

## Different Phenolic Compounds Activate Distinct Human Bitter Taste Receptors

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**S** Supporting Information

**ABSTRACT:** Bitterness is a major sensory attribute of several common foods and beverages rich in polyphenol compounds. These compounds are reported as very important for health as chemopreventive compounds, but they are also known to taste bitter. In this work, the activation of the human bitter taste receptors, TAS2Rs, by six polyphenol compounds was analyzed. The compounds chosen are present in a wide range of plant-derived foods and beverages, namely, red wine, beer, tea, and chocolate. Pentagalloylglucose (PGG) is a hydrolyzable tannin, (–)-epicatechin is a precursor of condensed tannins, procyanidin dimer B3 and trimer C2 belong to the condensed tannins, and malvidin-3-glucoside and cyanidin-3-glucoside are anthocyanins. The results show that the different compounds activate different combinations of the ~25 TAS2Rs. (–)-Epicatechin activated three receptors, TAS2R4, TAS2R5, and TAS2R39, whereas only two receptors, TAS2R5 and TAS2R39, responded to PGG. In contrast, malvidin-3-glucoside and procyanidin trimer stimulated only one receptor, TAS2R7 and TAS2R5, respectively. Notably, tannins are the first natural agonists found for TAS2R5 that display high potency only toward this receptor. The catechol and/or galloyl groups appear to be important structural determinants that mediate the interaction of these polyphenolic compounds with TAS2R5. Overall, the EC<sub>50</sub> values obtained for the different compounds vary 100-fold, with the lowest values for PGG and malvidin-3-glucoside compounds, suggesting that they could be significant polyphenols responsible for the bitterness of fruits, vegetables, and derived products even if they are present in very low concentrations.

**KEYWORDS:** polyphenols, bitterness, tannins

### ■ INTRODUCTION

It has been well-known and common sense that the consumption of diets rich in fruits and vegetables and with a moderate intake of red wine (i.e., polyphenol-rich foods) is inversely correlated to cardiovascular disease incidence and cancer risk, probably because the polyphenols modulate several biological reactions that lead to these disease conditions.<sup>1</sup> In fact, during the past years polyphenol compounds have been suggested as chemoprevention agents.

Polyphenol compounds result from plant secondary metabolism as chemical defense against predators and are usually divided in nonflavonoids and flavonoids, the latter being the most relevant ones. Flavonoids include very diverse compounds such as anthocyanins and flavan-3-ols. Although they are potentially beneficial to human health in small doses, many of these compounds are toxic in high doses. Several of these compounds are responsible for major organoleptic properties of vegetables and fruits, namely, color and taste. In fact, some of them are known to have a bitter taste and so, despite their healthy properties, many people do not like to eat vegetables and other plant-derived food because of the bitterness associated with polyphenols.<sup>2</sup> Debittering of food has been a longstanding major sensory challenge for the food industry<sup>2</sup> that requires identification of those compounds making food and beverages taste bitter. Therefore, studying the sensorial properties of polyphenol compounds is a prominent

subject relevant for people's food choices and the chemopreventive potential of food.

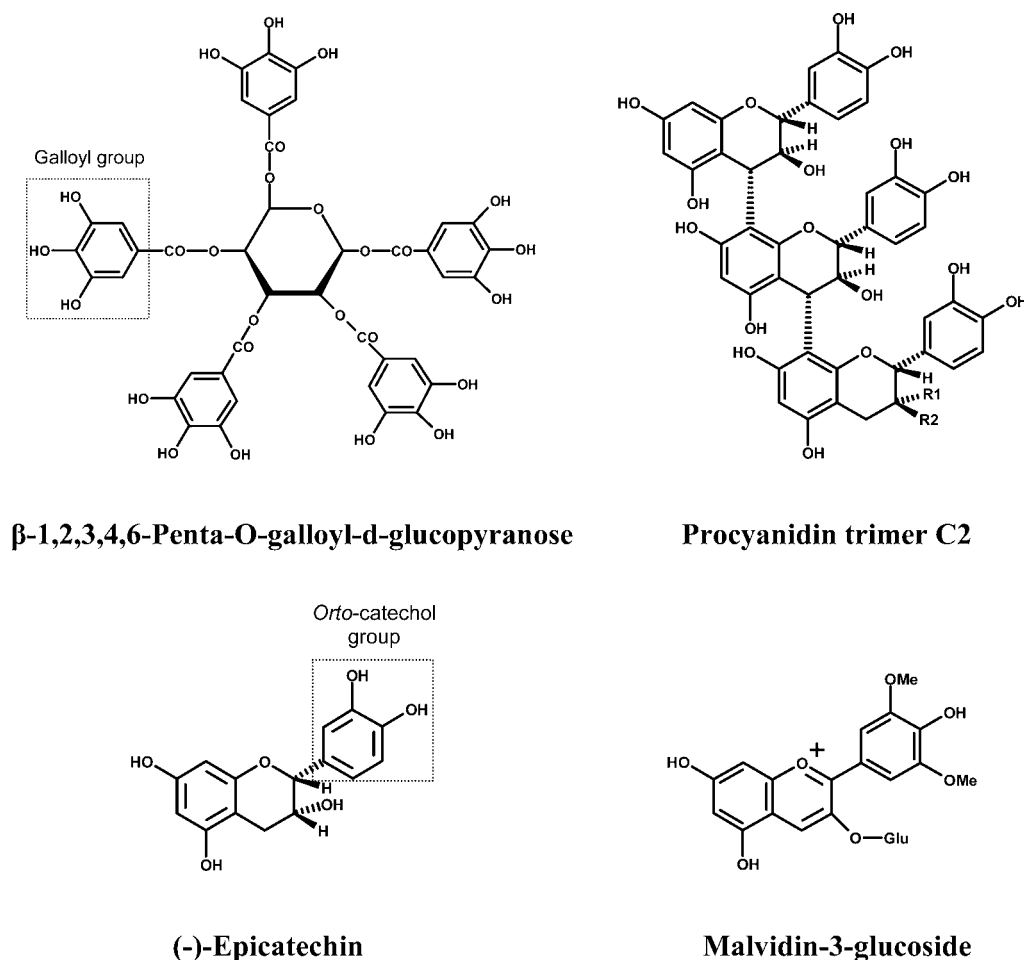
Although bitterness in foods is usually unpleasant, there are some foodstuffs in which it is a wanted sensory attribute, for example, red wine and beer. In fact, most of the sensory analysis data regarding polyphenol bitterness are related to red wine. It is thought that the bitterness of red wine is mainly induced by polyphenol compounds (e.g., tannins).<sup>2,3</sup> However, the limited available data regarding polyphenol's structure/bitterness relationship are rather inconsistent.<sup>3–7</sup> In general, these works studied the bitterness of polyphenol compounds, such as polymeric fractions of tannic acid and tannins, as well as flavan-3-ol monomers, dimers, and trimers, and demonstrated that larger molecules tend to be less bitter and more astringent.<sup>4,6</sup> Peleg and co-workers<sup>4</sup> found that (–)-epicatechin was more bitter than the stereoisomer (+)-catechin and that these both were more bitter than the procyanidin trimers, catechin-(4–8)-catechin-(4–8)-catechin and catechin-(4–8)-catechin-(4–8)-epicatechin. Robichaud and colleagues<sup>6</sup> found that tannic acid, a commercial hydrolyzable pentagalloylglucose-rich tannin, was more bitter than both (+)-catechin and a grape seed extract, which is rich in polymeric procyanidins.

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**Figure 1.** Chemical structures of the four polyphenol compounds studied.

However, other studies have shown that bitterness of polyphenols increases with molecular weight.<sup>3,5,8</sup> Hufnagel and Hofmann<sup>3</sup> found that procyanidin dimers and a trimer were more bitter than (-)-epicatechin. These authors also demonstrated by taste reconstruction and omission experiments that the bitterness of the red wine could be induced by subthreshold concentrations of phenolic acid ethyl esters and flavan-3-ols. Besides these inconsistencies, there is insufficient knowledge about the bitter taste of polyphenol compounds that do not belong to tannin classes, such as anthocyanins.

Bitter taste is elicited by a specific subset of taste receptor cells (TRC) localized in the oral cavity in groups of cells called taste buds, which are embedded in the epithelium of the gustatory papillae on the tongue and palate. These TRC are characterized by the expression of members of the TASTE 2 Receptor (*TAS2R*) gene family encoding bitter taste receptors.<sup>9–11</sup> In humans, this gene family codes for ~25 taste receptors (*TAS2Rs*) that are G protein-coupled receptors. So far, 20 of them have been orphaned, meaning that cognate bitter compounds have been assigned to them.<sup>10,12–20</sup> In general, *TAS2Rs* are sensitive to several or multiple different bitter compounds, a property that has been proposed to be the basis for recognition of the countless bitter chemicals.<sup>12,20,21</sup> Due to the presence of numerous nonsynonymous single-nucleotide polymorphisms (SNPs), the *TAS2R* genes encode functionally distinct receptor variants that form the basis for variations in bitterness perception in the population.<sup>15,16,22,23</sup>

To understand better structure/bitterness relation, to overcome the inconsistencies associated with sensorial analysis, and to identify which *TAS2Rs* are activated by the various polyphenol compounds, an objective, robust, and reliable method to analyze their bitterness is required. Few recent studies used heterologous expression experiments to successfully measure *TAS2R* activation by purified bitter chemicals that are normally present in food and beverage sources such as beer hops, cheese, and soy products.<sup>17–20,24</sup> In the present paper this method was used to examine the activation of the *TAS2Rs* by several polyphenol compounds widely spread in foods and beverages derived from plants and commonly present in our daily diet (Figure 1).

On the basis of their extensive prevalence in vegetables, fruits, and derived products, the following six substances that belong to some of the most important classes of polyphenols (anthocyanins, flavan-3-ols, and hydrolyzable tannins) were chosen:<sup>25</sup> (-)-epicatechin, procyanidin dimer B3, and trimer C2 (flavan-3-ols); malvidin-3-glucoside and cyanidin-3-glucoside (anthocyanins); and pentagalloylglucose (PGG) (hydrolyzable tannins). (-)-Epicatechin (structural unit of condensed tannins) and a number of different procyanidin dimers and trimers belong to the condensed tannins class and are present in a variety of frequently ingested foods including red wine, cocoa, grape seeds, tea, beer, and cereals.<sup>25</sup> Malvidin-3-glucoside and cyanidin-3-glucoside belong to the anthocyanin class, being highly present in red fruits and their derived products, red wine, and olives.<sup>25</sup> Although PGG is not

commonly found in a wide range of foodstuffs, it belongs to the other class of tannins (hydrolyzable tannins) and is present in pomegranate and green tea, and it can also be incorporated in red wine during the winemaking process (by addition of commercial tannic acid).<sup>26</sup>

Our aim was to elucidate if the selected polyphenol compounds are activators of human bitter taste receptors and to examine if different polyphenols activate different TAS2Rs.

## MATERIALS AND METHODS

**Chemicals.** All reagents used were of analytical grade. (+)-Catechin and (–)-epicatechin were purchased from Sigma-Aldrich, malvidin aglycon was purchased from Extrasynthèse, sodium borohydride and tartaric acid were purchased from Aldrich, tannic acid was purchased from Fluka Biochemica (Switzerland), taxifolin was purchased from Extrasynthèse (Genay, France), and Toyopearl HW-40(s) gel was purchased from Tosoh (Tokyo, Japan).

**Malvidin-3-glucoside and Cyanidin-3-glucoside (Anthocyanins) Isolation.** Malvidin-3-glucoside and cyanidin-3-glucoside were isolated as described in the literature.<sup>27</sup> Briefly, these compounds were isolated from a grape skin extract that was applied to a TSK Toyopearl HW-40(s) gel column. The elution was made with 10% CH<sub>3</sub>OH/CH<sub>3</sub>COOH (v/v), and then the solvent was evaporated. The resulting residue was applied to a C18 gel column, and the compounds were eluted with 10% methanol acidulated. The solution was again evaporated, and the resulting solution was mixed with distilled water, frozen, and lyophilized. The compound's purity was assessed by HPLC-MS, direct MS, and NMR analysis and was >99%. Spectroscopical data were in accordance with the literature.<sup>28,29</sup>

**Procyanidin Dimer (B3) and Trimer (C2) (Condensed Tannins) Synthesis.** The synthesis of procyanidin dimer B3 (catechin-(4–8)-catechin) and trimer C2 (catechin-(4–8)-catechin-(4–8)-catechin) followed the procedure described in the literature.<sup>30</sup> Briefly, taxifolin and (+)-catechin (ratio 1:3) were dissolved in ethanol, and the mixture was treated with sodium borohydride (in ethanol). The pH was then lowered to 4.5 by addition of CH<sub>3</sub>COOH/H<sub>2</sub>O 50% (v/v), and the mixture stood under argon atmosphere for 30 min. The reaction mixture was extracted with ethyl acetate. After evaporation of the solvent, water was added, and the mixture was passed through C18 gel, washed with water, and recovered with methanol. After evaporation of methanol, the fraction was passed through a TSK Toyopearl HW-40(s) gel column (300 mm × 10 mm i.d., 0.8 mL min<sup>-1</sup>, methanol as eluent) coupled to a UV–vis detector. Several fractions were recovered and analyzed by ESI-MS (Finnigan DECA XP PLUS) yielding procyanidins dimers (B3 and B6) and trimer (C2). The structure was elucidated by HPLC-MS and NMR analysis. Spectroscopical data were in accordance with the literature.<sup>31</sup>

**β-1,2,3,4,6-Penta-O-galloyl-D-glucopyranose (PGG) (Hydrolyzable Tannin) Synthesis.** PGG was synthesized according to the method of Chen and Hagerman.<sup>32</sup> Briefly, 5.0 g of tannic acid was methanolized in 70% methanol in acetate buffer (0.1 M, pH 5.0) at 65 °C for 15 h. The pH of the reaction mixture was immediately adjusted to 6.0 with NaOH. Methanol was evaporated under reduced pressure at <30 °C, and water was added to maintain the volume. The solution was extracted with 3 volumes of diethyl ether and 3 volumes of ethyl acetate. The ethyl acetate extracts were combined and evaporated, with addition of water to maintain the volume. The resulting suspension was centrifuged, and the precipitate was redissolved by heating in 2% methanol solution. PGG precipitated as the solution cooled to room temperature and was collected by centrifugation. PGG was washed twice with an ice-cold 2% methanol solution and once with ice-cold distilled water. The final material was lyophilized to yield a white powder with an overall mass yield of 23%. The purity of the obtained PGG was assessed by HPLC analysis<sup>33</sup> and <sup>1</sup>H NMR spectroscopy, and it was ≤99%. Spectroscopical data were in accordance with the literature.<sup>32</sup>

**Test Compound Preparation.** The compounds to be tested were dissolved either in buffer C1 [130.0 mM NaCl, 5.0 mM KCl, 10.0 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 2.0 mM CaCl<sub>2</sub>,

10.0 mM glucose (pH 7.4)] or in a mixture of dimethyl sulfoxide (DMSO) and buffer C1 not exceeding a final DMSO concentration of 0.1% (v/v) to avoid toxic effects on the transfected cells.

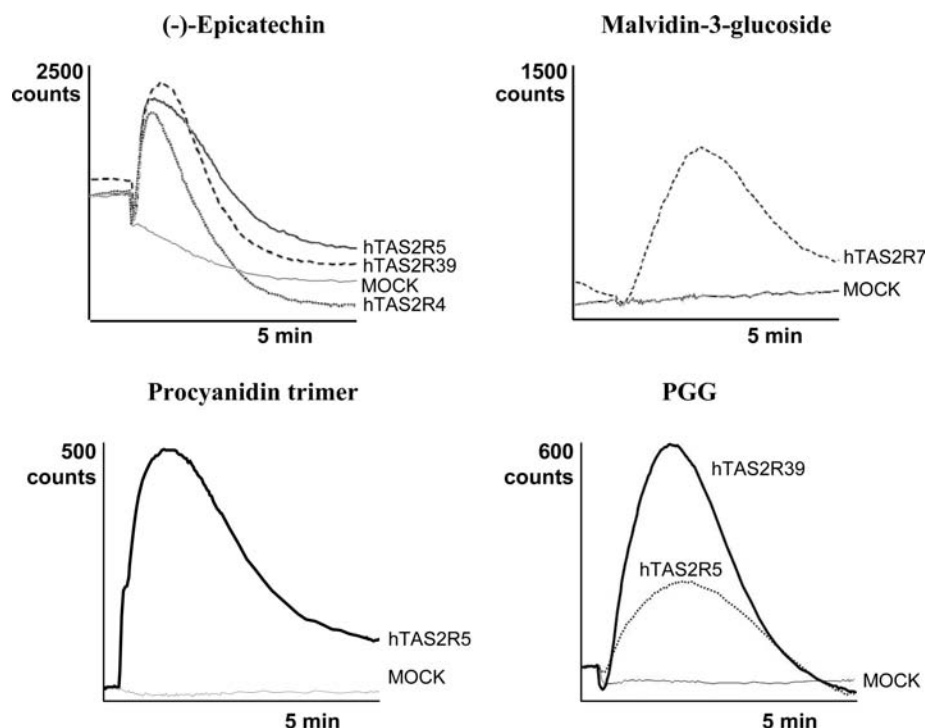
**Cell Transfection and Expression of TAS2Rs in Heterologous Cells.** Functional expression studies were carried out as described before.<sup>19,20</sup> Human embryonic kidney (HEK)-293T cells stably expressing the chimeric G protein subunit Gα16gust44 were seeded into poly-D-lysine-coated (10 μg mL<sup>-1</sup>) 96-well plates (Greiner Bio-One, Frickenhausen, Germany) under regular cell culture conditions [Dulbecco's modified Eagle medium (DMEM), 10% FCS, 1% penicillin/streptomycin; 37 °C, 5% CO<sub>2</sub>, 95% humidity]. After 24–26 h, cells were transfected transiently with 150 ng expression plasmids based on pEAK10 (Edge BioSystems) or pcDNA5/FRT (Invitrogen) using 300 ng of Lipofectamine2000 (Invitrogen). In addition to the TAS2R coding sequences, the plasmids contained the first 45 amino acids of rat somatostatin receptor 3 for cell surface localization followed by the herpes simplex virus (HSV) glycoprotein D epitope for immunocytochemical detection of the receptor. The TAS2R sequences are according to the literature.<sup>20</sup>

**Calcium Imaging analysis.** Twenty-four to 26 h after transfection, cells were loaded with the calcium-sensitive dye Fluo-4-acetoxymethyl ester (2 μM, Molecular Probes) in serum-free DMEM. Probencid (Sigma-Aldrich GmbH), an inhibitor of organic anion transport, was added at a concentration of 2.5 mM, to minimize the loss of the calcium indicator dye from cells. One hour after loading, the wells were washed three times with C1 buffer using a Denley Cell Washer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were incubated in washing buffer in the dark for 30 min between the washing steps. Fluorescence changes were recorded at 510 nm following excitation at 488 nm by a fluorometric imaging plate reader (FLIPR, Molecular Devices) before and after application of the test compounds in each well. A second application of 100 nM somatostatin-14 (Bachem) activating the endogenous somatostatin receptor type 2 was used to assess cell vitality. All experiments were performed at least in duplicates. Mock-transfected cells (cells transfected with empty pcDNA5/FRT vector used as negative control) were always measured in parallel on the same microtiter plates using the same compound concentrations used to examine the cells expressing the various TAS2Rs. All compounds were initially tested at different concentrations for unspecific calcium responses in untransfected HEK293T Gα16gust44 cells. On the basis of this pilot experiment, the used maximal compound concentrations were always lower than those concentrations [(–)-epicatechin, unknown, PGG, 100 μM, malvidin-3-glucoside, 30 μM, procyanidin trimer, 300 μM] that generated unspecific responses in the absence of transfected receptor DNAs.

**Determination of Half-Maximal Effective Concentrations (EC<sub>50</sub>) and Statistical Analysis.** Having identified responsive TAS2Rs, their concentration-dependent activation was examined and half-maximal effective concentrations (EC<sub>50</sub>) values for their bitter agonists were established. To calculate the concentration–response curves, the fluorescence changes of mock-transfected cells were subtracted from the corresponding values of receptor-expressing cells by means of the FLIPR384 software (Molecular Devices, Munich, Germany). To compensate for differences in cell density, signals were normalized to background fluorescence for each well. Signals were recorded in at least duplicate wells and the data averaged. Signal amplitudes were then plotted versus log agonist concentration. EC<sub>50</sub> values were calculated using SigmaPlot 9.01 (Systat Software GmbH, Erkrath, Germany) by nonlinear regression using the function

$$f(x) = \min + \frac{(\max - \min)}{1 + (x/EC_{50})^{-nH}} \quad (1)$$

where  $x$  is the test compound concentration and  $nH$  the Hill coefficient. Statistical significance of the difference between the several calculated EC<sub>50</sub> and of the signal amplitudes was evaluated by one-way analysis of variance, followed by the Bonferroni test. Differences were considered to be statistically significant when  $P < 0.05$ .



**Figure 2.** Fluorescence changes of Fluo4-AM-loaded HEK293T-G $\alpha$ 16gust44 cells expressing the TAS2Rs indicated in the graphs following administration of 10.0 mM (–)-epicatechin, 20.0  $\mu$ M malvidin-3-glucoside, 100.0  $\mu$ M procyanidin trimer, or 10.0  $\mu$ M PGG. Responses of mock-transfected cells (empty plasmid) are indicated by the thinner solid line.

## RESULTS

### Identification of TAS2Rs That Respond to Polyphenol Compounds.

To identify the TAS2Rs that are sensitive to the selected prototypical polyphenols, the 25 TAS2Rs, TAS2R1, TAS2R3, TAS2R4, TAS2R5, TAS2R7, TAS2R8, TAS2R9, TAS2R10, TAS2R13, TAS2R14, TAS2R16, TAS2R38, TAS2R39, TAS2R40, TAS2R41, TAS2R42, TAS2R43, TAS2R31, TAS2R45, TAS2R46, TAS2R30, TAS2R19, TAS2R20, TAS2R50, and TAS2R60, were expressed individually in HEK293T cells stably expressing the chimeric G protein G $\alpha$ 16gust44. This construct was designed to couple the activation of TAS2Rs in heterologous cells to the release of Ca<sup>2+</sup> from intracellular stores, which can be measured using calcium-sensitive fluorescence dyes.<sup>34</sup> Receptor-mediated changes in intracellular calcium concentrations were detected as fluorescence changes by means of a fluorometric imaging plate reader.

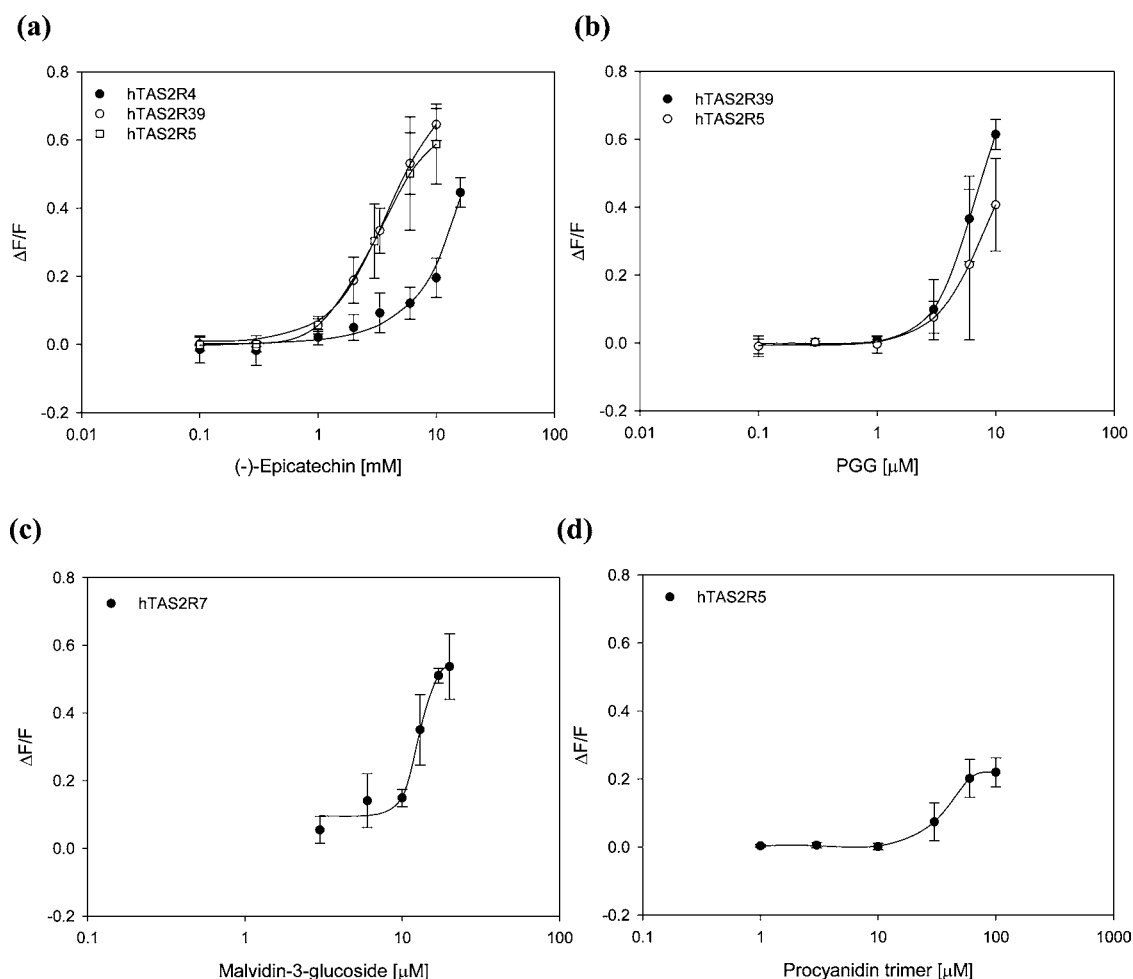
The six polyphenol compounds to be tested, (–)-epicatechin, procyanidin dimer and trimer, malvidin-3-glucoside, cyanidin-3-glucoside, and PGG, were administered, at the highest possible concentrations (predetermined in pilot experiments, data not shown), by bath application to the 25 different receptor-expressing cell populations. (–)-Epicatechin was employed up to 16.0 mM, whereas all the other compounds were employed only at micromolar range [procyanidin trimer, 100.0  $\mu$ M; malvidin-3-glucoside, 20.0  $\mu$ M; PGG, 10.0  $\mu$ M (Figure 2)]. By tracing cytosolic calcium levels, it was found that cells expressing TAS2R4, TAS2R5, or TAS2R39 responded to challenge with (–)-epicatechin. Cells transfected with DNA for TAS2R5 or TAS2R39 were also sensitive to PGG. Elevation of calcium levels was also seen in cells expressing TAS2R5 that have been exposed to procyanidin trimer. Finally, it was found that malvidin-3-glucoside elicited signals specifically in cells transfected with DNA for TAS2R7.

Cells expressing TAS2R1, TAS2R3, TAS2R8, TAS2R9, TAS2R10, TAS2R13, TAS2R14, TAS2R16, TAS2R38, TAS2R40, TAS2R41, TAS2R42, TAS2R43, TAS2R31, TAS2R45, TAS2R46, TAS2R30, TAS2R19, TAS2R20, TAS2R50, and TAS2R60 did not respond to the tested compounds (data not shown). It is also important to note that procyanidin dimer and cyanidin-3-glucoside did not activate any receptor, at least in the highest possible concentrations tested (100  $\mu$ M).

**Functional Characterization of Activated TAS2Rs.** To investigate the activation of the bitter receptors TAS2R4, TAS2R5, TAS2R7, and TAS2R39 by the polyphenols in greater detail, concentration–response functions were recorded and the half-maximal effective agonist concentrations (EC<sub>50</sub>) established (Figure 3). The concentration–response curves followed sigmoid functions. The calculated EC<sub>50</sub> values for the compound–receptor pairs are presented in Table 1, where the values with “ $\geq$ ” are estimates because the concentration–response curves did not saturate and so we were unable to determine an EC<sub>50</sub> value.

Collectively, the data demonstrate that the EC<sub>50</sub> values for the different agonist/receptor pairs vary from the micromolar to the millimolar range. Whereas the EC<sub>50</sub> values for PGG at both TAS2R5 and TAS2R39 are  $\geq$ 8.5 and  $\geq$ 6.6  $\mu$ M, respectively, those for (–)-epicatechin are  $\sim$ 1000-fold higher and vary between 3.2 and 3.8 mM. Like the EC<sub>50</sub> values for PGG, those for malvidin-3-glucoside and procyanidin trimer are also in the micromolar range, being 12.6  $\mu$ M at TAS2R7 and 35.6  $\mu$ M at TAS2R5, respectively.

Besides these pronounced differences in the EC<sub>50</sub> values, the observed threshold concentrations, defined as the lowest concentration that resulted in calcium signals in receptor-transfected cells, are also largely different (Table 2). Whereas PGG induced robust receptor responses already at a



**Figure 3.** Concentration–response curves for HEK293T-Gα16gust44 cells transfected with DNA for the indicated TAS2R following stimulation with the indicated test compound. Error bars represent the confidence interval ( $P = 0.05$ ). Dose-dependent activation was observed for TAS2R4, TAS2R5, and TAS2R39 by (–)-epicatechin (a), for TAS2R5 and TAS2R39 by PGG (b), for TAS2R7 by malvidin-3-glucoside (c), and for TAS2R5 by procyanidin trimer (d).

**Table 1.** EC<sub>50</sub> Values for the Test Compounds and Respective Receptors<sup>a</sup>

	EC <sub>50</sub> (μM)			
	TAS2R4	TAS2R5	TAS2R7	TAS2R39
(–)-epicatechin	≥30151.0	3210.0 ± 42.0a		3800.0 ± 200.0bc
procyanidin trimer		35.6 ± 0.7d		
PGG		≥8.5		≥6.6
malvidin-3-glucoside			12.6 ± 0.7	

<sup>a</sup>Values with the same letter are not significantly different ( $P < 0.05$ ). Values with  $\geq$  are estimates because the dose-response curves did not saturate.

**Table 2.** Threshold Concentrations, Defined as the Lowest Concentration That Resulted in Calcium Signals in Receptor-Transfected Cells, for the Test Compounds

	threshold concentrations (μM)			
	TAS2R4	TAS2R5	TAS2R7	TAS2R39
(–)-epicatechin	2000.0	1000.0		1000.0
procyanidin trimer		30.0		
PGG		3.0		3.0
malvidin-3-glucoside			6.0	

concentration of 3.0 μM at both TAS2R5 and TAS2R39, a 300–1000-fold higher concentration of (–)-epicatechin was required to evoke receptor responses at TAS2R4, TAS2R5, and

TAS2R39. Like PGG, threshold values for malvidin-3-glucoside and procyanidin trimer were in the micromolar range. The lowest concentrations that induced detectable receptor responses were 6.0 μM malvidin-3-glucoside for TAS2R7 and 30.0 μM procyanidin trimer for TAS2R5.

Also, signal amplitudes, which are related to the efficiency of the receptor activation, differ across receptors and the polyphenols (Table 3). (–)-Epicatechin activated TAS2R5 cognate receptor as well as TAS2R4 and TAS2R39 with high efficacy as revealed by the change in fluorescence, that is, the corresponding  $\Delta F/F$  values. Similarly, PGG was an efficient stimulus for TAS2R39, and also malvidin-3-glucoside showed high efficiency at its cognate receptor. In contrast, the observed

Table 3. Signal Amplitudes (Given as Relative Fluorescence Changes  $\Delta F/F$ ) for the Test Compounds

	signal amplitudes			
	TAS2R4	TAS2R5	TAS2R7	TAS2R39
(-)-epicatechin	0.44 ± 0.04a	0.59 ± 0.11a		0.65 ± 0.04a
procyanidin trimer		0.22 ± 0.04c		
PGG		0.36 ± 0.16d		0.61 ± 0.04d
malvidin-3-glucoside			0.54 ± 0.09	

efficacy of procyanidin trimer at its cognate receptor is relatively low.

## DISCUSSION

It has been well-known and common sense that the consumption of diets with high content of foods rich in vitamins, minerals, fibers, and polyphenols, as well as low levels of saturated fats, is directly associated with a low incidence of cardiovascular and cancer diseases. The well-known “French paradox” that appeared in 1992 is a classic example of this association.<sup>35</sup> Since that time, an exponential growth of epidemiological, clinical, and experimental data has supported the importance of a diet rich in polyphenol-rich foods.<sup>1</sup> In fact, these compounds have been suggested as chemoprevention agents. For instance, pentagalloylglucose has been shown in vivo to have anticancer effects against prostate, lung, and breast cancers as well as to have antidiabetes activity.<sup>36</sup> Also, cyanidin-3-glucoside has been shown to be able to revert human melanoma cells from the proliferating to the differentiated state.<sup>37</sup> Because of polyphenols’ beneficial effects on human health, increasing the phytonutrient content of plant foods is a potent dietary option for preventing diseases and a challenge for the design of functional foods. However, many people do not like to eat vegetables and/or derived products especially because of their bitter taste.<sup>2</sup> Therefore, the study of the bitterness properties of polyphenol compounds is a prominent subject that could reflect in people’s food choices and last in people’s food chemoprevention.

Therefore, in this paper the activation of human bitter receptor by six polyphenol compounds commonly present in human diet [(–)-epicatechin, procyanidin dimer B3 and trimer C2, malvidin-3-glucoside, cyanidin-3-glucoside, and PGG] was studied, and the data indicate that four of the six polyphenol compounds tested may mediate their bitterness through activation of at least four human bitter taste receptors, TAS2R4, TAS2R5, TAS2R7, and TAS2R39.

The agonist–receptor pairs display an interesting activation pattern. First, different compounds activate the same receptor. For example, (–)-epicatechin and PGG activate TAS2R39, whereas (–)-epicatechin, PGG, and procyanidin trimer activate TAS2R5. Second, different receptors are activated by the same compound. This is evidenced by (–)-epicatechin, which activates TAS2R4, TAS2R5, and TAS2R39, and PGG, which stimulates TAS2R5 and TAS2R39. These results conform well with the combinatorial activation patterns of TAS2Rs previously reported<sup>13,20</sup> (Table 1 in the Supporting Information).

Furthermore, by the structurally diverse compounds, these data also have implications for structure–activity relationships. They suggest that the catechol or galloyl group (which has only one more hydroxyl group than catechol) is a critical feature (but not essential) for the interaction of polyphenol compounds with TAS2R5. First, the compounds that activated this receptor, (–)-epicatechin, procyanidin trimer, and PGG,

have at least one of these groups, whereas the other tested compound, malvidin-3-glucoside, did not. Second, procyanidin trimer and PGG, which possess three *o*-catechol groups and five galloyl groups, respectively, activate TAS2R5 at 100-fold lower concentrations than does (–)-epicatechin, which has only one *o*-catechol group. However, this structural feature is not required for other compounds that activate TAS2R5 because the synthetic ligand 1,10-phenanthroline lacks these groups completely. In fact, it is evident that the presence of these groups is not sufficient for the responsiveness of this receptor because the procyanidin dimer (which has two *o*-catechol groups) does not activate this receptor. However, it seems that, when a compound activates TAS2R5, the presence of these groups could be essential for higher responses/interaction. The importance of the hydroxyl groups in bitter receptor activation is also referenced by Roland and co-workers,<sup>24</sup> who have studied the activation of receptor TAS2R39 by the soy isoflavone genistein and structurally similar isoflavonoids. These authors concluded that the presence of three hydroxyl groups in the isoflavonoid ring system, which resembles that of the polyphenols, seemed to be more favorable for TAS2R39 activation than the presence of fewer hydroxyl groups.

Notably, the above chemicals are the first natural bitter compounds found for TAS2R5. So far, this specialist receptor responded only to the synthetic compound phenanthroline and may therefore be the only TAS2R that is activated (“specific”) by natural tannins.

Roland and co-workers<sup>24</sup> also noted the importance of glucose residues attached to their test compounds for TAS2R39 activation. The present study demonstrated that TAS2R39 responded to PGG, which also contains a glucoside moiety. However, malvidin-3-glucoside and cyanidin-3-glucoside did not activate this receptor. Interestingly, the glycosylated compounds did not activate the TAS2R16, which has been suggested to be specific for  $\beta$ -glucopyranosides.<sup>12</sup> However, these authors<sup>12</sup> also found that the hydrophobicity and size of the aglycons are critical for receptor activation. The great structural differences between the compounds employed by Bufe and co-workers and those tested here easily explain the absence of responses of TAS2R16.

A receptor for which the presence of glucose residues seems to be important is TAS2R7. Whereas malvidin-3-glucoside activated TAS2R7, its aglycon form (without glucose residue) failed to do so, at least at the tested concentrations. Our results for malvidin-3-glucoside oppose those of a sensory study that showed that this anthocyanin does not taste bitter.<sup>38</sup> These authors studied the bitterness of malvidin-3-glucoside in a mixture with cyanidin-3-glucoside made in a solution saturated with tartaric acid (pH 3.6) and containing ethanol. It is also important to mention that anthocyanins coexist in solution in different forms because of their well-known pH dependence:<sup>39</sup> at low pH (1–2) they are essentially present in the red cationic form (AH<sup>+</sup>), but as the pH increases rapid proton transfer reactions occur, leading to the formation of blue quinonoid

bases (A and A-) and chalcones. Therefore, the two data sets appear not to be comparable because the use of other compounds and distinct experimental conditions could influence the perceived bitterness and receptor responses differently. It is important to point out that ethanol could not be tested in the *in vitro* assay because it is toxic for cells already at 1%.

Even though the factors that determine bitter intensity are still unknown, the concentration-response functions of the five polyphenols and their cognate receptors predict that PGG appears to be a strong bitter compound in foodstuffs if present at least in low micromolar concentrations. First, it activates both receptors, TAS2R5 and TAS2R39. Second, it displays high potency evident as low  $EC_{50}$  values at both cognate receptors. Third, it shows also high efficacy as revealed by the high signal amplitudes of the activated receptors, in particular for TAS2R39. Malvidin-3-glycoside and procyanidin trimer are also of high potency; that is, they have low  $EC_{50}$  values at their receptors. However, both compounds activate one receptor with relatively low efficacy, TAS2R7 and TAS2R5, respectively. This could mean that these polyphenol compounds elicit a weaker bitter taste than PGG, if they are present in food in similar concentrations as PGG. (-)-Epicatechin also appears to be a strong bitter chemical. It activates three receptors, TAS2R4, TAS2R5, and TAS2R39, with high efficacy. The potencies of this compound at its cognate receptors may be low relative to the other four polyphenols because its  $EC_{50}$  values are in the millimolar range. However, if (-)-epicatechin is present in food in this concentration range, it would function as a strong activator of bitterness due to its high efficacy. This is supported by data about  $\beta$ -glucopyranosides, which have millimolar potencies at only one receptor, hTAS2R16, yet elicit strong bitterness and avoidance behavior.<sup>12,40</sup> Thus, the data clearly demonstrate that PGG, malvidin-3-glucoside, and procyanidin trimer act as high-potency agonists on TAS2Rs, whereas (-)-epicatechin is a low-potency stimulus.

Polyphenol concentrations in vegetables, fruits, and derived products depend on many factors, for example, culture conditions and degree of ripeness. The tested polyphenols are present in numerous plant-derived products commonly present in our diet (e.g., red wine, beer, cocoa, red fruits), even though their concentrations vary widely.<sup>25</sup> For instance, (-)-epicatechin has been identified in 125 foods and beverages, and it is known to be present in dark chocolate (mean content of 0.070 mg g<sup>-1</sup>), apple (mean content of 0.008 mg g<sup>-1</sup>), and blackberry (mean content of 0.012 mg g<sup>-1</sup>). Malvidin-3-glucoside has been identified in 21 foodstuffs including black grapes (mean content of 0.039 mg g<sup>-1</sup>) and beans (mean content of 0.0006 mg g<sup>-1</sup>). Procyanidin trimer C2 has been identified in beer (0.3  $\mu$ g mL<sup>-1</sup>). The wide range of concentrations is particularly evident in red wine.<sup>41,42</sup> (-)-Epicatechin has been reported to be present in the range of 5.0–88.0 mg L<sup>-1</sup> (= 17.0–302.0  $\mu$ M) in over 800 types of red wines<sup>43</sup> and malvidin-3-glucoside is present in the range of 40.0–510.0 mg L<sup>-1</sup> (= 81.0–1035.0  $\mu$ M) in Port wine.<sup>41</sup> Thus, the concentrations of (-)-epicatechin are about 10-fold below its  $EC_{50}$  values at all cognate receptors, suggesting that this polyphenol may contribute little to the bitterness of red wine. Malvidin-3-glucoside is present at concentrations 6–80-fold higher than its  $EC_{50}$  value at TAS2R7, suggesting that this receptor is robustly activated when consuming these types of wine. With regard to procyanidin trimer and PGG used herein, there are no reported concentrations in red wine but, as stated

previously, this trimer has been reported to be present in beer at 0.3  $\mu$ g mL<sup>-1</sup>.<sup>25</sup> However, procyanidin trimer C1 is known to be present in red wine at 25.6 mg L<sup>-1</sup> (29.6  $\mu$ M).<sup>42</sup>

Although the existing results are controversial, some sensory evaluations perceive smaller polyphenol molecules as more bitter than larger ones.<sup>4,6</sup> Peleg and co-workers<sup>4</sup> studied the bitterness of several compounds including (-)-epicatechin and procyanidin dimers and trimers at the concentration of 0.9 g L<sup>-1</sup> in aqueous ethanol (1% v/v). This concentration corresponds to 3093.0  $\mu$ M (-)-epicatechin and to 1040.0  $\mu$ M procyanidin trimer, which in the case of (-)-epicatechin lies in the concentration range used herein but for procyanidin trimer is much higher than the concentration range used herein. At that concentration, Peleg<sup>4</sup> found that monomers were significantly more bitter than the dimers, which in turn were significantly more bitter than the trimers.

Hufnagel and Hofmann<sup>3</sup> studied the astringency and bitterness of numerous compounds from a red wine by sensorial analysis and demonstrated that the bitterness of that red wine could be induced by phenolic acid ethyl esters and flavan-3-ols [including (-)-epicatechin, procyanidin dimers, and C1 trimer]. But in contrast to Peleg, they found that the larger molecules were more bitter than smaller ones. In fact, the bitter taste threshold for (-)-epicatechin was found to be 930.0  $\mu$ M, and so these data correlate with our observations for TAS2R39. However, the bitter taste thresholds they found for procyanidins were between 400 and 500  $\mu$ M, which are much higher than the values found in this work (30.0  $\mu$ M). This observed correlation between the *in vivo* and *in vitro* analyses, with sensory data showing mostly higher thresholds values in comparison to *in vitro* data, is common and has been reported previously.<sup>19</sup> One possible explanation for this fact is the formation of complexes with proline-rich proteins in the saliva and/or adsorption of these compounds by the oral epithelium, which could alter their effective oral concentrations differently and explain the different concentration-response functions recorded *in vivo* and *in cell culture*.<sup>19</sup> It is also not possible to evoke the possibility that a particular fully activated TAS2R evokes bitter perception to a lesser degree than another one.

Also, a sensory analysis of fractions of grape seed phenolics showed that bitterness increases along with molecular size.<sup>5</sup> Moreover, Lea and Arnold<sup>8</sup> showed that bitterness is associated with oligomeric procyanidins reaching a maximum with the epicatechin tetramer. This is in agreement with our results because (-)-epicatechin monomer is less bitter than procyanidin trimer.

Apart from the molecular size of the bitter polyphenols under study, other factors could affect the bitterness of the compounds in sensory evaluations, as referred previously. For example, whereas ethanol enhances bitterness intensity, varying wine pH has little or no effect on perceived bitterness.<sup>44</sup> Fischer and Noble<sup>44</sup> prepared 18 wines, varying in ethanol (8, 11, and 14% v/v), pH (2.9, 3.2, and 3.8), and (+)-catechin (100.0 and 1500.0 mg L<sup>-1</sup>), using a dealcoholized white wine concentrate. In a completely randomized design, bitterness intensity was rated by 20 subjects, and it was found that bitterness increased by all three components. These authors have shown that an increase of 3% v/v ethanol elevated bitterness more (around 50%) than adding 1400.0 mg/L catechin to the same wine (which increased bitterness by 28%). Such an increase in catechin is not common in the winemaking industry; however, differences of 3% v/v ethanol occur frequently in table wines and will produce a significant increase in bitterness.

Unfortunately, it is impossible to examine this interesting effect of ethanol by means of the receptor assay, because ethanol is highly toxic for cells above 1%.

In summary, the results show that different polyphenol compounds activate different combinations of the ~25 TAS2Rs. (–)-Epicatechin activated three receptors, TAS2R4, TAS2R5, and TAS2R39, whereas only two receptors, TAS2R5 and TAS2R39, responded to PGG. In contrast, malvidin-3-glucoside and procyanidin trimer stimulated one receptor, TAS2R7 and TAS2R5, respectively. Overall, the EC<sub>50</sub> values obtained for the different compounds vary 100-fold, with the lowest values for the compounds that belong to the hydrolyzable tannin and anthocyanins classes, suggesting that these polyphenols could be the major compounds responsible for the bitterness of fruits and derived products, such as red wine.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Additional table. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS USED

DNA, DNA; DMSO, dimethyl sulfoxide; <sup>1</sup>H NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; PGG, pentagalloylglucose; TRC, taste receptor cells

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