichen antagonistischen Eigenschaften von (Iso-)Flavonoiden präsentiert und Geschmacksbewertungen durch sensorische Tests, Rezeptor-Assays und Modellieren verglichen. Des Weiteren werden Strategien zur Reduzierung von Bitterkeit evaluiert und auf Soja-Produkte und Tee angewandt.

Die systematische Untersuchung von (Iso-)Flavonoid-Aglykonen zeigte, dass ihr Substitutionsprofil von größerer Bedeutung für die Bitter-Rezeptor-Aktivierung ist als die Struktur ihres Molekülskeletts. Für die Funktion von Bitter-Rezeptor-Antagonisten sind jedoch sowohl ihr Substitutionsprofil als auch die Struktur ihres Molekülskeletts essenziell. Das Bitterrezeptor-Assay konnte als hilfreiches Instrument sowohl für die Identifizierung von Bitterrezeptor-Agonisten und -Antagonisten als auch die Identifizierung von reduzierter Rezeptor-Aktivierung durch Komplexbildner beschrieben werden. Basierend auf den Ergebnissen dieser Arbeit konnte die Komplexbildung mit Lebensmitteleiweißen als die erfolgversprechendste Strategie für die Reduzierung des bitteren Geschmacks von in Tee vorkommenden Flavonoiden identifiziert werden. Für in Sojabohnen vorkommende Isoflavone scheint jedoch die Entbitterung durch die Verwendung von Bitterrezeptor-Blockern die erfolgversprechendste Strategie zu sein. Neben der Verwendung von Rezeptor-Blockern konnten auch Anpassung von Prozessmethoden (dergestalt, dass für eine nur geringe Bildung von Isoflavon-Aglykonen gesorgt wird) und die sorgfältige Auswahl von Rohstoffen (z.B. Sorten mit niedrigem Genisteingehalt) zur Vermeidung eines bitteren Geschmacks empfohlen werden. Abschließend kann festgestellt werden, dass die Wahl der Entbitterungsstrategien von der molekularen Struktur des bitteren Lebensmittelinhaltsstoffes abhängt. Dies wurde an Hand von soja-basierten Produkten und Tee gezeigt. Offensichtlich ist für jedes Lebensmittelprodukt eine maßgeschneiderte Entbitterungsstrategie nötig.

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About the author

CURRICULUM VITAE

Wibke Silke Ute Roland was born on December 23rd 1983 in Berlin, Germany. After graduating from high school (Gymnasium Steglitz, Berlin) in 2002, she entered the Technical University of Berlin. In 2003/2004 she completed an internship at the coffee factory Deutsche Extrakt Kaffee GmbH, Berlin, in the divisions spray drying, agglomeration and filling. She received her "Vordiplom" (comparable to BSc degree) in Food Technology in 2005 and went to the Netherlands to participate in the Erasmus exchange program of the European Union at Wageningen University. From this point onwards, she combined her studies at Wageningen University and TU Berlin. Wibke completed an internship at Cargill Texturizing Solutions in Hamburg, Germany, working in process and product development of lecithins. Afterwards, she continued her studies of Food Technology in Berlin. In 2007, she returned to the Netherlands for her MSc thesis entitled "Flavour binding properties of coffee brew melanoidins" at the Laboratory of Food Chemistry under the supervision of Koen Bekedam and Dr. Henk Schols. She finished her studies at Wageningen University with an MSc degree in Food Technology, specialization Ingredient Functionality, and at TU Berlin with a "Diplom-Ingenieur" diploma (comparable to MSc degree) in Food Technology, specialization Dairy Technology, in 2008. Wibke was offered the opportunity to work as a PhD student in the Laboratory of Food Chemistry at Wageningen University under the supervision of Dr. ir. Jean-Paul Vincken and Prof. dr. ir. Harry Gruppen, which she started in 2009. Most of the practical work was conducted at Unilever R&D Vlaardingen, the Netherlands, in the department Molecular Aspects of Health under the supervision of Dr. Robin Gouka. The results of her PhD research are presented in this thesis. In 2013, she continued as a researcher in the Laboratory of Food Chemistry. In January 2014, she started working as project manager flavour-texture interactions at NIZO food research in Ede, the Netherlands.

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LIST OF PUBLICATIONS

Roland, W.S.U.; Vincken, J.-P.; Gouka, R.J.; van Buren, L.; Gruppen, H.; Smit, G. Soy isoflavones and other isoflavonoids activate the human bitter taste receptors hTAS2R14 and hTAS2R39. ***J Agric Food Chem***. **2011**, 59 (21):11764-11771.

Bohin, M.C.; **Roland, W.S.U.**; Gruppen, H.; Gouka, R.J.; van der Hijden, H.T.W.M.; Dekker, P.; Smit, G.; Vincken, J.-P. Evaluation of the bitter-masking potential of food proteins for EGCG by a cell-based human bitter taste receptor assay and binding studies. ***J Agric Food Chem***. **2013**, 61 (42):10010-10017.

Roland, W.S.U.; van Buren, L.; Gruppen, H.; Driesse, M.; Gouka, R.J.; Smit, G.; Vincken, J.-P. Bitter taste receptor activation by flavonoids and isoflavonoids: Modeled structural requirements for activation of hTAS2R14 and hTAS2R39. ***J Agric Food Chem***. **2013**, 61 (44):10454-10466.

Roland, W.S.U.; Gouka, R.J.; Gruppen, H.; Driesse, M.; van Buren, L.; Smit, G.; Vincken, J.-P. 6-Methoxyflavanones as bitter taste receptor blockers for hTAS2R39. - ***accepted for publication in PLOS ONE***

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Courses

- Advanced Food Analysis[†] (VLAG), Wageningen (The Netherlands), 2010
- Gut-Brain Communications (ABS), Kuopio (Finland), 2011
- International Chemical Design and Discovery course, Nijmegen (The Netherlands), 2012

Conferences and meetings

- 8th Pangborn Symposium of Sensory Science, Florence (Italy), 2009
- Mini-Symposium Sensory Science, Vlaardingen (The Netherlands), 2010
- Mini-Symposium Food Fortification, Vlaardingen (The Netherlands), 2010
- GPCR symposium[‡], Vlaardingen (The Netherlands), 2011
- Lecture Sensory Engineering, Vlaardingen (The Netherlands), 2011
- Process-Net Annual Meeting, Vlaardingen (The Netherlands), 2011
- 2nd International Conference on Food Oral Processing[†‡], Beaune (France), 2012
- Mini-Symposium "The Chemistry between Science and Industry", Vlaardingen (The Netherlands), 2013

General courses

- PhD introduction week (VLAG), Maastricht (The Netherlands), 2009
- Speed reading (KLV), Wageningen (The Netherlands), 2010
- Philosophy and Ethics of Food Science and Technology (VLAG), Wageningen (The Netherlands), 2010
- Techniques for writing and presenting a scientific paper (WGS), Wageningen (The Netherlands), 2011
- Teaching and supervising thesis students (WU), Wageningen (The Netherlands), 2011
- Career orientation (WGS), Wageningen (The Netherlands), 2012
- Project and time management, (WGS), Wageningen (The Netherlands), 2013

Optionals

- FCH study trip to Ghent, Belgium, WU (FCH), 2009
- PhD trip FCH Switzerland / Italy[†‡], WU (FCH), 2010
- PhD trip FCH Singapore / Malaysia[†‡], WU (FCH), 2012
- Organisation PhD trip FCH Singapore / Malaysia, WU (FCH), 2011-2012

- BSc / MSc thesis students presentations and colloquia, WU (FCH), 2009-2013
- PhD presentations, WU (FCH), 2009-2013
- Phytonutrient meetings, WU (FCH), 2009-2013
- Molecular aspects of health meetings, URDV, 2010-2012
- Nutrition and health meetings, URDV, 2010-2012
- Science update presentations, URDV, 2010-2012
- PhD research proposal

† Poster presentation; ‡ Oral presentation

Abbreviations used:

ABS: Applied Bioscience Graduate School

FCH: Laboratory of Food Chemistry

KLV: Koninklijke Landbouwkundige Vereniging Wageningen Alumni Network

URDV: Unilever R&D Vlaardingen

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Science

WGS: Wageningen Graduate Schools

WU: Wageningen University

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